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Use of the Sulphite Adduct of Nicotinamide–Adenine Dinucleotide to Study Ionizations and the Kinetics of Lactate Dehydrogenase and Malate Dehydrogenase

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1. The formation of the non-enzymic adduct of NAD^+ and sulphite was investigated. In agreement with others we conclude that the dianion of sulphite adds to NAD^+. 2. The formation of ternary complexes of either lactate dehydrogenase or malate dehydrogenase with NAD^+ and sulphite was investigated. The u.v. spectrum of the NAD–sulphite adduct was the same whether free or enzyme-bound at either pH6 or pH8. This suggests that the free and enzyme-bound adducts have a similar electronic structure. 3. The effect of pH on the concentration of NAD–sulphite bound to both enzymes was measured in a new titration apparatus. Unlike the non-enzymic adduct (where the stability change with pH simply reflects $HSO_3^- = SO_3^{2-} + H^+$), the enzyme-bound adduct showed a bell-shaped pH–stability curve, which indicated that an enzyme side chain of $pK = 6.2$ must be protonated for the complex to form. Since the adduct does not bind to the enzyme when histidine-195 of lactate dehydrogenase is ethoxycarbonylated we conclude that the protein group involved is histidine-195. 4. The pH-dependence of the formation of a ternary complex of lactate dehydrogenase, NAD^+ and oxalate suggested that an enzyme group is protonated when this complex forms. 5. The rate at which NAD^+ binds to lactate dehydrogenase and malate dehydrogenase was measured by trapping the enzyme-bound NAD^+ by rapid reaction with sulphite. The rate of NAD^+ dissociation from the enzymes was calculated from the bimolecular association kinetic constant and from the equilibrium binding constant and was in both cases much faster than the forward $V_{max.}$. No kinetic evidence was found that suggested that there were interactions between protein subunits on binding NAD^+.

Both Klinman (1975) and Blankenhorn (1977) have now presented evidence that the dehydrogenase-catalysed reduction of NAD^+ to NADH is not only stereospecific but is mediated by the attack of the H^- anion on the C-4 position of the nicotiniumamide ring. The purpose of the present paper is to investigate whether and to what extent NAD^+ bound to two dehydrogenases of known structure is activated for attack by anions, in particular sulphite.

In attempting to assay LDH in the forward direction Meyerhof *et al.* (1938) added Na_2SO_3 in an attempt to remove the pyruvate formed as a bisulphite adduct, but were surprised to find that the sulphite inhibited the enzyme by forming an adduct with NAD^+. Pfleiderer *et al.* (1956) first used the NAD–sulphite adduct to measure the number of NAD^+-binding sites on the enzyme and also recognized that the dianion SO_3^{2-} added to enzyme-bound NAD^+ much more readily than to free NAD^+ (Pfleiderer *et al.*, 1960). The present paper reports measurements of the pH-dependence of the stability of enzyme-bound NAD–sulphite and the recognition that an enzyme group must be protonated for the complex to form. Information on the nature of the enzyme groups that line the coenzyme-binding pocket of a protein may also be obtained by systematically varying the structure of the coenzyme, and Woenckhaus (1974) has reviewed the results that have been obtained with coenzyme analogues.

Abbreviations used: LDH, lactate dehydrogenase (L-lactate–NAD^+ oxidoreductase, EC 1.1.1.27); MDH, supernatant malate dehydrogenase (L-malate–NAD^+ oxidoreductase, EC 1.1.1.37).

Experimental

Pig heart LDH was purified as a by-product from the preparation of MDH from pig heart by method 2 of Lodola *et al.* (1978*a*). LDH was recovered by washing the DEAE-cellulose used in the batch-absorption step with 0.3M-H_3PO_4 adjusted to pH6.5 with 10M-NaOH. The enzyme was subsequently purified by gradient elution from DEAE-cellulose and by crystallization from $(NH_4)_2SO_4$ solution by using well-established techniques. The

preparation had a specific enzyme activity of 340 μmol of NADH oxidized/min per mg of protein when assayed as ΔA_{340} in a solution of 0.15 mM-NADH, 0.3 mM-pyruvate and 67 mM-H_3PO_4 adjusted to pH 7.2 with 10 M-NaOH. Protein was determined by assuming $A_{280}^{0.1\%} = 1.39$ (Jecsai, 1962). Dogfish M_4 LDH was a kind gift from Professor M. G. Rossmann (Purdue University, W. Lafayette, IN, U.S.A.). Pig heart cytoplasmic MDH was purified by method 2 of Lodola *et al.* (1978*a*) and had a specific activity of 120 μmol of malate oxidized/min per mg of protein when assayed as ΔA_{340} in a solution of 0.1 M-L-malate, 3 mM-NAD^+ and 95 mM-glycine adjusted to pH 10 with 10 M-NaOH at 25°C. Protein was determined by assuming $A_{280}^{0.1\%} = 1.5$ (Lodola *et al.*, 1978*a*). Some enzyme concentrations are expressed by assuming a subunit mol.wt. of 36000 (LDH) and 35000 (MDH). The enzymes were stored as crystalline suspensions in 3 M-$(NH_4)_2SO_4$ and were prepared for each experiment by sedimenting the crystals in a bench centrifuge, redissolving the protein in dilute buffer and filtering the solution through a Sephadex G-50 column equilibrated with the buffer required in the planned experiment. In some cases the protein solution was also cleared of dust by filtering it through a Millipore filter disc (8 μm pore size). Sodium pyruvate, NADH and NAD^+ (grade II) were purchased from Boehringer Corp. (London), Lewes, East Sussex BN7 1LG, U.K. AnalaR-grade Na_2SO_3 and oxalic acid were from BDH Chemicals, Poole, Dorset, U.K. The Na_2SO_3 solutions were prepared from large (0.5 cm) crystals and were used within 2 h, but were not further standardized. L-Malic acid was from Sigma (London) Chemical Co., Kingston on Thames, Surrey, U.K. NAD^+ was purified as described by Holbrook & Wolfe (1972).

Instrumentation

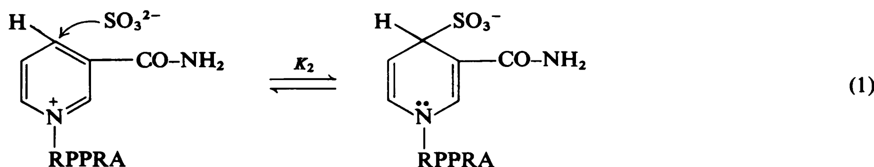
The dual-wavelength titrating spectrophotometer and fluorimeter and the pH-titrator have been previously described in outline (Shore *et al.*, 1975; Lodola *et al.*, 1978*b*). The pH-titrator used in this present work consisted of a 5 mm combined glass/calomel pH electrode and a stirring paddle mounted in an alloy block in such a way that they could be inserted into a 2 cm × 1 cm × 4 cm fluorescence cuvette above the optical light-path. Two micro-bore stainless-steel tubes were used to add either 2 M-acetic acid or 2 M-NaOH to the solution (6 ml) to vary the pH smoothly in the range pH 3–10. The volume change was never greater than 1%. Signals from the photomultiplier, either as absorbance or as fluorescence, and the pH electrode were sampled every 0.2 s and were stored in a 2 kbyte of 10 bits digital store (Transidyne General Corp., Ann Arbor, MI, U.S.A.). The records of experiments, which usually lasted 200 s, could then be displayed as a plot of optical signal (y) against pH (x) by using an oscilloscope in the x–y mode.

For stopped-flow measurements in a 2 mm × 2 mm cuvette, the excitation wavelength for protein fluorescence was 297 nm and was derived from an Hanovia 200VA Hg–Xe arc. Emission was collected by a light-guide and measured after passage through a Kodak-Wratten 18A filter by an 11-stage photomultiplier. Absorption measurements of NAD–sulphite were made at 327 nm by using a tungsten–halogen car headlamp bulb as light-source and a monochromator in a path length of 1 cm. The dead time with the fluorescence cuvette was less than 1 ms, and 2.3 ms with the absorption cuvette.

Results and Discussion

Non-enzymic reaction of NAD^+ and sulphite

There have been several investigations of the reaction of NAD^+ with Na_2SO_3 at neutral pH to give a compound that absorbs at 327 nm. It has been suggested that the reaction is due to the addition of HSO_3^- (Meyerhof *et al.*, 1938; Colowick *et al.*, 1951; Ciaccio, 1966); however, the pH-dependence of the reaction (Pfleiderer *et al.*, 1960) and recent kinetic and n.m.r. evidence (Johnson & Smith, 1976) rather suggest that the reaction should be formulated (RPPRA is adenosine diphosphate ribose moiety):



(1)

To satisfy ourselves that eqn. (1) could represent the reaction at all pH values and sulphite concentrations, we carried out an optical titration. A solution (6 ml) of a mixture of NAD^+ and Na_2SO_3 was placed in the titration apparatus and the A_{327} was monitored as the pH was varied from 4 to 10. Titration curves

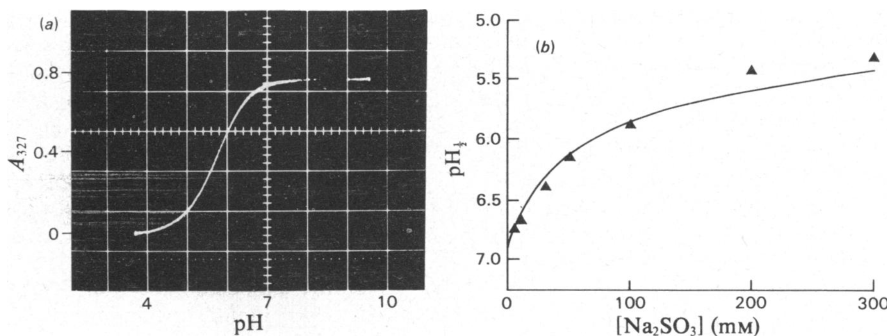


Fig. 1. *pH-Dependence of the non-enzymic equilibrium* $NAD^+ + SO_3^{2-} = NAD\text{–}SO_3^-$

(*a*) shows the manner in which the A_{327} of a solution containing 0.35mM-NAD^+ and 0.1M-Na_2SO_3 varied as the pH of the solution was raised from 3.7 to 9.6. (*b*) shows the variation of the pH for half-maximum saturation ($pH_{\frac{1}{2}}$) as the concentration of Na_2SO_3 in the solution was changed. ▲, Experimental points; ——, curve calculated by using $K_2 = 10$mM and $K_{SF} = 10^{-6.9}$M.

similar to the example shown as Fig. 1 were obtained. The curve is characterized by $pH_{\frac{1}{2}}$, the pH at which the change in absorbance is half complete and at which $dA/d(pH)$ is a maximum. For the reaction shown in eqn. (1) we predicted that $[H]_{\frac{1}{2}}$ ($[H]_{\frac{1}{2}} = 10^{-pH_{\frac{1}{2}}}$) would increase ($pH_{\frac{1}{2}}$ decrease) with increased total concentration of total sulphite $[S]_T$ according to:

$$[H]_{\frac{1}{2}} = K_{SF}\left(1 + \frac{[S]_T}{K_2}\right)$$

where K_{SF} is the acid dissociation constant of $HSO_3^- = SO_3^{2-} + H^+$ ($10^{-6.9}$M). The correspondence between the calculated curve and the experimental points in Fig. 1(*b*) is satisfactory. However, the curve through the points was calculated by using $pK_{SF} = 6.9$ and $K_2 = 10$mM, and it will be shown below that the kinetically determined value of K_2 is about 15mM. This extent of disagreement might be expected, since we assume that the activity coefficients of Na^+ and SO_3^{2-} ions are unity in the 0.1–0.3M range.

Equilibrium binding of sulphite to enzyme–NAD⁺ complexes at a fixed pH

When Na_2SO_3 (up to 0.02mM) is slowly titrated into a solution of 10μM-LDH and 2mM-NAD^+ at pH7.0 (that is when the enzyme is saturated with NAD^+) there is an increase in A_{327} and a 70% decrease in protein fluorescence (similar to that produced by the binding of NADH). Under these conditions the formation of free $NAD\text{–}SO_3^-$ (which also absorbs at 327nm) is negligible relative to the amount bound to the enzyme, since the equilibrium is so unfavourable with the small quantities of Na_2SO_3 added to saturate the enzyme. The increase in A_{327} with both LDH and MDH corresponded to the formation of 1 mol of bound $NAD\text{–}SO_3^-$/mol of enzyme subunits. This was not unexpected for LDH (Pfleiderer *et al.*, 1960) and is consistent with the results for nucleotide binding to cytoplasmic MDH from this laboratory (Lodola *et al.*, 1978*a*). With LDH, the change in protein fluorescence is not, and indeed is not expected to be, superimposable on the change in absorbance. However, if the degree of saturation of binding sites ($\bar{v}$) is obtained from protein fluorescence (F) as described by Holbrook (1972), that is by using $\bar{v} = (1 - F^{\frac{1}{2}})/0.3$, then the saturation function from protein fluorescence and that from the absorbance change coincide. Owens & Teale (1976) have examined some structural models which give rise to the geometric quenching implicit in the use of the equation of Holbrook (1972). With malate dehydrogenase, the two processes are indistinguishable in form. The different behaviour of these two enzymes has been discussed in relation to NADH binding (Lodola *et al.*, 1978*a*).

In the present paper we have used protein fluorescence to monitor the reactions, because it is very sensitive and enables measurements to be made at protein concentrations that are similar to dissociation constants (1μM). Equilibrium titrations were performed by the continuous addition of Na_2SO_3 in 50mM-sodium phosphate buffers adjusted to pH6.6 with 5M-NaOH. The data obtained in this way were plotted by using the equation:

$$\frac{K_{app.}}{(1-\bar{v})} = \frac{[L]_T}{\bar{v}} - [E]_T \text{ (Holbrook, 1972)}$$

where $\bar{v}$ = the degree of saturation of the total enzyme-binding sites, $[E]_T$, at a given total concentration of added ligand $[L]_T$, and $K_{app.}$ is the observed equilibrium constant. Plots of $1/(1-\bar{v})$ against $[L]_T/\bar{v}$ were linear, and the inverse of the slope of the

plot describes a single dissociation constant, $K_{app.}$, for complex-formation.

The measured dissociation constant ($K_{app.}$) for the binding of sulphite to E–NAD$^+$ (where E is the enzyme) became weaker as the concentration of NAD$^+$ present in each titration was decreased below the value required effectively to saturate the enzyme (Fig. 2). When these values of $K_{app.}$ were plotted against NAD$^+$ concentration, they gave a rectangular hyperbola corresponding to the relationship:

$$K_{app.} = K_{Na_2SO_3}\left(1 + \frac{K_{NAD+}}{[NAD^+]}\right)$$

The linearized plot of $K_{app.}$ against 1/[NAD$^+$] gives an intercept equal to $K_{Na_2SO_3}$, the sulphite-binding constant at pH6.6 at an infinite concentration of NAD$^+$ (which was $1.25 \pm 0.25\,\mu$M for LDH and $1.5 \pm 0.5\,\mu$M for MDH). The ratio of slope to intercept gives the dissociation constant for NAD$^+$ binding to the enzymes (K^+_{NAD}) and was found to be 0.18mM for LDH and 0.8mM for MDH, both in good agreement with the reported values obtained by different techniques (Stinson & Holbrook, 1973; Holbrook & Wolfe, 1972). The plot for LDH is shown in Fig. 2(*b*).

The true dissociation constant for sulphite from NAD$^+$ when bound to the enzyme is given from a consideration of the ionic equilibria responsible for binding. We have demonstrated above that only the SO_3^{2-} dianion adds to the 4-position of the nicotinamide ring, so the apparent dissociation constant will be greater than the pH-independent constant by the factor $1 + ([H^+]/K_{SF})$ (to correct for the actual concentration of dianion actually present in solution). However, a further factor involving the ionization of a group on the enzyme must also be taken into consideration.

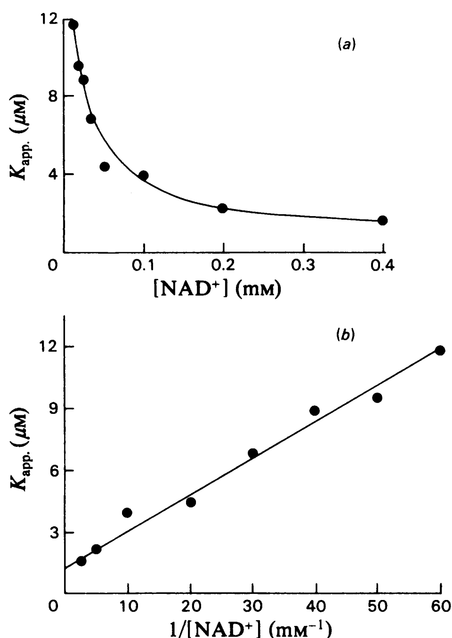


Fig. 2. *Equilibrium binding of sulphite to the complex of LDH and NAD$^+$*

(*a*) shows the variation in the apparent equilibrium dissociation constant for the addition of sulphite at pH6.6 to solutions of LDH (1μM) and NAD$^+$ (16–400μM). The formation of the complex LDH–NAD–SO_3^- was followed by the decrease in the intrinsic fluorescence of the enzyme and was transformed to the degree of saturation of the coenzyme-binding sites ($\bar{v}$) by using $\bar{v} = (1 - F^{\pm})/0.3$ (Holbrook, 1972). $K_{app.}$, the equilibrium binding constant, was obtained as the reciprocal slope of linear plots of $1/(1 - \bar{v})$ against $[Na_2SO_3]/\bar{v}$. (*b*) shows the plot of $K_{app.}$ against 1/[NAD$^+$] which was used to estimate the binding constant for sulphite in the presence of saturating NAD$^+$.

Role of histidine-195 in binding NAD–SO_3^- to LDH

LDH was 90% inhibited by the reaction of the essential histidine residue with a limited concentration of diethyl pyrocarbonate at pH6.6 exactly as described by Holbrook & Ingram (1973). When fresh inhibited enzyme (1μM) was titrated at pH6.6 with Na_2SO_3 in the presence of 0.1mM-NAD$^+$ we observed no formation of enzyme–NAD–SO_3^- other than the 0.1μM expected from the residual unchanged enzyme in the preparation. Since the chemical modification of histidine-195 considerably changes the ability of the enzyme to complex NAD–sulphite, we decided to investigate whether the ionization of histidine-195 (pK = 6.7; Holbrook & Ingram, 1973) was also reflected in the stability of the enzyme–NAD–sulphite complex.

Effect of pH on the stability of the NAD–SO_3^- complexes of LDH and MDH

When either dehydrogenase, NAD$^+$ and Na_2SO_3 are mixed together in a solution of low buffer capacity and the pH of this mixture is varied over the range pH5.5–10, a bell-shaped curve of protein fluorescence as a function of pH is produced, with an optimum saturation of NAD$^+$ and sulphite at pH6.55 (pH$_{opt.}$). An example of such a curve for dogfish M_4 LDH is shown in Fig. 3. Once again, for LDH, the non-linear geometric quench correction must be used to compute the degree of saturation

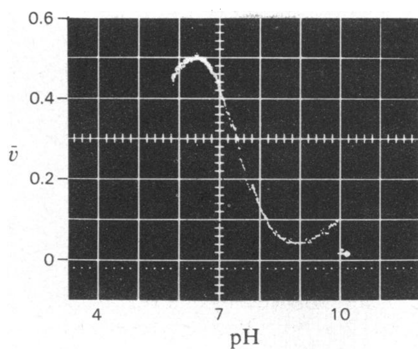


Fig. 3. *pH-dependence of the stability of the ternary complex LDH–NAD–SO_3^-*
The pH of a solution containing 1 μM-dogfish M_4 LDH, 0.1 mM-NAD^+ and 20 μM-Na_2SO_3 was varied from pH 5.8 to 10 while the intrinsic fluorescence of the protein was continuously measured. The degree of saturation of the coenzyme-binding sites was calculated from the observed fluorescence as described in the text and was displayed on an oscilloscope as a plot of $\bar{v}$ against pH. Such curves were characterized by SATMAX (maximum value of $\bar{v}$), $pH_{opt.}$ (the pH at SATMAX) and HALF (the pH span between the two values when $\bar{v}$ = SATMAX/2, actually obtained as twice the difference between $pH_{opt.}$ and the high pH when $\bar{v}$ = SATMAX/2).

from the quenching of protein fluorescence as the ternary complex is formed. For MDH, $pH_{opt.} = 6.6$.

An unusual feature of these curves with both LDH and MDH is that as the pH is raised and more sulphite dianion becomes available, less ternary complex is formed. This effect is not due to the weakening of NAD^+ binding to the enzymes, as this is known to be approximately independent of pH for both enzymes over this pH range [0.18 mM for LDH (Stinson & Holbrook, 1973) and 0.6 mM for MDH (Holbrook & Wolfe, 1972)]. There must therefore be another ionization at or near the enzyme active centre whose deprotonation decreases the stability of the E–NAD–SO_3^- complex. With two independent ionizations (pK_1 and pK_2) the unknown pK can be discovered by the general formula $pH_{opt.} = (pK_1 + pK_2)/2$. Thus if we substitute $pK_1 = pK_{SF} = 6.9$ and $pH_{opt.} = 6.55$, $pK_2 = pK_E$ (pK of enzyme group) = 6.2. From this result and a consideration of the result with the ethoxycarbonylated enzyme, we are led to the conclusion that the group responsible for stabilizing this ternary complex in LDH is protonated histidine-195, the group previously identified by Woenckhaus *et al.* (1969) as being in the active centre of the molecule and is the group that is protonated in the enzyme–NADH–oxamate complex (Holbrook & Stinson, 1973).

Electronic absorption spectra of NAD–SO_3^- and enzyme–NAD–SO_3^-

The protonated stable ternary complex could be either (I) $E^{BH^+}_{NAD^+\cdot SO_3^{2-}}$ or (II) $E^{BH^+}_{NAD\text{-}SO_3^-}$ in which the charges, summed over the base, B (histidine-195 in LDH), the nicotinamide ring and the sulphite are compensatory. In interpreting our results we will imply that the structure of NAD–SO_3^- is that shown as (II) above, whether it is free in solution or bound to the enzyme. We support this implication by the observation that the shape of the electronic absorption spectrum (300–400 nm) of 78 μM-enzyme–NAD–sulphite is identical at both pH 6 and 9 and is identical with that of 78 μM-free NAD–sulphite at pH 9. Protonation of the sulphur oxygen atom would, if it occurred, change the electron distribution on the pyridine ring and thus change the absorption spectrum. Thus there is no appreciable change in structure of the enzyme-bound adduct with pH. Compound (I) would have very little long-wave u.v. absorption and is not likely to be present in measurable proportion.

Activation of NAD^+ for reaction with sulphite by LDH and MDH

To interpret correctly the magnitude of the measured dissociation constant ($K_{app.}$) of sulphite from NAD^+ bound to either dehydrogenase, it is necessary to correct for (i) the proportion of the total sulphite that is present as SO_3^{2-} and (ii) the proportion of the total enzyme–NAD^+ complex in which the essential histidine is protonated. We define K_{SULF} as the pH-independent dissociation constant for the sulphite dianion dissociation from enzyme–NAD–sulphite to give the protonated enzyme–NAD^+ compound. This constant is related to $K_{app.}$ by eqn. (2):

$$K_{app.} = K_{SULF}\left(1 + \frac{K_E}{[H^+]}\right)\cdot\left(1 + \frac{K_{NAD^+}}{[NAD^+]}\right)\cdot\left(1 + \frac{[H^+]}{K_{SF}}\right) \quad (2)$$

Knowing (the values are for LDH) pK_E (6.2), pK_{SF} (6.9), K_{NAD^+} (0.18 mM) and $[NAD^+]$ in any experiment it is then possible to calculate K_{SULF} from $K_{app.}$ at pH 6.6: the value for MDH is 0.1 μM and the value for LDH is 0.09 μM. We contrast these dissociation constants for SO_3^{2-} with the protonated enzyme–NAD^+ (both about 0.1 μM) with the dissociation constant for SO_3^{2-} in the non-enzymic reaction (15 ± 5 mM) and conclude that the binding of NAD^+ to both LDH and MDH activates the nicotinamide ring for attack by sulphite dianion by a factor of 1×10^5–2×10^5.

To reinforce this result a program was compiled

and run on a PDP-11/45 computer which simulated on a cathode-ray tube a plot of fractional saturation of an enzyme with NAD–sulphite ($\bar{v}$) as a function of pH according to:

$$\bar{v} = \frac{[\text{sulphite}]_T/K_{SULF}}{\left(1+\frac{K_E}{[H^+]}\right)\cdot\left(1+\frac{K_{NAD^+}}{[NAD^+]}\right)\cdot\left(1+\frac{[H^+]}{K_{SF}}\right)+\frac{[\text{sulphite}]_T}{K_{SULF}}} \quad (3)$$

The values of all six variables other than $\bar{v}$ and H^+ could be altered by touching and rotating labelled potentiometers and it was then simple to examine visually their effect on the shape of the $\bar{v}$/pH plot. Each plot was characterized by $pH_{opt.}$ (the pH at which $\bar{v}$ was at its maximum), SATMAX (the maximum value of $\bar{v}$) and HALF (the difference between the two pH values when $\bar{v}$ = SATMAX/2). $pH_{opt.}$ was found to depend only on pK_E and pK_{SF} [as $pH_{opt.} = (pK_E + pK_{SF})/2$] and was quite independent of the values given to the other variables. It was possible to exactly match the experimental records by using (for LDH) $pK_E = 6.2$, $pK_{SF} = 6.9$, $K_{NAD^+} = 0.18$ mM, the values of $[\text{sulphite}]_T$ and $[NAD^+]$ used in a titration by choosing the unknown, $K_{SULF} = 0.1\,\mu$M. As a second quantitative criterion that the model matched the experiments we compared the simulated and measured values of the half-widths of the bell-shaped curves. For the example given above, the values were 2.9 and 3 respectively. We observed similar good agreement for MDH [e.g. at 0.05mM-NAD^+ and 0.17mM-Na_2SO_3 the experimental and simulated half-widths were both 2.6 (taking $pK_E = 6.3$)].

Although in the chloride buffer $pH_{opt.}$ was 6.6 ± 0.2, we observed that in a buffer in which 20mM-Na_2SO_4 replaced 20mM-NaCl the values of $pH_{opt.}$ were marginally higher (6.9 ± 0.3). A possible explanation for this variation may be the crystallographic finding that the crystalline apoenzyme at low pH contains a sulphate ion at the active centre close to histidine-195 (Adams *et al.*, 1973). Simple statements, such as 'the pK of the histidine-195 in the apoenzyme is 6.7' (Holbrook & Ingram, 1973) should ideally therefore be qualified to include the nature of the potential counterion.

The observation that MDH, like LDH, has a group with $pK = 6.3$ which is protonated in stable ternary complexes with NAD^+ and SO_3^{2-} would certainly be consistent with the idea that MDH contains a histidine residue at its active centre which fulfils the same function as histidine-195 in LDH. We know that MDH can be inhibited by reaction of a single histidine residue, but must await the complete primary amino acid sequence before the correspondence between the structures and mechanisms of these two enzymes may be considered to be fully established.

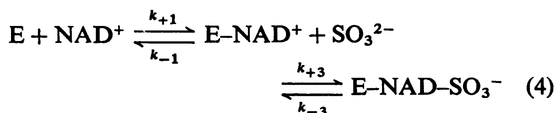
LDH–NAD^+–oxalate: pH titrations

In the sulphite pH titrations we noted that there was a very stable complex formed when the two positive charges (enzyme histidine and the pyridine ring of NAD^+) were balanced by the two negative charges of SO_3^{2-}. We thus decided to evaluate whether another stable ternary complex exhibited the same charge balance, and chose a ligand (oxalate) that was not subject to a changed ionization in the interesting pH range. The dianion of oxalate had previously been used as a competitive inhibitor of lactate, as it forms a catalytically inactive (pH > 7) complex with NAD^+ (Novoa & Schwert, 1961). We decreased the pH of solutions of LDH ($2\,\mu$M) and NAD^+ ($10\,\mu$M) with and without oxalate ($10\,\mu$M) from pH9.5 to 5. Both the complete mixture and the blank showed a decrease in protein fluorescence from 1.0 to 0.8 with decreasing pH. In the blank the change was half complete at pH5.7; in the presence of oxalate the change was half complete at pH6.4. When the fluorescence of the experimental solution was divided by that of the blank the result was a sigmoidal decrease in protein fluorescence (from 1.0 to 0.8) with $pK_{app.} = 7.1 \pm 0.5$. Because the protein fluorescence change on formation of the complex is so small it is unlikely that we can improve the precision of the estimate of the pK. However, the result is sufficiently precise to demonstrate that the formation of the complex depends on the protonation of an enzyme group with a pK in the same range as was observed for the NAD–sulphite complex (above) and for the complex of LDH–NADH–oxamate (Holbrook & Stinson, 1973).

Rate of reaction of NAD^+ with LDH and MDH

It has previously been impossible to obtain direct measurements of NAD^+ association to these enzymes, because there is no useful optical change when the complexes form and because the reactions are too fast to study at NAD^+ concentrations where a reaction with an equilibrium constant of 0.2mM is appreciably in favour of the complex. These limitations can be overcome if the bound NAD^+ can be trapped by a second reaction which does give an optical change (e.g. pyrazole for horse alcohol dehydrogenase and NAD^+; Shore & Gilleland, 1970). The method that we have used to determine these association rate constants depends on the use of sulphite dianion as a trapping reagent and the

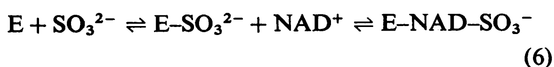
consequential protein-fluorescence quenching as a monitor of NAD^+ binding.



$$E + NAD^+ \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E\text{–}NAD^+ + SO_3^{2-} \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} E\text{–}NAD\text{–}SO_3^- \quad (4)$$

For the trapping technique to be valid, it is important that the value of $k_{+3} \cdot [SO_3^{2-}]$ is much greater than $k_{+1} \cdot [NAD^+]$ and k_{-1}. The value of k_{-3} will be insignificant, as sulphite binding is very tight. Also it is necessary to examine two potential competing reactions

$$NAD^+ + SO_3^{2-} \rightleftharpoons NAD\text{–}SO_3^- + E \rightleftharpoons E\text{–}NAD\text{–}SO_3^- \quad (5)$$



$$E + SO_3^{2-} \rightleftharpoons E\text{–}SO_3^{2-} + NAD^+ \rightleftharpoons E\text{–}NAD\text{–}SO_3^- \quad (6)$$

Non-enzymic rate of formation of $NAD\text{–}SO_3^-$ addition product. The addition rate of sulphite dianion to NAD^+ was measured by the observation of the increase in A_{327} produced when solutions of 50μM-NAD^+ were rapidly mixed with various concentrations of Na_2SO_3 (10–180mM) at pH8.0 in the stopped-flow spectrophotometer. Pseudo-first-order conditions were used. The results were stored in a transient recorder and analysed by an analogue computer which enabled a plot of the logarithm of the data to be displayed on an oscilloscope and from which the individual first-order rate constants can be read. A plot of the apparent first-order rate constants against sulphite concentration was linear. The slope and intercept allowed the second-order 'on'-constant and the 'off'-constant for the reaction to be calculated. In close agreement with Johnson & Smith (1976), we found $k_{+2} = 2.4 \times 10^3 M^{-1} \cdot s^{-1}$, $k_{-2} = 35 s^{-1}$. The kinetically determined dissociation constant for the reaction k_{-2}/k_{+2} is 14mM.

Association rate of sulphite to $E \cdot NAD^+$ binary complexes. We observed a decrease in the protein fluorescence after rapidly mixing sulphite with the binary complexes of MDH and LDH with NAD^+ at pH8.0 and 7.2 respectively in a stopped-flow fluorimeter. For LDH, the curve was first corrected for non-linear quenching (Holbrook, 1972) and then for both enzymes the apparent pseudo-first-order rate constants for the change were measured. Routinely, a mixture of 2μM-enzyme and saturating NAD^+ was mixed with various concentrations of Na_2SO_3 in 50mM-sodium phosphate buffer. Plots of the apparent pseudo-first-order rate constants against [sulphite] were linear and, making no allowance for the concentration of dissociated SO_3^{2-} ions present, nor the effect of the enzyme ionization, gave an apparent second-order rate constant of $1 \times 10^6 M^{-1} \cdot s^{-1}$ for LDH at pH7.2 and $1 \times 10^5 M^{-1} \cdot s^{-1}$ for MDH at pH8.0. The pH-independent second-order rate constant for the above reaction, calculated as

$$k_3 = k_{app.}\left(1 + \frac{K_E}{[H^+]}\right)\left(1 + \frac{[H^+]}{K_{SF}}\right)$$

was $1.65 \times 10^7 M^{-1} \cdot s^{-1}$ for LDH and $7 \times 10^6 M^{-1} \cdot s^{-1}$ for MDH. Using the equilibrium constants (K_{SULF}) and k_{+3} it may be calculated that k_{-3} is $1.5 s^{-1}$ (LDH) and $0.7 s^{-1}$ (MDH).

Rate of association of NAD^+ with LDH and with MDH. Having measured the rate at which sulphite would add to enzyme-bound NAD^+ it was possible to design experiments in which $k_{+3} \cdot [SO_3^{2-}]$ was much greater than $k_{+1} \cdot [NAD^+]$, k_{-1} or k_{-3} (eqn. 4). This was achieved by mixing enzyme (2μM) with Na_2SO_3 (20mM for LDH, 250mM for MDH) in one syringe and various concentrations of NAD^+ in the second syringe of a stopped-flow device. The calculated rate constant at which enzyme-bound NAD^+ would be trapped by reaction with sulphite was $15000 s^{-1}$ for LDH (at pH6.6) and $25000 s^{-1}$ for MDH (at pH8). Thus the rate at which the reactions of eqn. (4) reach equilibrium (all as $E\text{–}NAD\text{–}SO_3^-$) is limited by $k_{+1} \cdot [NAD^+]$. The progress of the reaction was followed from the decrease in the intrinsic protein fluorescence as the ternary complex formed. The apparent first-order rate constant for the increase in the concentration of the ternary complex was measured at each NAD^+ concentration. A single example for LDH is shown in Fig. 4(*a*) and the plot of $k_{app.}$ against $[NAD^+]$ is shown as Fig. 4(*b*) (the extent of formation of ternary complex for LDH was obtained after the degree of saturation had been calculated from the non-linear protein fluorescence change as described by Holbrook, 1972). The slope of a plot such as that in Fig. 4(*b*) describes k_{+1}, the 'on' rate for NAD^+, and was $8.8 \times 10^6 M^{-1} \cdot s^{-1}$ for LDH at pH6.6 and $4 \times 10^6 M^{-1} \cdot s^{-1}$ for MDH at pH8. The plots almost pass through the origin, as would be expected from the relatively small values of k_{-3} in comparison with $k_{+1} \cdot [NAD^+]$. We were careful to avoid contaminating the solutions with lactate from the skin, although the extent of production of NADH when the LDH was mixed with NAD^+ in the absence of Na_2SO_3 was never more than −5% change in protein fluorescence in 5s.

Consideration of alternative routes for ternary-complex formation. Only if the pathways shown as eqns. (5) and (6) are not followed can we ascribe the rates in the last section to the bimolecular association of the enzymes with NAD^+. Were eqn. (5) followed, then, as there is no $NAD\text{–}SO_3^-$ present immediately after mixing, one should observe a lag phase before the rate of formation of enzyme-bound $NAD\text{–}SO_3^-$ reached a steady-state rate.

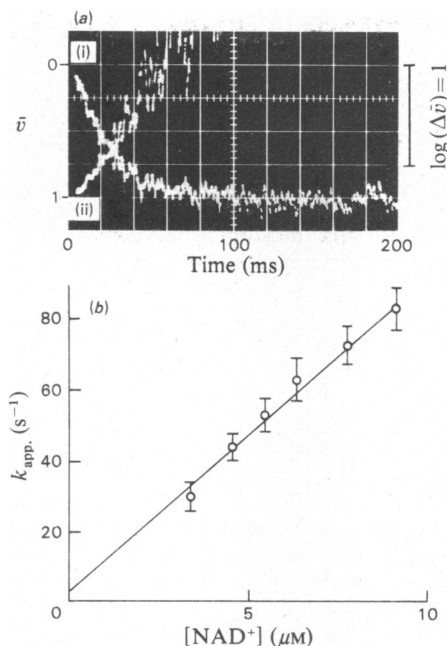


Fig. 4. *Stopped-flow measurements of the rate of NAD$^+$ binding to LDH*

(*a*) (i) Rate of increase (note the inverted scale) in the proportion of the NAD–sulphite-binding sites of LDH that are occupied ($\bar{v}$) after mixing equal volumes of two solutions, one containing 2μM-LDH and 20mM-Na_2SO_3 and the other containing 9.2μM-NAD$^+$. The values of $\bar{v}$ were obtained from the decrease in protein fluorescence during the reaction as described in the text. (*a*) (ii) Linear plot of $\log(\Delta\bar{v})$, from which the apparent first-order rate constant for the change was read as $44 s^{-1}$. (*b*) Dependence of the apparent first-order rate constant for the binding of NAD$^+$ to pig LDH on [NAD$^+$]. The slope of the plot was used to obtain $k_{+1} = 8.8 \times 10^6 M^{-1} \cdot s^{-1}$.

Careful examination of many curves similar to Fig. 4(*a*) failed to reveal a lag phase.

We have not been able to distinguish the reactions shown by eqn. (4) from those shown by eqn. (6), that is to distinguish between the bimolecular reaction of NAD$^+$ with free enzyme and with a putative enzyme–sulphite complex. Both equations, of course, represent the 'on' constant for NAD$^+$. There are indications that eqn. (6) should be given consideration. There is crystallographic evidence that LDH will form weak complexes with a number of anions (Adams *et al.*, 1973), and the enzyme complex with oxalate had been studied in some detail (Kolb & Weber, 1975). Protection experiments have shown that the apoenzyme will interact with Na_2SO_3 (Rajewsky, 1966; Pfleiderer *et al.*, 1968) at pH 8, although it is not known whether the sulphite binds at the active centre or is simply a general counterion for positively charged protein groups.

The ability to follow the rate of association of NAD$^+$ with the two dehydrogenases has shown that all the subunits in each oligomer appear to be equivalent towards NAD$^+$. This is not unexpected from the presently known properties of LDH (Holbrook *et al.*, 1975) and it is consistent with the report from Banaszak's group (Weininger *et al.*, 1977) that the differential occupancy of the two NAD$^+$-binding sites in the crystals of the enzyme MDH is due to intermolecular contacts which are present in the crystal but which would be absent from the enzyme in solution.

From the association rate constants and from the known equilibrium dissociation constants for NAD$^+$ from the enzymes it is possible to calculate the rate constants (k_{-1}) for the dissociation of NAD$^+$ from the enzyme–NAD$^+$ complexes. The value $1400 s^{-1}$, for pig heart LDH, is satisfactorily much higher than $V_{max.}$ ($200 s^{-1}$) in the reverse reaction, as would be expected from the observation that the steady-state complex of the enzyme in that reaction is an enzyme–NADH–pyruvate compound (Stinson & Holbrook, 1973; Holbrook & Stinson, 1973; Whittaker *et al.*, 1974).

The value of k_{-1} for MDH (from $k_{+1} = 4 \times 10^6 M^{-1} \cdot s^{-1}$ and $K_{E \cdot NAD} = 0.6 M$; Holbrook & Wolfe, 1972) is $2400 s^{-1}$ and $V_{max.}$ for the cytosol enzyme in the reverse (oxaloacetate reduction) direction is $500 s^{-1}$. Those results are clearly not consistent with the conclusion of Frieden & Fernandez-Souza (1975) that $V_{max.}$ represents the rate of dissociation of the enzyme–NAD$^+$ complex. This disagreement is not serious, since the NAD$^+$ 'off' rate is not obtained directly in the experiments of Frieden & Fernandez-Souza (1975) but is the result of computations many steps removed from the original kinetic constants. The fact that the NAD$^+$ 'off' rate is much higher than $V_{max.}$ is quite consistent with the direct measurements of the protein fluorescence of the steady-state enzyme complex by Lodola *et al.* (1978*c*), which show that the steady-state complex of cytosolic MDH in the reverse direction has quenched protein fluorescence and is either a binary complex with NADH or a ternary complex with NADH and oxaloacetate.

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