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KINETICS OF THE PROTECTION OF LACTATE DEHYDROGENASE BY SUBSTRATES AGAINST HEAT INACTIVATION

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SUMMARY

1. NAD^+, NADH, lactate, pyruvate and the substrate analogues oxamate and oxalate all protected pig lactate dehydrogenase (EC 1.1.1.27) isoenzymes M_4 and H_4 against inactivation at 52–64° in Tris–HCl buffer (pH 7.5). The time-course of inactivation could be described as a single first-order reaction, both in the presence and absence of substrates. Protection by substrates was dependent on substrate concentration.

2. For kinetic treatment of the data, protection was defined as the difference between the first-order rate constants of inactivation measured in the absence and in the presence of substrate (Δk), respectively. On the assumption that protection was due to the equilibrium formation of enzyme–substrate complexes, simple equations were derived for calculation of the dissociation constants involved. A convenient procedure was found for distinguishing between the random order and compulsory order formation of a ternary complex.

3. Protection, defined as Δk, was a hyperbolic function of the concentration of NAD^+ and NADH indicating the expected proportionality between protection and coenzyme binding. The dissociation constants calculated from these hyperbolic functions were of the order of $1 \cdot 10^{-5}$–$1 \cdot 10^{-4}$ M which, in turn, conformed with the idea that protection was due to binding of coenzyme to the specific binding sites. Lactate, pyruvate, oxamate and oxalate, when added to lactate dehydrogenase in the absence of coenzyme and at concentrations lower than about $1 \cdot 10^{-2}$ M, did not protect lactate dehydrogenase against heat inactivation. The protection observed at higher concentrations was most marked with oxalate and it was definitely a non-hyperbolic function of oxalate concentration.

4. Lactate, pyruvate, oxamate and oxalate, when added to lactate dehydrogenase in the presence of coenzyme, had a marked protective effect at concentrations as low as $1 \cdot 10^{-4}$ M. Detailed investigations were carried out with the lactate dehydrogenase-H_4 + NAD^+ + pyruvate ternary system. Assuming that protection was due to the equilibrium formation of a ternary enzyme–substrate complex, an attempt was made to determine the reaction sequence and to calculate the dissociation constants involved. By applying the above kinetic equations, we found that the ternary complex was formed in a compulsory order, with pyruvate being bound only to the lactate dehydrogenase–NAD^+ complex and not to the free enzyme. A dissociation constant

of $0.75 \cdot 10^{-3}$–$0.96 \cdot 10^{-3}$ M was calculated for pyruvate in the ternary complex, whilst the dissociation constant obtained for NAD^+ was $1.5 \cdot 10^{-3}$ M.

INTRODUCTION

It is a more or less general experience that binding of substrates affects the rate at which enzymes are irreversibly inactivated by various treatments. Heat inactivation is one of the most widely applied procedures and it is well known that various substrates and substrate analogues of lactate dehydrogenase (EC 1.1.1.27) do protect this enzyme at elevated temperatures[1,2].

The term heat inactivation implies that the chemical mechanism whereby activity is (irreversibly) lost is not identified. Neither can it be decided whether this protection by substrates is due to direct blocking of some groups primarily involved in the process of inactivation, or to some indirect effects mediated through a structural change in the protein. However, in any case, protection is due to an interaction between substrate and enzyme. Consequently, if specificity of the protection can be demonstrated, it can rightly be assumed that protection is due to the formation of specific enzyme–substrate complexes.

In this paper we report studies on the use of this indirect approach for a quantitative description of the formation of enzyme–substrate complexes involving lactate dehydrogenase. BURTON[3] seems to have been the first to show that a dissociation constant can be calculated for enzyme–substrate complexes from data on the overall rate of inactivation in the presence of increasing concentrations of substrate. He has considered the simplest possible mechanism only[3]. Recently, there has been a renewed interest in heat inactivation as a means of demonstrating the specific binding of various ligands to enzymes present in partially purified preparations[4–6] or to chemically modified and inactive enzyme proteins[7,8]. However, it seems that BURTON's ideas have still not been further developed (see, *e.g.* ref. 9 or ref. 4)*.

The use of lactate dehydrogenase in these studies had the following advantages: (1) We have found[11] that the time-course of heat inactivation of pig lactate dehydrogenase isoenzymes M_4 and H_4 was a single first-order reaction, the rate of which could be determined with agreeable precision. (2) Lactate dehydrogenase has been known to be an enzyme to which the two substrates were bound and from which the two products were released in a compulsory order[12–14], and for which much additional information was available on the formation of various binary and ternary enzyme–substrate complexes[15–19].

MATERIALS AND METHODS

Pig heart and pig muscle lactate dehydrogenase isoenzymes were prepared as already described[11]. The repeatedly recrystallized preparations were electrophoretically homogeneous and had a maximal specific activity. NAD^+ (Reanal), NADH (Reanal), sodium pyruvate (British Drug Houses), sodium lactate (Riedel de Haen),

* During the preparation of this paper our attention was drawn to a paper by GUANG-CHOU AND CHEN-LU[10] in which the term Δk, as defined by Eqn. 3 of this communication, was apparently used for the first time.

disodium oxalate (British Drug Houses) and potassium oxamate (Fluka) were analytical grade preparations.

Protein content and enzyme activity were measured and heat inactivation carried out as already described[11]. Treatments at different temperatures were carried out in the same 0.2 M Tris–HCl buffer, adjusted to pH 7.5 at 25°. Protein concentration is given in this paper in terms of moles/l and refers to the molecular weight of the monomer (mol. wt. 36 000). In all experiments the initial molar concentration of the protein was at least 10 times lower than the initial concentration of ligand(s).

RESULTS AND KINETIC MODELS

(1) Comparison of the protective effect of various substrates

From representative results shown in Figs. 1 and 2 it is seen that all substrates and substrate analogues tested markedly reduce the first-order rate constant of inactivation of lactate dehydrogenase. It is apparent (Fig. 1) that reduced and oxidized

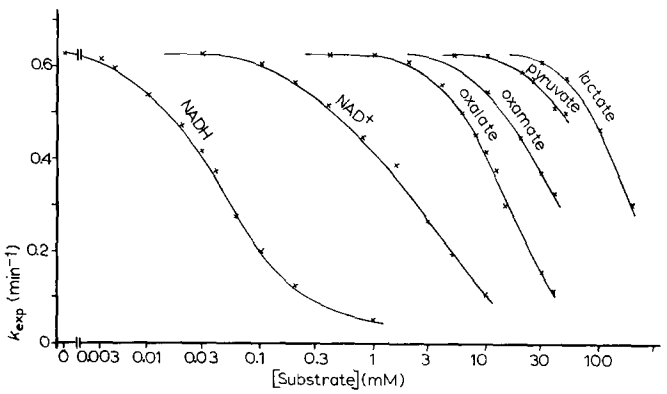


Fig. 1. Effect of substrates on the first-order rate constant of inactivation (k_{exp}). Lactate dehydrogenase–M_4 was incubated at 60° in the presence of the indicated concentrations of NADH, NAD^+, oxalate, pyruvate, oxamate and lactate. The concentration of lactate dehydrogenase was $1 \cdot 10^{-5}$ M, except in the case of NADH ($1 \cdot 10^{-6}$ M).

coenzymes (NADH and NAD^+) are effective at much lower concentrations than reduced and oxidized metabolites (lactate and pyruvate) or the metabolite analogues oxalate and oxamate. Fig. 2 demonstrates that when increasing concentrations of oxalate (an analogue of lactate) are added to lactate dehydrogenase in the presence of various constant concentrations of NAD^+, there is a downward shift in the critical range of oxalate concentrations.

The same conclusions could be drawn from a series of further experiments in which the experimental conditions were varied as follows: (i) Lactate dehydrogenase-H_4 was substituted for lactate dehydrogenase-M_4. (ii) The concentration of enzyme protein was varied over the range $1 \cdot 10^{-6}$–$1 \cdot 10^{-4}$ M. (iii) The temperature of heat treatment was varied over the range 52–64°. (iv) In experiments analogous to those shown in Fig. 2, the coenzyme *plus* metabolite (analogue) combination involved NAD^+ + pyruvate, NADH + oxamate or NADH + oxalate.

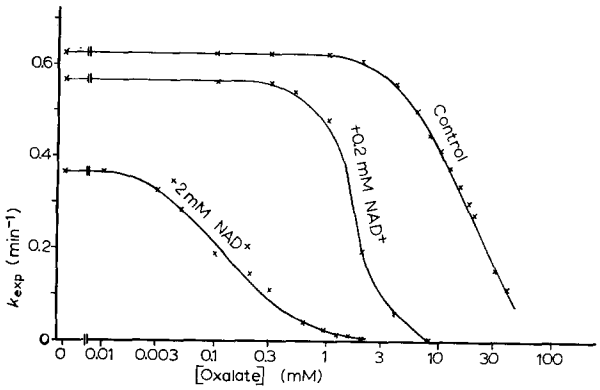


Fig. 2. Effect of oxalate on the first-order rate constant of inactivation in the presence of NAD^+. The constant concentrations of NAD^+ are given on the graph. $1 \cdot 10^{-5}$ M lactate dehydrogenase-M_4 was incubated at 60°.

(2) Models for and kinetic description of protection by substrates

The results described in Section 1 indicated that protection by substrates was due to the formation of specific enzyme–substrate complexes, coupled with an increased heat stability of such complexes. Three important kinetic schemes based on this consideration are shown in Table I. In these reaction schemes, the overall reaction of inactivation ($v = k_{exp} \cdot E_t$) is resolved into equilibrium steps in which enzyme–substrate complexes are formed, followed by the parallel inactivation of the different forms of the enzyme in the resulting equilibrium state.

It is reasonable to assume that our experiments from which k_{exp} has been determined meet the requirements of the two conditions under which the concentration of the enzyme–substrate complex(es) can be expressed in terms of E_t and the corresponding dissociation constants. These conditions are that (a) the rate of inactivation is too slow to interfere with the maintenance of equilibrium concentrations and (b) the concentration of free substrate is constant during the course of inactivation. (With reference to Condition b, see MATERIALS AND METHODS.)

When these equilibrium considerations are applied to Eqns. 1A–1C (Table I), and both sides of the resulting equations are divided by E_t, Eqns. 2A–2C are obtained. These equations define the experimentally obtained first-order rate constant (k_{exp}) as a function of substrate concentration. In the case in which one substrate is bound (Scheme A),

$$k_{exp} = \frac{k_0 K_A + k_A A}{K_A + A} \tag{2A}$$

In the case in which two substrates are bound in a compulsory order (Scheme B),

$$k_{exp} = \frac{k_0 K_A K_B + k_A K_B A + k_{AB} AB}{K_A K_B + K_B A + AB} \tag{2B}$$

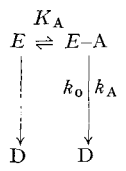
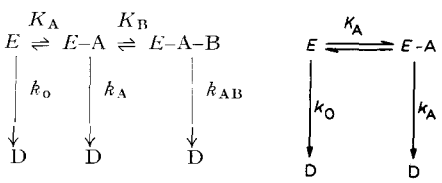
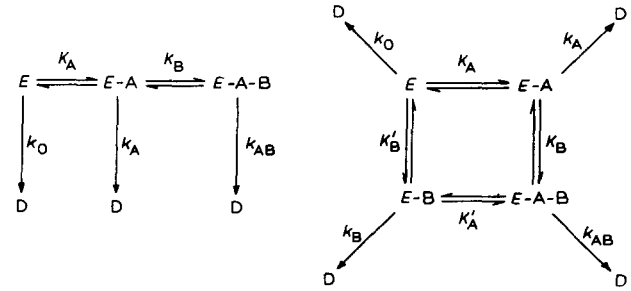
In the case in which two substrates are bound in a random order (Scheme C),

$$k_{exp} = \frac{k_0 K_A K_B + k_A K_B A + k_{AB} AB + k_B K'_A B}{K_A K_B + K_B A + AB + K'_A B} \tag{2C}$$

TABLE I

THREE IMPORTANT EQUILIBRIUM SCHEMES DESCRIBING ENZYME INACTIVATION IN THE PRESENCE OF SUBSTRATE(S)

E = free enzyme; E–A = binary complex of enzyme and substrate A; E–B = binary complex of enzyme and substrate B; E–A–B = ternary complex of enzyme, substrate A and substrate B; D = inactive denatured form of the enzyme; K_A = dissociation constant for A in the E–A complex; K_B' = dissociation constant for B in the E–B complex; K_A' = dissociation constant for A in the E–A–B complex; K_B = dissociation constant for B in the E–A–B complex; k_0, k_A, k_{AB} and k_B are the first-order rate constants with which the four forms of the enzyme are inactivated; k_{exp} = apparent first-order rate constant of the overall reaction (obtained experimentally); E_t = total residual active enzyme.

(A) One substrate	*Two substrates*	
	(B) Compulsory sequence	*(C) Random sequence*
		
$k_{exp}E_t = k_0E + k_AE\text{–A}$ (Eqn. 1A)	$k_{exp}E_t = k_0E + k_AE\text{–A} + k_{AB}E\text{–A–B}$ (Eqn. 1B)	$k_{exp}E_t = k_0E + k_AE\text{–A} + k_{AB}E\text{–A} - \text{B} + k_BE\text{–B}$ (Eqn. 1C)

Eqns. 2A–2C can be conveniently used for the determination of the dissociation constants involved after a simple transformation. To demonstrate this, let us first consider the one-substrate scheme (A in Table I). If both sides of Eqn. 2A are subtracted from k_0, we obtain

$$\Delta k = k_0 - k_{exp} = (k_0 - k_A) \frac{1}{1 + K_A/A} \tag{3}$$

and

$$\frac{1}{\Delta k} = \frac{1}{k_0 - k_A} + \frac{K_A}{k_0 - k_A} \cdot \frac{1}{A} \tag{4}$$

By definition, k_0 is the first-order rate constant of inactivation in the absence of substrate, and this value can easily be obtained experimentally. Accordingly, Δk is easily calculated for different concentrations of A (see Fig. 3). The graphical solution for K_A is analogous to that of a Lineweaver–Burk plot.

The same principles can also be applied to the two-substrate schemes in Table I. In this case, results of experimental series in which the concentration of one of the substrates is kept constant and the concentration of the second substrate is varied are required. In turn, Δk will have to be defined as the difference between the overall

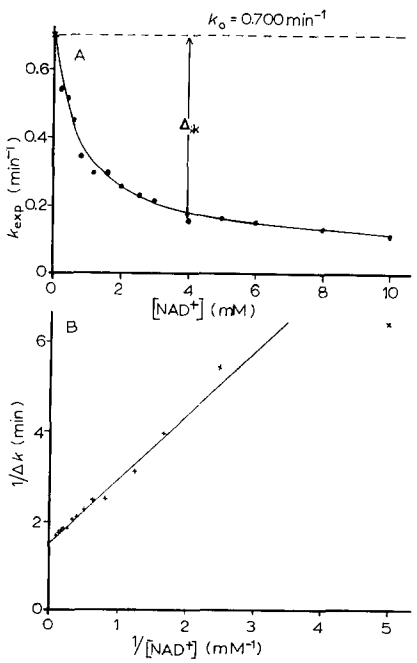


Fig. 3. Kinetic treatment of the protective effect of NAD$^+$. $8 \cdot 10^{-6}$ M lactate dehydrogenase–H_4 was incubated at 64°. In Fig. 3A, the experimentally obtained rate constants are plotted against NAD$^+$ concentration. The solid line has been calculated from Fig. 3B; the dashed line shows the value of k_0. The observed difference between k_0 and k_{exp} at the given concentration of NAD$^+$ is Δk. In Fig. 3B these Δk values are plotted according to Eqn. 4. From the straight line drawn tc the points, $k_A = 0.05$ min^{-1} and $K_A = 0.83 \cdot 10^{-3}$ M. (For definitions see Table I).

rate constants obtained in the absence and in the presence of a certain concentration of the constant substrate.

Two substrates, compulsory order

If the concentration of A is constant and that of B is varied, the following equation is obtained from Eqn. 2B:

$$\Delta k = k_{\mathrm{exp,B=0}} - k_{\mathrm{exp}} = \frac{K_A(k_0 - k_{AB}) + A(k_A - k_{AB})}{K_A + A} \cdot \frac{1}{1 + \frac{K_B(K_A + A)}{A} \cdot \frac{1}{B}} \quad (5)$$

The formal analogy between Eqns. 3 and 5 is obvious. It is seen that the two complex constants in Eqn. 5 are both dependent on the constant concentration of A. A similar equation is obtained for the case when B is constant and A is varied, *i.e.*

$$\Delta k = k_{\mathrm{exp,A=0}} - k_{\mathrm{exp}} = \frac{K_B(k_0 - k_A) + B(k_0 - k_{AB})}{K_B + B} \cdot \frac{1}{1 + \frac{K_A K_B}{K_B + B} \cdot \frac{1}{A}} \quad (6)$$

Two substrates, random order

The equations obtained from Eqn. 2C are, for constant A and varied B:

$$\Delta k = k_{\mathrm{exp,B=0}} - k_{\mathrm{exp}} =$$

$$= \frac{(A)^2(k_A - k_{AB}) + K'_A A(k_A - k_B) + K_A A(k_0 - k_{AB}) + K_A K'_A(k_0 - k_B)}{(K_A + A)(K'_A + A)} \cdot$$

$$\cdot \frac{1}{1 + \frac{K_B(K_A + A)}{K'_A + A} \cdot \frac{1}{B}} \quad (7)$$

and for constant B and varied A:

$$\Delta k = k_{\mathrm{exp,A=0}} - k_{\mathrm{exp}} =$$

$$= \frac{(B)^2(k_B - k_{AB}) + K_B B(k_B - k_A) + K'_B B(k_0 - k_{AB}) + K_B K'_B(k_0 - k_A)}{(K_B + B)(K'_B + B)} \cdot$$

$$\cdot \frac{1}{1 + \frac{K'_A(K'_B + B)}{K_B + B} \cdot \frac{1}{A}} \quad (8)$$

It should be noted that the random order scheme (C in Table I) is symmetrical and, therefore, Eqns. 7 and 8 are analogous.

Kinetic distinction between compulsory order and random order binding of substrates

It can be seen that Eqns. 5–8 are formally analogous and that they all contain two complex constants. With reference to the Michaelis–Menten equation, these constants may be referred to as the v_{max}-type constant (or Δk_{max}) and the K_m-type constant (or apparent Michaelis constant, "K_m"), respectively. Obviously, both can be determined from a double reciprocal plot of Δk *versus* substrate. The K_m-type constants in Eqns. 5–8 are simple functions of the concentration of the constant substrate. It is further seen from Table II that these secondary functions are different

TABLE II

DEPENDENCE OF THE APPARENT MICHAELIS CONSTANTS ("K_m") OF EQNS. 5–8 ON THE CONCENTRATION OF THE CONSTANT SUBSTRATE

Experimental arrangement	*Compulsory order*	*Random order**
A is constant, B is varied	$"K_m" = K_B + K_A K_B \cdot \frac{1}{A}$	$"K_m" = \frac{K_B(K_A + A)}{K'_A + A}$
B is constant, A is varied	$\frac{1}{"K_m"} = \frac{1}{K_A} + \frac{1}{K_A K_B} \cdot B$	$\frac{1}{"K_m"} = \frac{K_B + B}{K'_A(K'_B + B)}$

* In the case in which $K_A = K'_A$ and $K_B = K'_B$ (since $K_A K_B = K'_A K'_B$ either both or none of the two equations should hold), the "K_m" values for A and B will equal K_A, and K_B, respectively.

for the first and second substrates in the compulsory order scheme, and that there is a linear form for these equations in both experimental arrangements whereby the compulsory order mechanism can be distinguished from the random order mechanism. Table II also shows that a graphical determination of K_A and K_B should be possible from secondary plots, in case the compulsory order mechanism is operating. An experimental application of these suggestions is demonstrated in Fig. 5 below (Section 3.3).

(3) Kinetics of the binding of substrates to lactate dehydrogenase

We discuss three examples in which the kinetics of substrate binding have been studied in detail.

(3.1) Binding of NAD^+ to lactate dehydrogenase-H_4

Results obtained at 64° are seen in Fig. 3. Fig. 3A shows the first-order rate constants obtained at increasing concentrations of NAD^+ and the way in which Δk values have been calculated. Fig. 3B is double reciprocal plot from the same data for $1/\Delta k$ against $1/NAD^+$. It should be noted that from Fig. 3B all the constants in Eqn. 4 can easily be determined.

(3.2) Binding of oxalate to lactate dehydrogenase-M_4

Results obtained at 53°, where k_0 is as low as 0.0102 min^{-1}, are shown in Fig. 4. It is seen that protection, defined as Δk, is definitely a nonhyperbolic function of oxalate concentration. Similarly, nonhyperbolic functions were also obtained at higher temperatures, where the overall rate of heat inactivation was much higher[11], and also with further combinations involving lactate dehydrogenase-M_4 or lactate dehydrogenase-H_4 and one or two substrates.

It should be noted that the simple equilibrium scheme (A in Table I) cannot account for a nonhyperbolic Δk-function. The operation of a nonequilibrium, steady-state scheme is certainly one of the theoretically available possibilities[20–23]; however, in the case of the experiment in Fig. 4, where $k_0 = 0.0102$ min^{-1}, the irreversible steps seem to be too slow for the steady-state assumption. It is more likely that, lactate dehydrogenase being a tetrameric enzyme with four binding sites per tetramer, there is a cooperative interaction between the subunits. Cooperative interaction in the binding of substrate to the specific binding site of identical subunits of an oligomeric enzyme is certainly possible[4–6,24]. Alternatively, the inactivation of subunits

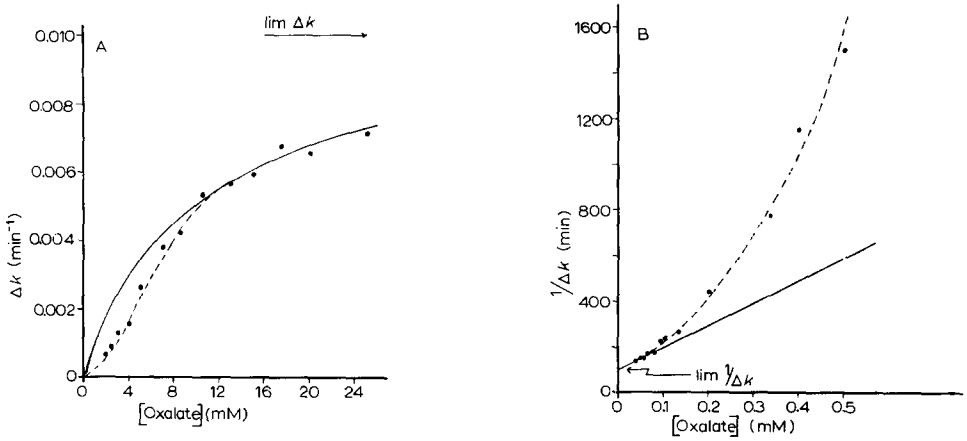


Fig. 4. Protection by oxalate. $1.1 \cdot 10^{-5}$ M lactate dehydrogenase–M_4 was incubated at 53°. In Fig. 4A, the solid line is calculated from the solid line in Fig. 4B. The arrow indicates the limiting value for Δk, which cannot be higher than k_0(if $A \rightarrow \infty$ and $k_A = 0$, see Eqn. 3). In Fig. 4B, the same points are shown in a double reciprocal plot. Lim Δk is marked on the ordinate. From the best straight line drawn through points with lowest 1/[oxalate] values, $K_A = 9.70 \cdot 10^{-3}$ M, and $\Delta k_{max} = 0.0102$ min^{-1}.

may not be independent of each other[11,25] and this should lead to cooperative protection if we consider the formal analogy between a series of hybrid tetramers and a series of homotetramers binding 0, 1, 2, 3 and 4 molecules of substrate per tetramer (see DISCUSSION).

(3.3) Formation of the lactate dehydrogenase-NAD$^+$–pyruvate ternary complex

It has already been stated in Section 1 that pyruvate markedly increased the heat stability of lactate dehydrogenase when added in the presence of NAD$^+$. This indicates that an abortive ternary lactate dehydrogenase–NAD$^+$–pyruvate complex is formed, an assumption which is supported by convincing independent evidence[18,26]. Our present studies were aimed at deciding, with the use of the kinetic procedure outlined in Section 2, whether the ternary complex was formed through a random order binding of NAD$^+$ and pyruvate to lactate dehydrogenase (Scheme C in Table I) or through a compulsory sequence of binding (Scheme B in Table I).

In Fig. 5, the predictions of Table II for the compulsory order scheme were tested in secondary plots. The apparent Michaelis constants ("K_m"), obtained from experimental series in which the concentration of either NAD$^+$ (Fig. 5A) or pyruvate (Fig. 5B) was constant, were replotted according to the corresponding linear function in Table II. It is seen that the predicted linear relationships have been obtained, supporting the compulsory order scheme. It is even possible to calculate the dissociation constants K_A and K_B from Fig. 5. The values obtained by using the corresponding equations in Table II are $K_A = 1.57 \cdot 10^{-3}$ M, $K_B = 0.75 \cdot 10^{-3}$ M (Fig. 5A) and $K_A = 1.43 \cdot 10^{-3}$ M, $K_B = 0.96 \cdot 10^{-3}$ M (Fig. 5B). The agreement between the two independent estimates is satisfactory and this justifies the application of the described kinetic procedure.

It is interesting to note that the reciprocal of the dissociation constant for NAD$^+$ found in the absence of pyruvate (point on the ordinate, Fig. 5B) is significantly different from the reciprocal of the dissociation constant obtained in the presence of

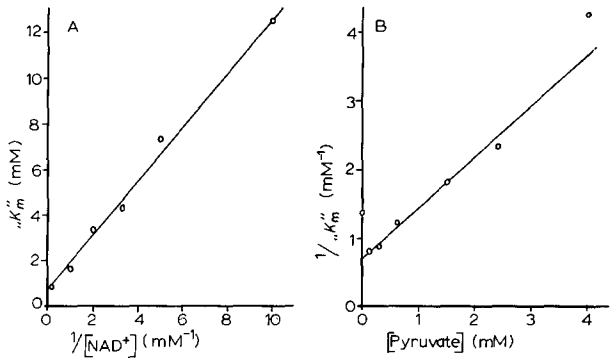


Fig. 5. Secondary plots of protection by NAD$^+$ *plus* pyruvate. Dependence of the apparent Michaelis constants ("K_m") on the concentration of the constant substrate. $2 \cdot 10^{-5}$ M lactate dehydrogenase–H_4 was incubated at 64° in the presence of a constant concentration of NAD$^+$ *plus* increasing concentrations of pyruvate (5A) or in the presence of a constant concentration of pyruvate *plus* increasing concentrations of NAD$^+$ (5B). The point shown on the ordinate of Fig. 5B gives the apparent dissociation constant obtained in the absence of pyruvate ("K_m" = K_A).

pyruvate (ordinate intercept, Fig. 5B). A similar difference was observed by CRIDDLE *et al.*[27] in the binding of NADH to lactate dehydrogenase in the presence and in the absence of NAD$^+$, respectively.

DISCUSSION

We have demonstrated the advantages of defining protection by substrates against heat inactivation in terms of Δk, *i.e.* the difference between the first-order rate constants of inactivation determined in the absence and in the presence of a certain concentration of substrate. From studying the concentration dependence of Δk, it was possible to distinguish between the compulsory order and random order reaction schemes for the formation of the ternary complex lactate dehydrogenase–NAD$^+$–pyruvate and, also, to calculate the two dissociation constants involved in the compulsory order scheme.

The main result described in this paper is a generally applicable kinetic procedure for a wide range of indirect studies on the binding of substrates (ligands) to enzymes. Application of the kinetic treatment presented is not limited either to heat denaturation or to the measurement of enzyme inactivation. The essential conditions of application are: (1) Formation of the reversible enzyme–substrate complex should alter the rate of an irreversible sequential reaction. The enzyme–substrate complex may be either more or less reactive than the free enzyme. (2) The second, irreversible, reaction should follow a first-order time-course. (3) The irreversible reaction step(s) should be rate limiting. For instance, all these conditions are fulfilled if the enzyme is treated with a chemical reagent, such as iodoacetamide, and if the experiment is run under such conditions that the reaction follows an apparently first-order time-course (see *e.g.* O'SULLIVAN AND COHN[28]).

What we have serious doubts about is the suitability of oligomeric enzymes composed of identical polypeptide chains, like lactate dehydrogenase, for indirect studies on the binding of substrates. In the present studies this has been indicated by

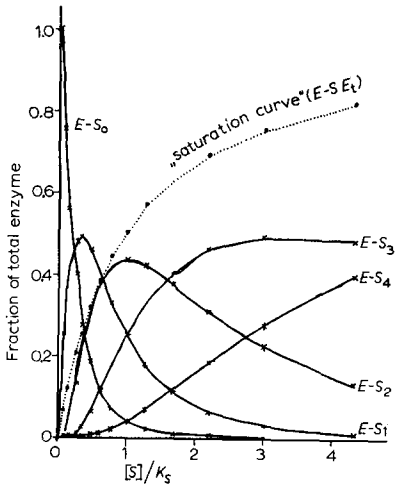


Fig. 6. Distribution of bound substrate among the four molecular forms E–S_1, E–S_2, E–S_3 and E–S_4 as a function of substrate concentration. Calculated curves. The four binding sites are assumed to be independent and equal. Accordingly, the probability of the four molecular species which contain an increasing number of bound substrates is obtained as the corresponding hypergeometric probability function:

$$p = \frac{\binom{M}{k}\binom{N-M}{n-k}}{\binom{N}{n}} \qquad (9)$$

where N = total number of binding sites (= subunits, ε_t), M = total number of binding sites occupied by substrate (E–S), $n = 4$, *i.e.* the number of subunits of the enzyme, k = 0, 1, 2, 3 and 4 correspond to the molecular species E–S_0, E–S_1, E–S_2, E–S_3 and E–S_4, respectively. The five probability functions obtained from Eqn. 9 give p_0, p_1, p_2, p_3 and p_4 as a function of M/N. The abscissa of Fig. 6 ($[S]/K_S$) is obtained from M/N from the equilibrium relationship $[S]/K_S = M/(N - M)$. The ordinate is transformed according to the relationship: $E\text{–}S = \sum_{i=1}^{i=4} (i \cdot p_i)$. Accordingly, the four curves E–S_1 to E–S_4 give $1p_1$ to $4p_4$. The abscissa is given in units of K_S and the ordinate in units of E_t.

the frequent occurrence of nonhyperbolic functions for the dependence of Δk on substrate concentration (*e.g.* see the binding of oxalate, Section 3.2). From such relationships no definite conclusion can be drawn about the binding of substrate.

Evidence recently obtained in our laboratory[11,25,29,30] indicates that we have to consider the binding of substrate to the tetrameric form of lactate dehydrogenase and that, as a rule, there is an interaction between the polypeptide chains during the course of inactivation. We have already pointed out, in Section 3.2, that there is a formal analogy between hybrid lactate dehydrogenase tetramers and homotetrameric lactate dehydrogenase species to which 0, 1, 2, 3 or 4 molecules of substrate are bound. The expected distribution of total enzyme among the latter five tetrameric forms as a function of substrate concentration is shown in Fig. 6. We should like to stress that overall protection of the tetrameric enzyme by substrate can only be a hyperbolic function of substrate concentration if protection of each molecular form is proportional to the number of substrate molecules bound to the tetramer. Since, in the

analogous case of hybrid isoenzymes, no such proportionality has been observed[11,25], we suggest that in the case of *e.g.* oxalate (Fig. 4) the macromolecular mechanism of inactivation[25] interferes with an estimation of the dissociation constant.

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