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Equilibrium Binding of Nicotinamide Nucleotides to Lactate Dehydrogenases

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1. No discontinuities were observed during the continuous titration with NADH of the lactate dehydrogenases of ox muscle, pig heart, pig muscle, rabbit muscle, dogfish muscle or lobster tail muscle. The binding was monitored by either the enhanced fluorescence of bound NADH or the quenched fluorescence of the protein. A single macroscopic dissociation constant, independent of protein concentration, could be used to describe the binding to each enzyme, and there was no need to postulate the involvement of molecular relaxation effects. 2. The affinity for NADH decreases only threefold between pH6 and 8.5. Above pH9 the affinity decreases more rapidly with increasing pH and is consistent with a group of about pK 9.5 facilitating binding. Muscle enzymes bind NADH more weakly than does the pig heart enzyme. 3. Increasing temperature and increasing concentrations of ethanol both weaken NADH binding. 4. NADH binding is weakened by increasing ionic strength. NaCl is more effective than similar ionic strengths derived from sodium phosphate or sodium pyrophosphate. 5. Commercial NAD^+ quenches the protein fluorescence of the heart and muscle isoenzymes. Highly purified NAD^+ does not, and its binding was monitored by competition for the NADH-binding sites. A single macroscopic dissociation constant is sufficient to describe NAD^+ binding at the concentrations tested. The dissociation constant is about 0.3mM and is not sensitive to changed ionic strength and to changed pH in the range pH6–8.5.

A satisfactory theory for the mechanism of action of lactate dehydrogenases should explain the observed pH-dependencies of reaction rates and binding constants in terms of the amino acid side chains at the active centre of the enzyme. Such a theory could be based on information obtained from structural, kinetic and equilibrium investigations. The present paper reports the affinity of the enzyme for pure NADH and NAD^+ over a wide range of conditions of pH, temperature and organic solvent composition. The investigation was originally undertaken to examine the report (Anderson & Weber, 1965) that NADH binding to ox muscle lactate dehydrogenase could not be satisfactorily described by a single macroscopic dissociation constant without postulating molecular relaxation effects in an enzyme that is composed of four chemically identical subunits.

The binding of NADH to lactate dehydrogenase is easily followed either by the increase in the fluorescence of the bound NADH or by the decrease in the protein fluorescence of the enzyme (Velick, 1958). In interpreting such fluorescence changes it is necessary to be able to relate the change in fluorescence to the fraction of binding sites occupied by NADH. These relationships have been recently re-examined, and although a linear relationship was confirmed between the increased fluorescence of NADH and the concentration of bound NADH, the decreased fluorescence of the protein was found to be non-linearly related to the concentration of bound NADH. However, an equation based on a concept of geometric quenching, $F^{1/n} = 1-\alpha(1-x)$, can be used to calculate the fractional saturation of the NADH-binding sites (α) from the measured protein fluorescence (Holbrook, 1972; Holbrook *et al.*, 1972). A continuously titrating fluorimeter was used in these studies. This method is particularly suitable to detect anomalous behaviour in ligand titration curves. Cahn *et al.* (1962) designated the isoenzymes of lactate dehydrogenase which predominate in heart and skeletal muscle respectively as H and M types. These two extreme isoenzymes will be referred to here as the heart and muscle enzymes respectively.

Experimental

Enzymes

Pig heart lactate dehydrogenase was obtained from Whatman Biochemicals, Maidstone, Kent, U.K., with a specific enzyme activity of 360 units/mg assayed in 0.15mM-NADH, 0.3mM-sodium pyruvate in 67mM-sodium phosphate buffer, pH7.2 at 25°C, and followed

as $-\Delta E_{340}$. The muscle isoenzymes were assayed similarly but with 1mM-pyruvate. Pig and rabbit muscle enzymes were prepared as described by Stinson & Gutfreund (1971) for the pig enzyme. Dogfish muscle enzyme was a gift from Professor M. G. Rossmann (Purdue University, Ind., U.S.A.). Ox muscle enzyme was from Boehringer Corp. (London) Ltd., London W.5, U.K. The muscle enzyme from lobster tails was partially purified by repeated fractionation with $(NH_4)_2SO_4$ and adsorption on DEAE-cellulose (J. J. Holbrook, unpublished work). Protein concentrations were determined from measurements of extinction at 280nm by assuming that a 1mg/ml solution of the heart enzyme had extinction 1.39 and 1mg/ml solutions of the muscle enzymes had extinction 1.29 (Jecsai, 1962). All enzyme concentrations are expressed as the concentrations of the subunits, by assuming a subunit molecular weight of 36000 for all enzymes. The specific enzyme activities of the various forms used were: pig heart, 360 units/mg; rabbit muscle, 630 units/mg; pig muscle, 610 units/mg; dogfish muscle, 437 units/mg; ox muscle, 382 units/mg; lobster tail muscle, 150 units/mg. Enzymes were prepared for the titrations either by dialysis against an appropriate dilute buffer solution or by gel filtration on Sephadex G-25. No low-molecular-weight u.v.-absorbing material was detected when the pig muscle enzyme was filtered through a column of Sephadex G-25 in 6M-urea. A small peak of u.v.-absorbing material was displaced from the pig heart enzyme by urea and corresponded to 5% of the concentration of the NADH-binding sites (calculated by assuming the same extinction coefficient at 260nm as for AMP).

Substrates and nucleotides

Sodium pyruvate, NADH and NAD^+ were from Boehringer Corp. (London) Ltd. NADH was purified by adsorbing a solution of 200mg of the crude material in water (5ml) on a column (10cm×16cm²) of DEAE-cellulose bicarbonate (Whatman, microgranular type DE-52), which had been well washed with water. Nucleotides were eluted by applying a linear gradient of water to 1M-NH_4HCO_3 in a total volume of 400ml. NADH was eluted as part of the major peak of u.v. absorption at between 0.7 and 0.9M-NH_4HCO_3. The E_{340}/E_{260} ratio was measured through the peak. A few fractions in the latter half of this peak had the high E_{340}/E_{260} ratio of 0.41 expected for NADH and were used for titrations within 12h.

NAD^+ contains dehydrogenase inhibitors (Babson & Arndt, 1970; Dalziel, 1963). In preliminary experiments it was found that NAD^+ appeared to quench 30% of the protein fluorescence of the pig heart enzyme and that some samples even fluoresced at 450nm like NADH. The NAD^+ was purified by eluting 0.5g of the sodium salt adsorbed to a column (15cm×20cm²) of Dowex 1 (Cl^- form) with 15mM-HCl. The NAD^+ was eluted as the acid emerged from the column, and was used in binding experiments after being neutralized with Na_2CO_3, care being taken to avoid local high concentrations of alkali. Attempts were made to freeze-dry the column eluate. However, the chances of obtaining pure NAD^+ were low, since any slight thaw yielded a yellow fluorescent impurity, which was thought to be responsible for quenching the protein fluorescence by the unpurified coenzyme. The operational criterion of purity adopted was that a 5mM solution of the coenzyme did not quench the fluorescence of lactate dehydrogenase by more than 10% when this was excited at 305nm. NAD^+ concentrations were determined from the increase in extinction at 340nm after conversion into NADH in 0.5M-ethanol in 0.1M-glycine buffer, adjusted to pH10 with 5M-NaOH containing a trace of alcohol dehydrogenase.

Detection of NADH binding by enhanced NADH fluorescence

A split-beam differential fluorimeter was used. The machine records the difference in fluorescence (ΔF) between NADH added at a constant rate ($5\mu l \cdot min^{-1}$) to two cuvettes, the one containing the enzyme and the other an equal volume (3ml) of the appropriate buffer at 20°C. The results were corrected for the changed absorption of the exciting radiation (320nm) by the added NADH and for a slight dilution (Holbrook, 1972). The fractional saturation (α) of the total concentration of the NADH-binding sites ($[E]_0$) in the solution was equated to the ratio $\Delta F/\Delta F_{max.}$, where $\Delta F_{max.}$ is the limiting value of the fluorescence difference (ΔF) approached at high concentrations of added NADH. The concentration of bound ligand ([EL]) is related to the concentration of unoccupied ligand-binding sites ([E]) and the concentration of free ligand ([L]) by the law of mass action and a dissociation constant ($K_{E,L}$). If the relations $[EL] = \alpha[E]_0$ and $[L] = [L]_0 - \alpha[E]_0$ are used, it may be readily shown that the fractional saturation of ligand-binding sites is related to the total concentration of ligand added ($[L]_0$) by eqn. (1):

$$\frac{K_{E,L}}{(1-\alpha)} = \frac{[L]_0}{\alpha} - [E]_0 \qquad (1)$$

Graphs based on this equation of $1/(1-\alpha)$ against $[L]_0/\alpha$ will be straight lines and have slope of $1/K_{E,L}$ and an intercept of $[L]_0/\alpha = [E_0]$ when $1/(1-\alpha) = 0$. Use of this equation in experiments when $[E_0]$ is chosen to be about equal to $K_{E,L}$ enables a demonstration that a whole titration curve is consistent with the expected concentration of binding sites and a unique dissociation constant. Use of this approach first showed that it was an impurity in the commercial

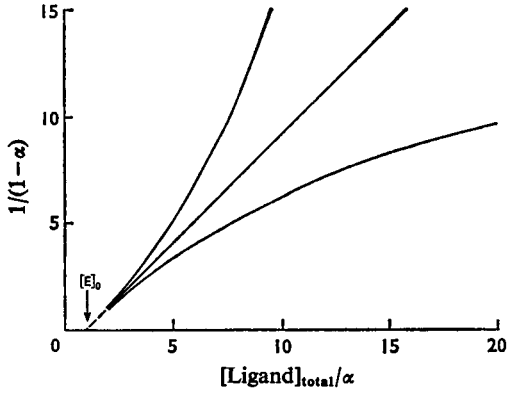

Fig. 1. *Use of a plot of* $1(1-\alpha)$ *against* $[ligand]_{total}/\alpha$ *to determine both the concentration of ligand-binding sites* ($[E]_0$) *in solution and their dissociation constant* (K)

The plot is based on the equation:

$$K/(1-\alpha) = [\text{ligand}]_{total}/\alpha - [E]_0$$

derived from the law of mass action. The slope of the graph is $1/K$ and the intercept with the axis is the value of $[E]_0$. The two curves shown simulate the result of errors in determining the limiting value to which the fractional saturation (α) approaches at infinite ligand concentration. The upper curve results from the end point being 5% too low and the lower curve from the end point being 5% too high.

NAD^+ that was responsible for quenching protein fluorescence, since the value of $[E]_0$ from this plot was 20 times the expected subunit concentration of the enzyme used.

The value of $\Delta F_{max.}$ was chosen by inspection of titration curves. It could easily be refined, since if the initial estimate was either 5% too low or too high the plot of $1/(1-\alpha)$ against $[L]_0/\alpha$ rapidly deviated from a straight line (Fig. 1). The value of $\Delta F_{max.}$ finally chosen was always consistent with the original recorder trace. To facilitate comparison with Anderson & Weber (1965), the results were sometimes plotted according to Brown & Hill (1922–23) as a graph of $\log[\alpha/(1-\alpha)]$ against $\log[L]$. [L] was calculated as $[L]_0 - \alpha[E]_0$ at each point.

Detection of NADH binding by quenched protein fluorescence

When NADH binds to lactate dehydrogenase from pig heart the fluorescence of the protein is decreased to 14% of its value in the absence of NADH. This method of monitoring ligand binding is easier and intrinsically more sensitive than observing the enhancement of NADH fluorescence, and was used as a routine to measure the affinity of the enzyme for NADH under varied conditions of solvent, temperature and competing ligand. The fractional saturation was obtained directly from the measured protein fluorescence (F) by using eqn. (2):

$$\alpha = (1-F^{1/n})/(1-x) \qquad (2)$$

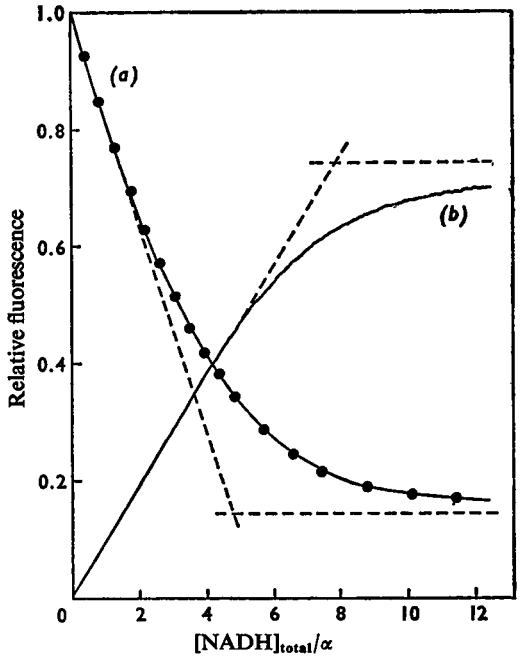

Fig. 2. *Non-linear quenching in protein fluorescence as NADH is bound to pig heart lactate dehydrogenase*

In two separate experiments (*a*) the protein fluorescence and (*b*) the NADH fluorescence was monitored as NADH (0.52mM) was continuously added to the enzyme (7.8μM). The affinity of the enzyme for the NADH was made high by choosing a low temperature (10°C), a low salt concentration (sodium pyrophosphate, I 0.03M) and pH 6.0. The curves are corrected for absorption of the incident radiation and for reabsorption of the protein fluorescence. The points superimposed on the protein-fluorescence trace are the values of the protein fluorescence calculated from the NADH fluorescence assuming the equation for geometric quenching, $F^{1/n} = 1-\alpha(1-x)$, with values of n (4) and x (0.615) determined as described by Holbrook (1972). ---- are tangents to the initial portions of the curves and the end points. These lines intercept at 4.8 and 7.9μM. The ratio of these values is 0.608 and is close to the ratio of 0.61 predicted from $(1-x^4)/4(1-x)$ with $x = 0.615$.

and values of $n = 4$ and $x = 0.615$. These constants were obtained as described by Holbrook (1972) by using an unrestrained curve-fitting procedure with the experimental results shown in Fig. 2. The best fit of these results to the equation $F^{1/n} = 1-\alpha(1-x)$ was at $n = 3.8$ and $x = 0.597$, in fair agreement with the known tetrameric structure of the enzyme. For the muscle enzyme α was obtained from eqn. (2) by using $n = 4.0$ and $x = 0.61$. Having obtained values of α at known total NADH concentrations, the dissociation constant for NADH and the concentration of NADH-binding sites in the solution was obtained from plots of $1/(1-\alpha)$ against $[NADH]_{total}/\alpha$. For protein fluorescence, the exciting wavelength was 305nm and the fluorescence was selected by a Kodak Wratten filter 18A. The correction for dilution during the titration and for the increased absorption of exciting and emitted light by the added NADH was made exactly as described by Holbrook (1972).

Competition between NAD^+ and NADH

Since purified NAD^+ did not either quench protein fluorescence nor fluoresce like NADH it is possible to detect NAD^+ binding by observing the effect of the presence of NAD^+ on the apparent affinity of the enzyme for NADH without having to introduce corrections for any signal contributed by the NAD^+ itself. Assume that enzyme is mixed with a concentration of NAD^+ that is much larger than the enzyme concentration. If α is the fractional saturation of the enzyme with NADH, monitored as described above, and if K_{E,NAD^+} and $K_{E,NADH}$ are the dissociation constants of NAD^+ and NADH to the same site on the enzyme, then the change in the fractional saturation (α) as NADH is added to the solution will be described by eqn. (3):

$$\frac{K_{E,NADH}\left(1+\frac{[NAD^+]}{K_{E,NAD^+}}\right)}{(1-\alpha)} = \frac{[NADH]_0}{\alpha} - [E_0] \quad (3)$$

This equation has exactly the same form as eqn. (1) except that the dissociation constant for NADH will be apparently greater. The apparent dissociation constant ($K_{app.}$) will be given by eqn. (4):

$$K_{app.} = K_{E,NADH}(1+[NAD^+]/K_{E,NAD^+}) \quad (4)$$

and a graph of $K_{app.}$ against $[NAD^+]$ will be a straight line of slope $K_{E,NADH}/K_{E,NAD^+}$. Thus K_{E,NAD^+} could be obtained if $K_{E,NADH}$ was known.

In a confirmatory experiment NAD^+ binding was monitored in 67mM-sodium phosphate buffer, pH7.2, at room temperature, by the gel-filtration method of Hummel & Dryer (1962). Pig heart enzyme samples (3.67mg) were filtered through a column (30cm × 0.8cm²) of Sephadex G-50 equilibrated with buffer containing 0.115, 0.28 or 0.575mM-NAD^+. The concentration of NAD^+ in the eluate from the column was continuously recorded at 260nm. The amount of NAD^+ bound was obtained from the area of the trough in the E_{260} trace, which appeared after the protein peak. The flow rate was constant at about $30ml \cdot min^{-1}$, and the troughs were calibrated by measuring the areas of troughs obtained when 0.2, 0.5 and 1ml of water were added to the column. A plot of the reciprocal of the amount of NAD^+ bound against the reciprocal of the free NAD^+ concentration was linear. The maximum NAD^+ binding was 0.96mol/mol of subunit and the dissociation constant was 0.19mM.

Results and Discussion

The use of the continuous-titration method to obtain binding constants has some advantages. The stirring keeps dust particles continually suspended and also ensures good thermal equilibration. This results in increased accuracy, since the fluorescence of free NADH is very sensitive to temperature. Increased precision results from the dynamic subtraction of the signals obtained from two solutions in identical environments. Since the method is rapid, any apparent deviation from a simple binding curve can be easily checked, and although some titrations were apparently anomalous, none of the anomalies could be reproduced if highly purified nucleotides and enzymes were used.

One of the reasons for this investigation was to examine whether the four NADH-binding sites on each lactate dehydrogenase molecule could be represented by a single macroscopic dissociation constant. Binding was studied by using the enhancement of NADH fluorescence, since there is little doubt that the degree to which NADH fluorescence is perturbed on binding is independent of the average number of NADH molecules bound to each enzyme molecule. Anderson & Weber (1965) justified this assumption on the grounds that there was an isoemissive wavelength. For the complex of the enzyme with NADH and oxamate a direct proof has been given (Holbrook, 1972). For the formation of the binary complex with NADH a direct proof is more difficult to furnish. By choosing conditions of low salt concentration, low temperature and pH6 the affinity of pig heart enzyme for NADH is greatest. Under the conditions of Fig. 2, where the concentration of binding sites (7.8μM) is over 30 times the dissociation constant (0.2μM), it was possible to demonstrate that the perturbation of NADH fluorescence was linearly related to the concentration of bound NADH up to an average of 70% saturation. A direct test was not made for the other isoenzymes because of their intrinsically lower affinity for NADH. The results in Fig. 2 also show that there is a non-linear change

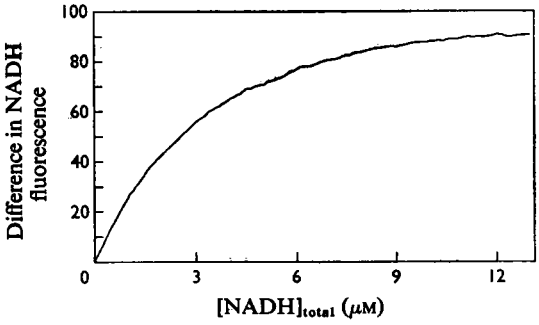

Fig. 3. *Trace showing the increase in the difference in NADH fluorescence during the titration of pig muscle lactate dehydrogenase* (1.56μM) *with NADH*

The signal recorded was the difference in the total concentration of NADH added to the enzyme solution and to an equal volume of 67mM-sodium phosphate buffer, pH7.2, at 20°C. Fluorescence was excited at 320nm and measured through a Kodak Wratten filter 18A. Only the trace up to the addition of 12μM-NADH is shown. The curve is uncorrected for dilution and change in intensity of the exciting radiation by the added NADH.

in protein fluorescence over the same range in which NADH fluorescence changes linearly. The total NADH concentration at which tangents to the initial and final limbs of titration curves monitored by non-linear protein-fluorescence quenching intersect was predicted (Holbrook *et al.*, 1972) to be lower than $K_{E,NADH}+[E]_0$ by the factor $(1-x^n)/[n(1-x)]$. This factor, for $n=4$ and $x=0.615$, is 0.61, in good agreement with the ratio of the intercepts (4.8μM/7.9μM) measured from Fig. 2. This agreement, and the fact that the equation $F^{1/n}=1-\alpha(1-x)$ fitted the experimental results best with $x=0.597$ and $n=3.8$, close to the value of $n=4$ expected for this tetrameric enzyme, was the justification for using this equation to obtain α from protein-fluorescence measurements.

A titration curve showing the enhanced fluorescence as NADH was continuously added to the pig muscle enzyme is shown in Fig. 3. The curve shows no discontinuities. Similarly smooth curves were obtained during titrations of the pig heart and ox muscle enzymes. Plots of these results according to Brown & Hill (1922–23) were single straight lines with slopes 1.0±0.03. No changes in slope with increasing average degree of saturation of the binding sites was observed (Fig. 4). The experiments of Anderson & Weber (1965), in which changes in slope were observed, were conducted over a wide range of protein concentrations. Although the NADH-dissociation constant for the pig muscle enzyme was

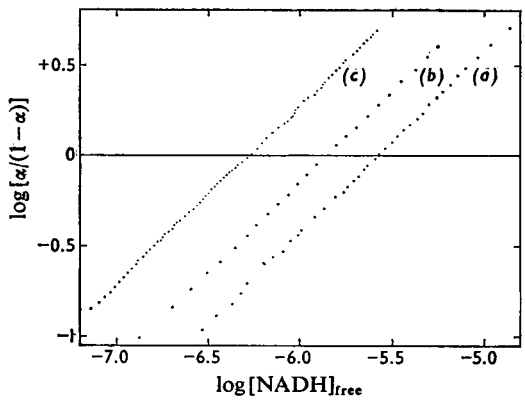

Fig. 4. *Plots according to Brown & Hill* (1922–23) *of the equilibrium binding of NADH to various lactate dehydrogenases*

The fractional saturation (α) was determined from the fluorescence of NADH when added to the enzymes. The conditions were: (*a*) 1.56μM pig muscle enzyme in 67mM-sodium phosphate buffer, pH7.2; (*b*) 0.8μM ox muscle enzyme in 0.05M-KH_2PO_4–0.1mM-EDTA adjusted to pH7.4 with 5M-NaOH; (*c*) 0.36μM pig heart enzyme in 67mM-sodium phosphate buffer, pH7.2. The temperature was 20°C in all experiments. Each set of points can be represented as a straight line of slope 1.0±0.03. The dissociation constants were: (*a*) 2.7μM; (*b*) 1.4μM; (*c*) 0.52μM.

independent of protein concentration, the value for the pig heart enzyme increased from 0.5μM at below 1μM-enzyme to 10μM at 20μM-enzyme. Such an effect has been predicted by Steinhard & Reynolds (1969) to be due to the presence of small concentrations of a tight-binding impurity in the added ligand (NADH). This explanation is probably correct, since if NADH that had been purified by chromatography was used, the increase in apparent dissociation constant with protein concentration was no longer marked. It is probable, if a clean separation of NADH from the impurity with no 340nm absorption could be obtained chromatographically, that the dissociation constant would be completely independent of protein concentration (Table 1). It should be pointed out that dissociation constants are best obtained from titrations when the concentration of binding sites is low compared with the dissociation constant but that accurate information about the concentration of binding sites in solution is best obtained from titrations at high enzyme concentration. It is concluded that NADH binding to pig lactate dehydrogenases can be described by single macroscopic dissociation constants independent of protein

Table 1. *Effect of protein concentration on the affinity of pig lactate dehydrogenases for NADH*

The results are from binding monitored by NADH-fluorescence enhancement in 67mM-sodium phosphate buffer, pH7.2 at 20°C. Chromatographically purified NADH was used to titrate the heart enzyme.

Enzyme	Subunit concentration from protein determination (μM)	Concentration of NADH-binding sites from titration (μM)	Dissociation constant (μM)
Pig muscle	1.56	1.5	2.8
	10	9.1	3.7
	20	19.2	4.3
Pig heart	0.12	(0.25)*	0.5
	0.46	0.4	0.4
	2.3	2.0	0.3
	4.6	4.5	0.5
	23	22	0.7

* $[E]_0$ is much less than the dissociation constant and this value has little meaning.

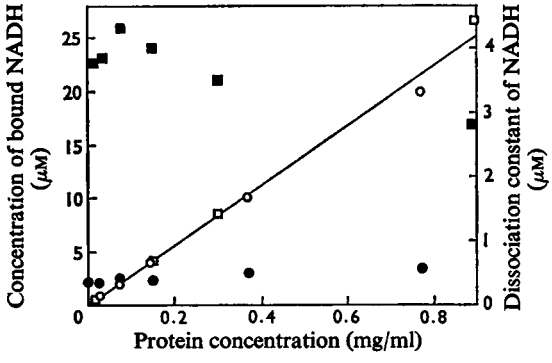

Fig. 5. *Effect of protein concentration on the dissociation constants of NADH from lactate dehydrogenases determined from protein-fluorescence quenching*

The fractional saturation (α) of enzymes with NADH was determined from the measured protein fluorescence (F) from the equation $\alpha = (1-F^{1/4})/(1-x)$, taking $x = 0.615$ for the pig heart (○, ●) and $x = 0.61$ for the pig muscle (□, ■) enzymes. The concentrations of NADH-binding sites (○, □) and their dissociation constants (●, ■) were then determined from plots of $1/(1-\alpha)$ against $[NADH]/\alpha$. The solvent was 67mM-sodium phosphate buffer, pH7.2, at 20°C. Chromatographically purified NADH was used in the heart-enzyme titrations.

concentration and of the fraction of the NADH-binding sites occupied (Fig. 5). There is thus no necessity to postulate molecular relaxations during the 5min necessary to obtain a binding curve. The disagreement between these results and those of Anderson & Weber (1965) is difficult to explain although it should be recalled that these workers used another method and measured the fluorescence of very dilute solutions of the enzyme which had been kept for some time. A summary of the results obtained on the binding of NADH to various isoenzymes and species of lactate dehydrogenase is given in Table 2.

A study of the effect of pH on the binding of NADH could give information on the nature of the amino acid side chains which contribute to the formation of the complex. Results of the experiments with the pig enzymes in pyrophosphate buffers at a constant ionic strength of 0.3M are shown in Fig. 6. Most of the results are from measurements of protein-fluorescence quenching although some additional points were obtained from enhancement of NADH fluorescence for the pig heart enzyme. Some difficulty was found in keeping the pH stable at the higher values, and it was necessary to include 10mM-Na_2CO_3 to ensure that the pH did not change during the titration. The enzyme was recovered after the titrations fully active in the region pH5.5–11. At pH5 both the enzyme activity recovered and the overall change in protein fluorescence were decreased. A similar pH profile was obtained for the muscle enzyme in a trisethylsulphonate–glycine–imidazole (each component was 0.05M) buffer system, although the dissociation constants were two- to three-fold higher. The results for the pig heart enzyme do not agree with those of Heck (1969), who found from relaxation experiments that NADH binding was weaker (1.2μM) at pH6 than at pH8 (0.56μM). However, the overall profile reported here is similar for both enzymes and is in broad agreement with the steady-state measurements of Schwert *et al.* (1967)

Table 2. *Dissociation constants of NADH from isoenzymes of lactate dehydrogenases of various species*

Solvents were 67mM-sodium phosphate buffer, pH7.2 at 20°C. The method used was either enhancement of NADH fluorescence (N) or quenching of protein fluorescence (P).

Species and isoenzyme	Fractional protein fluorescence when saturated with NADH (x^4)	Protein concentration range (mg/ml)	Method	Mean dissociation constant for NADH (μM)
Pig heart	0.14	0.004–0.7	N	0.51
		0.004–0.8	P	0.53
Ox muscle	0.17	0.03	N	1.4
		0.028	P	2.0
Pig muscle	0.13	0.05–0.72	N	3.7
		0.006–0.9	P	3.7
Dogfish muscle	0.16	0.028	P	3.6
Rabbit muscle	0.14	0.028	P	3.5
Lobster tail muscle*	(0.6)*	0.03*	P*	7*

* An impure preparation of the enzyme was used and the analytical values are only approximate.

Table 3. *Dissociation constants of NAD^+ from lactate dehydrogenase*

The ratio of the dissociation constant of NAD^+ to that of NADH was obtained from the slope of a graph of $[NAD^+]$ against the apparent dissociation constant of NADH according to eqn. (4) (see the text). Buffers were: at pH6 and 8.5, sodium pyrophosphate (I 0.15M); pH7.2, 67mM-sodium phosphate at 20°C. Values for K_{NAD^+} are ± likely error.

Enzyme	pH	$K_{E,NAD^+}/K_{E,NADH}$	$K_{E,NADH}$ (μM)	K_{E,NAD^+} (mM)
Pig muscle	6.0	270	1.7	0.4 ±0.2
	7.2	145	3.3	0.5 ±0.2
	8.5	104	5.7	0.6 ±0.2
Pig heart	6.0	310	0.6	0.2 ±0.05
	7.2	360	0.9	0.3 ±0.1
	8.5	310	1.8	0.5 ±0.2
	7.2*	184	1.2	0.2 ±0.05
	7.2*	280	0.9	0.3 ±0.1
	7.2*	340	0.65	0.2 ±0.05
	7.2†	—	—	0.19±0.05

* These three values are sodium pyrophosphate buffers of I 0.5, 0.2 and 0.05M respectively.
† From a gel-filtration experiment.

and the fluorescence studies of Winer (1963). Ionizations of the histidine residue at the substrate-binding site (pK = 6.8; Holbrook & Ingram, 1973) are not markedly reflected in changes in affinity of the enzyme for NADH. There is, at most, a threefold decrease in affinity for both isoenzymes between pH6 and 8. Ionization of the essential thiol group is not responsible for the rapid decrease in affinity above pH9, since the pH profile for the enzyme in which this thiol group is blocked still has this feature (Holbrook & Stinson, 1970). The rapid weakening above pH9 could reflect ionization of tyrosine-85, which is thought from the crystallographic studies on the dogfish enzyme to form a hydrogen bond to N^1 of the bound adenine ring (Rossmann *et al.*, 1971). Such a suggestion would be supported by the lack of lysine residues in the active-centre 'pocket', since these too might have a pK between 9 and 10.

The affinity of the pig heart enzyme decreases more rapidly with increasing temperature than does that of the pig muscle enzyme (Fig. 7). Both results can be described by a linear plot of log$K$ against $1/T$, which indicates temperature-invariant enthalpy of binding ($-52 kJ \cdot mol^{-1}$ for the heart and

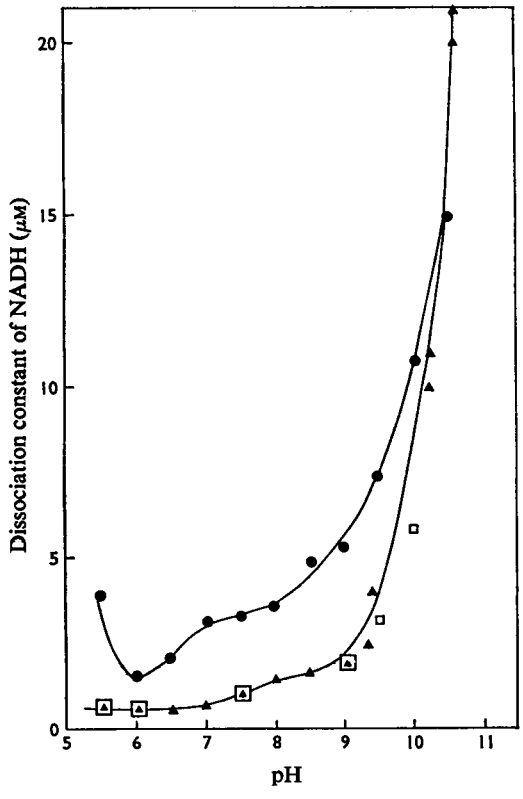

Fig. 6. *pH-dependence of the dissociation constant of lactate dehydrogenases for NADH*

The dissociation constants of the pig muscle enzyme were determined from protein-fluorescence quenching (●). Those for the pig heart enzyme were either from protein-fluorescence quenching (▲) or from NADH-fluorescence enhancement (□). The buffers were sodium pyrophosphate of I 0.15M at 20°C; 10mM-Na_2CO_3 was included in some of the high-pH buffers. The protein concentrations were about 0.03mg/ml.

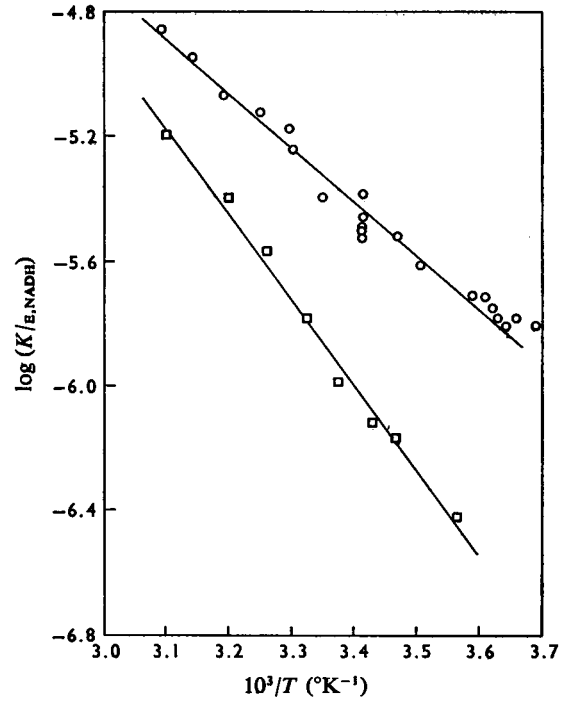

Fig. 7. *Temperature-dependence of the dissociation constant of NADH from pig heart* (□) *and pig muscle* (○) *lactate dehydrogenases*

Results were from titrations of 0.02–0.03mg/ml protein solutions in which NADH binding was monitored by protein-fluorescence quenching. The buffer was 67mM-sodium phosphate buffer, pH7.2 at 20°C. No loss of enzyme activity was noted up to 50°C. The lines are drawn for $-\Delta H = 52$ and 33kJ·mol^{-1} for the heart (□) and muscle (○) enzyme respectively.

-34kJ·mol^{-1} for the muscle isoenzyme). There was no temperature-induced change between two structures visible in this parameter.

The binding of NADH to both the pig heart and muscle enzymes is decreased by the inclusion of ethanol in the medium (Fig. 8). Enzymes recovered after titration in up to 30% (v/v) ethanol showed full enzyme activity when assayed without ethanol. Attempts to assay the pig muscle enzyme in 30% ethanol gave complex kinetics. There was an initial rapid decrease in extinction at 340nm followed by a progressively decelerating rate of decrease of extinction. However, 10% ethanol had no effect on the steady-state rate in either direction.

An increase in ionic strength weakens NADH binding to both the heart and muscle enzymes (Fig. 9). The magnitude of the effect is not simply a reflection of the ionic strength but is also dependent on the pH and the ions present. In pyrophosphate buffer, the weakening is much greater at pH5.5 than at 9.5. NaCl also seems to weaken NADH binding much more effectively than the same ionic strength from sodium phosphate at pH7.2. Such specific salt effects have been noted previously in the hybridizations of lactate dehydrogenase (Chilson *et al.*, 1964), although our knowledge of the structure of the enzyme is probably still too slight to attempt a specific interpretation.

No easily measured physical property of NAD^+ or of the enzyme is sufficiently perturbed on NAD^+ binding to allow direct determination of the affinity

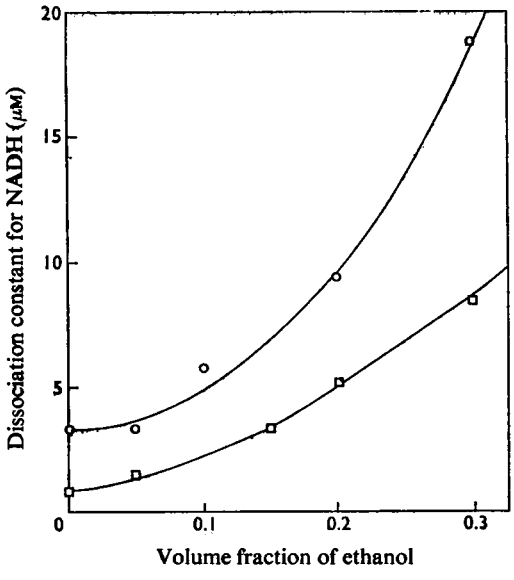

Fig. 8. *Effect of ethanol on the dissociation constant of NADH from lactate dehydrogenases*

Samples of pig heart (□, about 0.5 μM) and pig muscle (○, about 1 μM) enzyme were titrated with NADH at 20°C in buffers made by mixing 67 mM-sodium phosphate buffer, pH 7.2, with ethanol to obtain the volume compositions shown.

of the protein for NAD^+. One method to obtain such information is to allow NAD^+ to compete for the NADH-binding sites and observe the binding by an apparent decrease in the affinity of the enzyme for NADH. This method rests on the assumption that NADH and NAD^+ cannot bind at the same site at the same time. This seems a reasonable assumption for the active site of the enzyme, although binding of NAD^+ at other, non-catalytic sites on the protein could be missed. Also, since in these experiments the enzyme exists as the complex with either NAD^+ or NADH, any rearrangements of the apoprotein, at a lower rate than that at which the nucleotides bind, will be missed. In the Experimental section of the present paper it was noted that pure NAD^+ has little effect on protein fluorescence of the enzyme. Thus the linear plots (Fig. 10) of the apparent dissociation constant for NADH against the concentration of NAD^+ are interpreted to mean that the ratio of the dissociation constant of NADH to that of NAD^+ is independent of the fraction of the coenzyme-binding sites occupied by NAD^+. Since $K_{E,NADH}$ is independent of the fractional saturation with NADH, then so also must K_{E,NAD^+} be independent of fractional saturation. Thus NAD^+ binding may be described by a single macroscopic dissociation constant. The precision of these results is not great, and it is doubtful if heterogeneity in the four NAD^+-binding sites would have been detected if the relative dissociation constants from each set of sites differed by less than

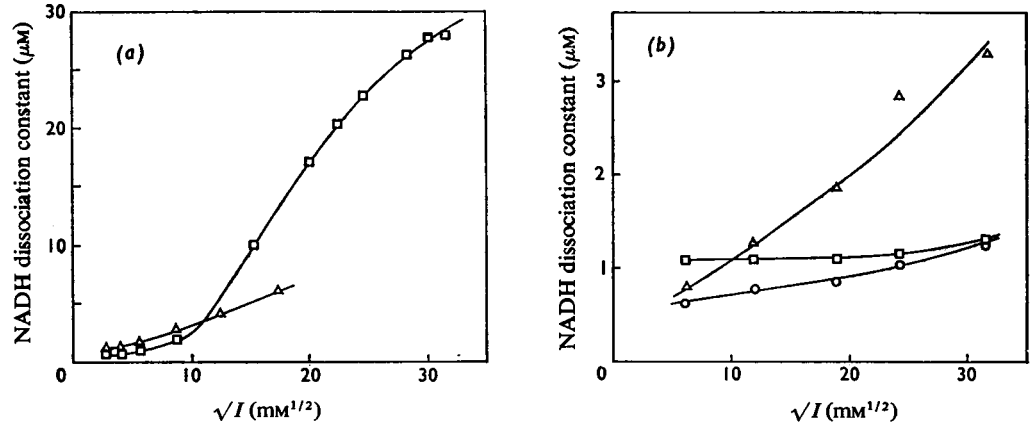

Fig. 9. *Effect of salt concentration on the affinity of the pig muscle (a) and pig heart (b) lactate dehydrogenases for NADH*

(*a*) Samples of the muscle enzyme (0.5 μM or 1 μM) were titrated in sodium phosphate buffer, pH 7.2 (△), or NaCl solution containing 5 mM-sodium phosphate buffer, pH 7.2, of the total ionic strengths shown (□). (*b*) Samples of the heart enzyme (0.5 μM) were titrated in sodium pyrophosphate buffers with the ionic strengths shown at pH 5.5 (△), pH 7.2 (○) and pH 9.5 (□) at 20°C.

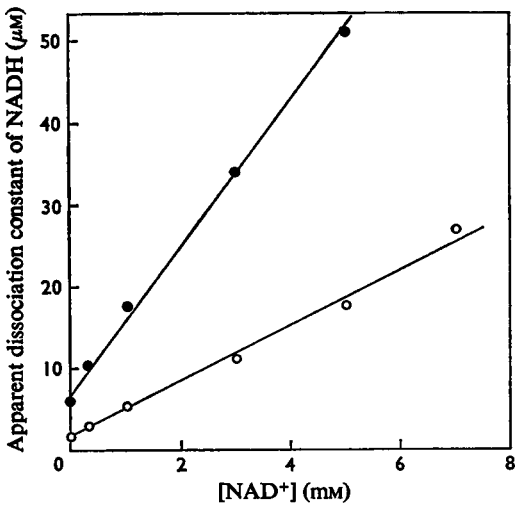

Fig. 10. *Competition between NADH and NAD$^+$ for the nucleotide-binding site of lactate dehydrogenase*

Either pig heart enzyme (○) or pig muscle enzyme (●) was titrated with NADH in sodium pyrophosphate buffer, pH8.5, *I* 0.15M, containing NAD$^+$ added to give the concentrations shown. The apparent dissociation constant for NADH was determined from the quenching of protein fluorescence during a titration with NADH at each of the NAD$^+$ concentrations shown.

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a factor of 5. Table 3 gives NAD$^+$-dissociation constants determined in this way. NAD$^+$ binding is tighter at pH6 than at 8.5, although the difference borders on the significant. The binding is not greatly changed with increasing ionic strength. These values are in broad agreement with the results from kinetic (Schwert *et al.*, 1967) and ultracentrifugal (Takenaka & Schwert, 1956) measurements. The single dissociation constant for the pig heart enzyme determined by the gel-filtration method also is in agreement with that determined by fluorimetry and shows that each subunit can bind one NAD$^+$.

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