The Identification of Intermediates in the Reaction of Pig Heart Lactate Dehydrogenase with its Substrates

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Pig heart lactate dehydrogenase was studied in the direction of pyruvate and NADH formation by recording rapid changes in extinction, proton concentration, nucleotide fluorescence and protein fluorescence. Experiments measuring extinction changes show that there is a very rapid formation of NADH within the first millisecond and that the amplitude of this phase (phase 1) increases threefold over the pH range 6-8. A second transient rate (phase 2) can also be distinguished (whose rate is pH-dependent), followed by a steady-state rate (phase 3) of NADH production. The sum of the amplitudes of the first two phases corresponds to 1 mol of NADH produced/mol of active sites of lactate dehydrogenase. Experiments that measured the liberation of protons by using Phenol Red as an indicator show that no proton release occurs during the initial very rapid formation of NADH (phase 1), but protons are released during subsequent phases of NADH production. Fluorescence experiments help to characterize these phases, and show that the very rapid phase 1 corresponds to the establishment of an equilibrium between $E_{\text{Lactate}}^{\text{NAD}} \rightleftharpoons H^+E_{\text{Pyruvate}}^{\text{NADH}}$. This equilibrium can be altered by changing lactate concentration or pH, and the $H^+E_{\text{Pyruvate}}^{\text{NADH}}$ species formed has very low nucleotide fluorescence and quenched protein fluorescence. Phase 2 corresponds to the dissociation of pyruvate and a proton from the complex with a rate constant of 1150 s^{-1} . The observed rate constant is slower than this and is proportional to the position of the preceding equilibrium. The $E_{\text{Pyruvate}}^{\text{NADH}}$ formed has high nucleotide fluorescence and quenched protein fluorescence. The reaction, which is rate-limiting during steady-state turnover, must then follow this step and be involved with dissociation of NADH from the enzyme or some conformational change immediately preceding dissociation. Several inhibitory complexes have also been studied including $E_{\text{Oxamate}}^{\text{NAD}^+}$ and $E_{\text{Oxamate}}^{\text{NADH}}$ and the abortive ternary complex $E_{\text{Lactate}}^{\text{NADH}}$. The rate of NADH dissociation from the enzyme was measured and found to be the same whether measured by ligand displacement or by relaxation experiments. These results are discussed in relation to the overall mechanism of lactate dehydrogenase turnover and the independence of the four binding sites in the active tetramer.

A satisfactory description of the mechanism of the reactions of lactate dehydrogenase with its substrates should define the number of intermediates that are present during enzyme catalysis, their chemical composition and their rates of inter-conversion. For this purpose it is important to measure the concentrations of intermediates formed and their rates of formation.

Previous studies on heart (H_4) and skeletal-muscle (M_4) lactate dehydrogenase by rapid-reaction techniques were summarized by Gutfreund (1971), and some additional experiments on the M_4 isoenzyme

were presented by Bennett & Gutfreund (1973). The present paper describes much more detailed investigations on the reactions of the H_4 isoenzyme. Bennett & Gutfreund (1973) showed that some of the earlier observations on the transient formation of NADH by lactate dehydrogenase, although qualitatively correct, were inaccurate because some NAD^+ had been reduced by substrate-like impurities in the enzyme solution before addition of substrate.

In the experiments described in the present paper precautions have been taken to avoid the formation of NADH before mixing by keeping the

enzyme separate from the substrates. To get accurate information about the amplitudes of different phases when studying changes in extinction, it is necessary to know the exact position of the baseline. The split-beam stopped-flow spectrophotometer designed by D. W. Yates, C. H. McMurray & H. Gutfreund (see Gutfreund, 1972, p. 180) compensates for changes in baseline, and allows amplitudes to be measured with great accuracy. A considerable amount of additional information is obtained if nucleotide and protein-fluorescence signals are determined, since intermediates, which cannot be distinguished by extinction changes, give different fluorescence signals. The experiments described in this paper are designed to characterize the intermediates formed during the first turnover of the enzyme.

Materials

Pig heart lactate dehydrogenase preparations were obtained from Whatman Biochemicals Ltd., Maidstone, Kent, U.K. (batches 8 and 14). Crystalline enzyme was separated from the $(\text{NH}_4)_2\text{SO}_4$ suspension by centrifugation and then dissolved in 0.1 M-phosphate buffer, pH 7. The solution of enzyme (10–20 mg/ml) was treated with charcoal to remove bound nucleotide, centrifuged and then desalted on a column (1 cm $\times$ 13 cm) of Sephadex G-50 (fine grade). The specific activity was 360 units/mg at pH 7.2 and 25°C (67 mM-phosphate, 0.3 mM-pyruvate, 0.15 mM-NADH). Protein concentration was determined from extinction measurements at 280 nm by assuming an extinction coefficient of 1.39 for a 1 mg/ml solution and a subunit mol.wt. of 36000. At an enzyme site concentration of 50 μM there was no detectable peak at 340 nm by difference spectroscopy against a solution of serum albumin and tryptophan with the same extinction as lactate dehydrogenase at 290 nm. Throughout this paper, concentrations of lactate dehydrogenase are given in terms of molarity of active sites, assuming 4 active sites/enzyme molecule.

NAD⁺ was obtained from P-L Biochemicals, Inc., Milwaukee, Wis., U.S.A. (lots 1208814 and 1208817). For use, 0.25 g was dissolved in 15 ml of water, the pH carefully adjusted to 5.5 with 2 M- Na_2CO_3 and the solution passed through a column (2 cm $\times$ 15 cm) of Dowex-1 (Cl^- form) anion-exchanger resin. After thorough washing of the column with water, the NAD⁺ was eluted with 15 mM-HCl (Stinson & Holbrook, 1973). For use at pH 6 or 7, solid KH_2PO_4 was added to the NAD⁺ solution to give a concentration of 0.1 M and the pH carefully adjusted to either 6 or 7 with NaOH. For use at pH 8, the pH was adjusted to 6 with Na_2CO_3 , so that the NAD⁺ had negligible buffering power. The concentration of NAD⁺ was determined from E_{260} , by assuming an extinction coefficient of $1.8 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

NADH (lot 7172114) and sodium pyruvate were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, and were used without further purification. Adenosine 5-diphosphoribose (ADP-ribose, sodium salt, lot 144001) was obtained from P-L Biochemicals Inc.; oxamic acid was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., and was recrystallized from water. Phenol Red and Chlorophenol Red were obtained from BDH Chemicals Ltd. L-(+)-Lactic acid (lot 82C-3450) was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A., and was used without further purification. DL-Lithium lactate was prepared from lactic acid (AnalaR, analytical reagent) by neutralization with LiOH (BDH Chemicals Ltd.), crystallization from water, and then by recrystallization from 85% (v/v) ethanol at room temperature. The first crop of crystals obtained from recrystallization did not contain contaminants capable of oxidizing NADH or of quenching the nucleotide-fluorescence enhancement which resulted from the formation of $\text{E}_{\text{Lactate}}^{\text{NADH}}$. Since L-lactate and DL-lactate gave identical transient rates and amplitudes, the DL-lactate was used in most experiments. The concentration of L-lactate was determined by the method described by Hohorst (1965) and the lactate concentrations given in the present paper are for the L-isomer.

Phosphate buffers, 0.1 M, were used at pH 6, 7 and 8 without ionic strength adjustment. These were prepared by weighing out KH_2PO_4 and Na_2HPO_4 in the appropriate proportions for each pH as described by Harned & Owen (1950). Changing ionic strength from 0.137 to 0.537 at pH 6 had no effect on transient rate or amplitude.

All solutions, except the buffers, were prepared fresh on the day used.

Instrumentation

Measurements of rapid changes in extinction were recorded on a split-beam stopped-flow spectrophotometer built by D. W. Yates, C. H. McMurray and H. Gutfreund, and described by Gutfreund (1972, p. 180). This machine measures light passing through two 0.5 cm cells before mixing and one 1 cm cell after mixing, and this results in the baseline of zero extinction remaining very stable. In the traces recorded on this machine, an increase in extinction caused a downward deflection on the oscilloscope. The initial portion of the traces shown is a record of extinction before flow stops, and under continuous flow conditions the mean age of the solution after mixing is 4 ms old. This has been determined in separate test reactions (Gutfreund, 1972, p. 179). Calculations on the concentration changes of NADH are based on

the assumption that the molar extinction of all forms of the reduced nucleotide (free and bound) is within 5% of $6.23 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 340 nm.

Measurements of rapid changes in nucleotide and protein fluorescence were recorded on a stopped-flow fluorimeter as described by Bagshaw *et al.* (1972). For nucleotide fluorescence, the excitation monochromator was set at 335 nm with 10 nm bandpass slits and NADH fluorescence was observed through Wratten 98 and 2B filters which have a peak transmission close to 435 nm, the peak-emission wavelength for NADH bound to lactate dehydrogenase. An increase in nucleotide fluorescence on this machine causes a downward deflection of the oscilloscope. The machine has a 2 mm-square observation chamber, and fluorescence is measured at 90° to the exciting light. The small volume of the observation chamber means that under flow conditions the age of the solution at the point of observation is 1 ms (calculated from experiments similar to that quoted by Gutfreund, 1972, p. 179).

For protein fluorescence the excitation monochromator was set at 300 nm, and the protein fluorescence was observed through Schott WG 345 and U.G. 11 filters.

Methods and Results

In all the experiments described in the present paper the enzyme was kept in one syringe of the stopped-flow apparatus, and NAD^+ and lactate were placed together in the other syringe. When $1 \mu\text{M}$ -enzyme and 2 mM - NAD^+ are mixed in the absence of added lactate, some slow enzyme turnover results with the formation of 0.2–0.5 site equivalent of NADH. The extent of the reaction depends on pH and the care taken to exclude contamination of the enzyme solution with sweat, dust etc. In all the experiments reported here plastic gloves were worn to keep this contamination to as low a value as possible. Similar problems have been reported by White *et al.* (1973).

Spectrophotometric observation of NADH formation

When lactate dehydrogenase was mixed with NAD^+ and lactate in the stopped-flow spectrophotometer, a rapid transient increase in extinction was seen at 340 nm. This transient was followed by a linear increase in extinction representing steady-state production of NADH. Such an experiment is illustrated in Fig. 1 with a line drawn to represent the steady-state rate. The slope of the derivative plot corresponds to an observed rate constant of 148 s^{-1} for the approach to the steady state and after correction for the instrument dead-time, as described

in the legend to Fig. 1, it is evident that 0.2 equiv. of NADH/enzyme site is produced at a very much faster rate than the observed transient. Similar

Fig. 1. Reaction of lactate dehydrogenase with NAD^+ and lactate monitored by measuring E_{340}

(a) The reaction mixture contained $15 \mu\text{M}$ -pig heart lactate dehydrogenase (final subunit concentration) from one syringe and 1.84 mM - NAD^+ and 100 mM -lithium lactate (final concentration) from another in 0.1 M -phosphate buffer, pH 7.0, at 21°C . The steady-state rate of NADH production is indicated by the lines on either side of Fig. 1(a). (b) The derivative plot shown represents the transient approach to the steady-state rate treated as a first-order reaction by the method of Gutfreund (1972, p. 202), by plotting the logarithm of the difference in extinction between the signal and the steady-state rate ($\log \Delta E$) against the time after flow stops. Projecting this line backwards 4 ms, the mean time taken by the solution to travel from the point of mixing to the observation chamber, allows the total amplitude of the first-order transient to be measured. The fractional saturation of enzyme sites (α) during this reaction is 0.80. The difference between the extinction baseline for enzyme and NAD^+ and the extrapolated derivative plot shows that some very rapid formation of NADH has occurred, amounting to 0.2 equiv./active site. The steady-state rate was 6.80 s^{-1} and k was 148 s^{-1} .

Fig. 2. Release of protons during the reaction of lactate dehydrogenase with NAD^+ and lactate monitored with Phenol Red

(a) The reaction mixture contained $14\text{ }\mu\text{M}$ -pig heart lactate dehydrogenase (final subunit concentration) from one syringe and 8 mM - NAD^+ and 20 mM -lithium lactate from another. Both syringes contained $25\text{ }\mu\text{M}$ Phenol Red in 0.6 mM -phosphate buffer, pH 8, with 100 mM -NaCl at 25°C . The change in extinction at 560 nm is observed (a). Proton release during the steady-state phase was equated with extinction changes at 340 nm on an identical solution. A change in extinction of 0.04 was equivalent to a change in proton concentration of $10\text{ }\mu\text{M}$. (b) The derivative plot shown represents the transient approach to the steady-state rate treated as a first-order reaction by using an extended Guggenheim (1926) procedure, where the readings R of the recorded extinction are taken at time t , and readings R' and R'' are taken at times $t+8\text{ ms}$ and $t+16\text{ ms}$ respectively. The slope of this line gives an observed rate constant of 240 s^{-1} . α is the total fraction of enzyme sites which are protonated during the reaction.

experiments were performed at pH 6 and at pH 8 and derivative plots from these experiments indicated that this very rapid formation of NADH, or 'burst', amounted to 0.1 equiv. of NADH/active site at pH 6, and to approx. 0.3 equiv. of NADH/active

site at pH 8. The sum of the amplitudes of the instant 'burst' and the first-order transient amounted to 1 equiv. of NADH/enzyme site. These values are taken from experiments with saturating substrate concentration and are calculated by taking the steady-state rate into account (see Gutfreund, 1972, p. 201). From these results we concluded that NADH production occurred in three phases: phase 1, the very rapid formation ('burst') of NADH; phase 2, a rapid but measurable transient; and phase 3, the steady-state rate of formation of NADH. The substrate concentration and pH-dependences of the amplitudes and rates of these three phases were subsequently determined more accurately from the measurements of the quenching of protein fluorescence, and the intermediates responsible for these phases were identified. Whereas each of the subsequent methods used gave more accurate information about one or more of the steps, the observation of extinction gave the most complete record of the sequence of events during the reaction of lactate dehydrogenase with its substrates.

Spectrophotometric observation of the production of H^+

In the overall reaction:

protons are taken up or released depending on the direction in which the reaction is observed. By using indicator dyes one can monitor the change in concentration of free H^+ in the stopped-flow spectrophotometer throughout the reaction of the enzyme with its substrates. The rate of proton production at pH 8 was monitored in the presence of Phenol Red at 560 nm under the conditions outlined in the legend of Fig. 2. The same reaction mixture was also used to monitor NADH production at 340 nm to ascertain that the presence of Phenol Red did not affect the enzyme reaction. The rate of NADH production in the steady state was used to calibrate the change at 560 nm in terms of H^+ equiv. The assumption was made that in the steady state the rate of production of NADH and protons is equivalent. The rate of transient liberation of protons and of NADH was the same (240 s^{-1}), but with protons there was no 'burst' before the observed transient process. If oxamate is added to the reaction mixture described above, it will bind very rapidly to E^{NADH} formed by:

This inhibits the rate of dissociation of NADH from the enzyme and thus the initial transient will still be present, but steady-state turnover will be inhibited.

Fig. 3. Reaction of lactate dehydrogenase with NADH and pyruvate monitored by measuring E_{340}

(a) The reaction mixture contained $14\mu\text{M}$ -pig heart lactate dehydrogenase (final subunit concentration) and $10\mu\text{M}$ -NADH from one syringe, and 0.4mM -pyruvate (final concentration) from another in 0.1M -glycine buffer, pH 9, at 22°C . In the derivative plot shown (b), the logarithm of the extinction change was plotted against time. The data thus obtained fits first-order kinetics corresponding to the first-order reaction of the ternary complex of $\text{H}^+\text{E}_{\text{Pyruvate}}^{\text{NADH}} \rightarrow \text{E}_{\text{Lactate}}^{\text{NAD}^+}$. k was 78s^{-1} .

When proton and NADH formation were monitored in a reaction mixture similar to that described in Fig. 2, but with oxamate added, no liberation of protons could be detected during the transient formation of NADH. However, the steady-state rate was equivalent for H^+ and NADH.

Single turnover experiments of pyruvate reduction

Shore & Gutfreund (1970), using horse liver alcohol dehydrogenase and Stinson & Gutfreund (1971), using skeletal-muscle lactate dehydrogenase, have shown that a number of interesting features

could be established from experiments in which a stoichiometric complex of enzyme with NADH is mixed with a large excess of substrate. Stinson & Gutfreund (1971) have shown that at high pyruvate concentrations the reaction

occurs within the dead-time of the stopped-flow spectrophotometer. The first-order process recorded in Fig. 3 corresponds to the interconversion:

Since there is no deuterium-isotope effect on the rate of this process when specifically deuterated reduced nucleotide is used as substrate, this process must occur in at least two steps. Similar results have been obtained with skeletal-muscle lactate dehydrogenase (Bennett & Gutfreund, 1973).

The observed rate of the above single-turnover reaction is equal to the true rate constant times the ratio $[\text{H}^+\text{E}_{\text{Pyruvate}}^{\text{NADH}}]/[\text{E}_{\text{total}}]$, where $[\text{E}_{\text{total}}]$ is the total concentration of enzyme sites. As the pyruvate concentration is increased the observed rate reaches a maximum. As with skeletal-muscle lactate dehydrogenase (Stinson & Gutfreund, 1971) the V_{max} , and the pyruvate concentration required for half V_{max} , during the first enzyme turnover are equal to the steady-state turnover rate and K_m for pyruvate reduction. Thus the interconversion discussed above is rate limiting under steady-state conditions.

If the enzyme concentration is increased to 3 equiv. of active sites/mol of NADH ($30\mu\text{M}:10\mu\text{M}$) the first-order reactions obtained are identical with the reactions with an active-site/[NADH] ratio of $12\mu\text{M}:10\mu\text{M}$, showing that all four sites of the enzyme react independently.

Observation of nucleotide fluorescence during lactate oxidation

NADH fluorescence is enhanced about 3.5 times over that of free NADH when the nucleotide is bound to pig heart lactate dehydrogenase (Velick, 1958). The wavelengths of the excitation and emission maxima are also shifted towards the blue end of the spectrum. When pyruvate or oxamate is bound to the E_{NADH} complex, the enhanced fluorescence is quenched to a value below that of free NADH; for example the fluorescence yield of $\text{E}_{\text{Oxamate}}^{\text{NADH}}$ complex is 0.25 that of free NADH (Holbrook & Stinson, 1970). Experiments in which nucleotide fluorescence was monitored during rapid mixing of

Fig. 4. Reaction of lactate dehydrogenase with NAD^+ and lactate monitored by nucleotide fluorescence at pH 6 (a), 7 (b) and 8 (c)

In each experiment the reaction mixture contained $1\text{ }\mu\text{M}$ -pig heart lactate dehydrogenase (final subunit concentration) from one syringe and 0.65 mM - NAD^+ and 20 mM -lithium lactate (final concentration) from another, in 0.1 M -phosphate buffer at 21°C . The derivative plot (a' , b' , c') for each experiment represents the first-order transient approach to the steady-state rate of NADH production, where the logarithm of the difference between the steady-state rate and the signal, $\log(V_1 - V_2)$, is plotted against time. At pH 6, k was 102 s^{-1} and the steady-state rate was 13.1 s^{-1} ; at pH 7, k was 181 s^{-1} and the steady-state rate was 22.4 s^{-1} ; and at pH 8.0, k was 253 s^{-1} and the steady-state rate was 37.3 s^{-1} .

enzyme-NADH complex with pyruvate show that the fluorescence is quenched by a similar amount. These differences in nucleotide-fluorescence yield of the various intermediates can be used in stopped-flow experiments to study changes in intermediate concentrations when pig heart lactate dehydrogenase reacts with lactate and NAD^+ to form pyruvate and NADH.

The experiments described below were performed on the stopped-flow fluorimeter. Before each set of experiments, calibration values were obtained for the fluorescence of buffer, NADH and E^{NADH} , by adding buffer, $1\text{ }\mu\text{M}$ -NADH, and $2\text{ }\mu\text{M}$ -NADH containing excess of enzyme ($3\text{ }\mu\text{M}$) respectively. The enhanced fluorescence value for fully liganded enzyme was then calculated, by using a dissociation

Table 1. *Transient rate and amplitude at pH 6, 7 and 8 for reaction of lactate dehydrogenase with NAD⁺ and lithium lactate*

The reaction mixtures contained 1 μ M-pig heart lactate dehydrogenase (15 μ M for nucleotide extinction) from one syringe and 0.65 mM-NAD⁺ and various concentrations of lithium lactate from another in 0.1M-phosphate buffers at 21°C. With the exception of nucleotide extinction values, the values were obtained by extrapolation to infinite lactate concentration (e.g. see Fig. 5 and 11).

	pH ...	Transient rate (k_0 , s ⁻¹)		
		6	7	8
Nucleotide extinction		88.7	180*	—†
Nucleotide fluorescence		97.0	167	262
Protein fluorescence		91.7	167	263

	pH ...	Transient amplitude (fraction of total change)		
		6	7	8
Nucleotide extinction		0.91	0.75	—†
Nucleotide fluorescence		2.27‡	2.08‡	1.0
Protein fluorescence		0.92	0.78	0.70

* These values ranged from 120 to 180 s⁻¹ because of the difficulty of getting consistent data in the absence of oxamate where the steady-state rate is appreciable at 15 μ M-enzyme.

† No transient was observed with 15 μ M-enzyme in absence of oxamate because of high steady-state rate.

‡ Higher than 1.0 because of the contribution of E_{NADH} to nucleotide fluorescence.

constant of 1 μ M (Holbrook, 1972) to calculate the percentage of liganded enzyme present.

The results of typical experiments performed at pH 6, 7 and 8 are shown in Fig. 4. At each pH an initial transient increase in fluorescence is followed by a steady-state rate of NADH production. The age of the solution in the observation chamber is 1 ms at the start of the record. When the derivative plots were extrapolated back to zero time, no appreciable amount of any faster phase of NADH production was detected. The rapid transients observed follow first-order kinetics, as can be seen from the derivative plots alongside the original data, and first-order rate constants calculated from a number of such plots at each pH are given in Table 1.

Experiments were next performed to measure the effect of various lactate concentrations on the rate of the initial transient by using similar conditions to those described above. The results of several such experiments are shown in Fig. 5, where the data are plotted as $1/k$ against $1/[\text{lactate}]$. At each pH value the observed rate constant (k) for the transient

Fig. 5. *Reaction of lactate dehydrogenase with NAD⁺ and lactate studied by measuring the transient rate of NADH production as a function of lactate concentration at pH 6, 7 and 8, monitored by nucleotide fluorescence*

Reactions were carried out as described in Fig. 4, but with variations in the lithium lactate concentrations. The transient first-order approach to the steady-state rate was measured for each reaction, and the reciprocal transient rate is plotted against reciprocal lactate concentration. The k_0 values represent the transient rates extrapolated to infinite lactate concentration. Δ , pH 6.0 (k_0 97.0 s⁻¹, K_m 1.93 mM); $\circ$, pH 7.0 (k_0 167 s⁻¹, K_m 0.80 mM); $\square$, pH 8.0 (k_0 262 s⁻¹, k_m 0.64 mM).

Table 2. K_m for lactate

Reaction mixtures contained 1 μ M-pig heart lactate dehydrogenase from one syringe and 0.65 mM-NAD⁺ and various concentrations of lithium lactate (1–50 mM at pH 6, 0.25–50 mM at pH 7 and 1–20 mM at pH 8) from another in 0.1 M-phosphate buffers at 21°C.

Method	pH ...	K_m (mm)		
		6	7	8
Protein fluorescence				
From transient rate		1.61	0.62	0.58
From transient amplitude		1.46	0.40	0.34
Nucleotide fluorescence				
From transient rate		1.70	0.80 (0.68)*	0.64
From transient amplitude		1.50	0.46 (0.38)*	0.65
From steady-state rate		2.24	0.60 (0.60)*	2.70

* As above except that the NAD⁺ concentration was 2 mM.

increases with increasing lactate concentration until saturation is reached. From the reciprocal plots the rate constant for the transient at infinite lactate concentration can be determined, and these constants are shown in Table 1. The concentration of lactate

Fig. 6. Reaction of lactate dehydrogenase with NAD^+ and lactate studied by measuring the transient amplitude of NADH production as a function of lactate concentration at pH 6, 7 and 8, monitored by nucleotide fluorescence

Reaction conditions for these experiments were identical with those described in Fig. 6. Transient amplitudes were measured and divided by the fluorescence value for an equal concentration of enzyme fully liganded with NADH giving the transient amplitude in terms of equivalents of enzyme sites. The reciprocal amplitude is plotted against reciprocal lactate concentration. The dotted portions of the trace represent lactate concentrations that cause formation of highly fluorescent abortive ternary complex $\text{E}^{\text{NADH}}_{\text{Lactate}}$. (a) pH 6.0 (K_m 1.50 mM); (b) pH 7.0 (K_m 0.46 mM); (c) pH 8.0 (K_m 0.65 mM).

needed to achieve half-maximal transient rate, K_m , can also be determined from the reciprocal plot ($K_m = \text{slope/intercept}$) and typical values are shown in Table 2.

Various lactate concentrations also affect the amplitude of the initial transient. The model put forward in the Discussion section of this paper dictates that a direct relationship should be found between transient amplitude and rate; a plot of $1/\text{transient amplitude}$ against $1/[\text{lactate}]$ should also give a straight line, with the amplitude at infinite lactate concentration being equivalent to the concentration of enzyme active sites and the slope/intercept ratio giving a K_m value for lactate.

Fig. 7. Steady-state rate of NADH production as a function of lactate concentration during enzyme turnover monitored by nucleotide fluorescence, at pH 6, 7 and 8

Reaction conditions were identical with those described in Fig. 5, with an additional experiment in which the 0.65 mM- NAD^+ ($\circ$) was replaced by 2.0 mM- NAD^+ (Δ) at pH 7. Steady-state rates were determined immediately after the transient and the reciprocal steady-state rate is plotted against reciprocal lactate concentration. The dotted portions of the trace represent inhibition of the steady-state rate by excess of substrate. $\square$, pH 6.0 (K_m 2.24 mM, V_{max} 14.1 s^{-1}); $\circ$ and Δ , pH 7.0 (K_m 0.60 mM, V_{max} 31.3 s^{-1}); $\bullet$, pH 8.0 (K_m 2.70 mM, V_{max} 62.5 s^{-1}).

Results of experiments at pH values of 6, 7 and 8 are given in Fig. 6, and a straight-line relationship does in fact occur at pH 8. Deviations from a straight-line relationship are seen with higher lactate concentrations at pH 7, and these are even more marked at pH 6. These deviations are caused by formation of an abortive ternary complex $\text{E}^{\text{NADH}}_{\text{Lactate}}$

with a much greater fluorescent yield than E^{NADH} ; this has been demonstrated in separate experiments when E^{NADH} was mixed with lactate. For example, when 3 μM -pig heart lactate dehydrogenase and 2 μM -NADH in 0.1 M-phosphate buffer, pH 7, were mixed with 200 mM-lithium lactate in the same buffer, the resulting fluorescent yield was about twice that of the control experiment in the absence of lactate. The enhanced fluorescence yield may also be measured by differential fluorescence titration of E^{NADH} with lactate (J. J. Holbrook, unpublished work).

Table 3. Formation of ternary complex $E_{\text{Lactate}}^{\text{NADH}}$ at pH 6, 7 and 8 as observed by enhanced nucleotide-fluorescence amplitude changes

Reaction mixtures contained $1\mu\text{M}$ -pig heart lactate dehydrogenase from one syringe, 0.65mM - NAD^+ and various concentrations of lithium lactate from another in 0.1M -phosphate buffers at 21°C .

Parameter	pH ... 6	7	8
K_i (mM)	20.8*	30.3*	$\sim 75^\dagger$
Maximum nucleotide-fluorescence amplitude (equiv. of E^{NADH})	2.27	2.08	1.0 ‡

* Determined from deviation of nucleotide-fluorescence amplitude from expected value at various concentrations of lithium lactate.

† Determined from deviation of steady-state rates from expected values at high lactate concentrations (see Fig. 6). The value at pH 8 is from 20mM -lactate only.

‡ No nucleotide-fluorescence enhancement was observed to 20mM -lactate.

Formation of this abortive ternary complex has been noted previously with ox heart lactate dehydrogenase (Winer & Schwert, 1959). If due allowance is made for the enhancement caused by abortive ternary-complex formation, K_m values can be calculated from the plots in Fig. 7 and these are given in Table 2. The K_m values obtained from transient-amplitude determination are in reasonable agreement with those obtained from transient rates.

The deviations of transient amplitude from a straight line, plotted in reciprocal form against $1/[\text{lactate}]$, give a straight line. The intercept on the y axis gives the maximum fluorescence enhancement at infinite lactate concentration and the slope/intercept ratio gives K_i for dissociation of the abortive ternary complex. These values are given in Table 3. The value of 2–2.2-fold maximum-fluorescence enhancement is in good agreement with the value of 2–3-fold reported by Winer & Schwert (1959) for ox heart lactate dehydrogenase at pH 6.

Experiments similar to those shown in Fig. 4 contain data on steady-state NADH production as well as on the initial transient. Steady-state data from experiments with various lactate concentrations at pH 6, 7 and 8 are shown in Fig. 7, where $1/(\text{steady-state rate})$ is plotted against $1/[\text{lactate}]$ and conventional Lineweaver–Burk plots are obtained. The values for K_m and V_{max} obtained from these plots are given in Table 2, and the K_m values at pH 6 and 7 agree well with the K_m values derived from the transient data.

Inhibition by excess of substrate can be seen at pH 7 and 8, and the deviations from a straight line

Fig. 8. Reaction of lactate dehydrogenase with NAD^+ and lactate in the presence of oxamate, monitored by nucleotide fluorescence

(a) The reaction mixture contained $1\mu\text{M}$ -pig heart lactate dehydrogenase (final subunit concentration) from one syringe, and 0.65mM - NAD^+ , 20mM -lithium lactate and $30\mu\text{M}$ -oxamate from another in 0.1M -phosphate buffer, pH 7, at 21°C . In (b) the results of several similar experiments are given, where the reaction conditions were the same with the exception of various oxamate concentrations. The initial transient amplitude was measured and converted into equivalents of $\text{NADH}/\text{enzyme site}$ as described in the legend to Fig. 6. The reciprocal amplitude is then plotted against oxamate concentration.

at high concentrations of lactate can be used (replotted as $1/(\text{steady state})$ against $[\text{lactate}]$ to obtain K_i for the apparent inhibition by lactate at high concentrations, assuming non-competitive inhibition}. The K_i value of 39mM at pH 7 is similar to the dissociation constant for lactate from $E_{\text{Lactate}}^{\text{NADH}}$ (30mM) and suggests that inhibition of the steady-state turnover is due to formation of the $E_{\text{Lactate}}^{\text{NADH}}$ abortive ternary complex. The apparent absence of inhibition of the steady-state rate at pH 6 (Fig. 7) where there is known to be marked enhancement of nucleotide fluorescence (Fig. 6) seems to be in contradiction

to this suggestion, but it is possible that marked inhibition occurs at all lactate concentrations used, such that the line drawn in Fig. 7 is in fact displaced upwards by an unknown amount from the true line in the absence of inhibition.

Oxamate has been effectively used to slow down the steady-state rate seen by nucleotide extinction (Bennett & Gutfreund, 1973), so that the initial transient can be seen more easily. When stopped-flow nucleotide-fluorescence experiments are performed in the presence of oxamate, however, the size of the initial transient observed decreases markedly with increasing oxamate concentration, as shown in Fig. 8. The results of a number of such experiments are also shown in Fig. 8, plotted as $1/(\text{transient amplitude})$ against $[\text{oxamate}]$, and a K_i value of $27.3 \mu\text{M}$ can be calculated from these data. The transient diminishes in the presence of oxamate because the $\text{E}^{\text{NADH}}_{\text{Oxamate}}$ complex formed has very low fluorescence (approx. 0.2 that of free NADH). The K_i value obtained represents the dissociation constant for oxamate from the $\text{E}^{\text{NADH}}_{\text{Oxamate}}$ complex.

The nucleotide fluorescence experiments described above show that the very rapid 'burst' of NADH observed by nucleotide extinction (phase 1) has low fluorescence and is likely to correspond to formation of the ternary complex $\text{H}^+\text{E}^{\text{NADH}}_{\text{Pyruvate}}$. Phase 2, the rapid observable transient, is likely to represent dissociation of pyruvate from the enzyme, to give highly fluorescent E^{NADH} . The elimination of the rapid observable transient in the presence of oxamate agrees with this interpretation because the inhibitory complex $\text{H}^+\text{E}^{\text{NADH}}_{\text{Oxamate}}$ formed also has low fluorescence.

Observation of protein fluorescence during lactate oxidation

The combination of purified NAD^+ with pig heart lactate dehydrogenase has very little effect on protein fluorescence, whereas combination of NADH with lactate dehydrogenase causes a decrease in fluorescence to 0.12 that of the unliganded enzyme (Holbrook, 1972). When the enzyme is saturated with NADH, combination with carboxylic acids, such as lactate or oxamate, does not change the protein fluorescence appreciably from that of the binary complexes. Thus, this sensitive technique can be used to measure rate(s) of step(s) involved in the conversion of $\text{E}^{\text{NAD}^+}_{\text{Lactate}}$ into $\text{E}^{\text{NADH}}_{\text{Pyruvate}}$ and in the association and dissociation of NADH from enzyme. Holbrook (1972) has shown that quenching

of protein fluorescence is a non-linear function of the degree of saturation of pig heart lactate dehydrogenase with NADH and that this non-linear relationship is adequately described by the equation $F^{1/n} = 1 - \alpha(1-x)$, where F is the observed fluorescence relative to the unliganded enzyme, n is the number of ligand-binding sites/molecule of enzyme (4), α is the degree of saturation, and x is the geometric quenching factor. We have used this relationship to analyse data of protein fluorescence obtained with pig heart lactate dehydrogenase. For this enzyme Holbrook (1972) determined x to be 0.59.

Protein fluorescence was excited at 300 nm (excitation at 280 nm gave the same results) and emission was measured at about 360 nm (filters Schott WG 345 and U.G. 11). Calibration runs were performed before each set of experiments. The fluorescence of buffer, $1 \mu\text{M}$ -enzyme and $1 \mu\text{M}$ each of enzyme and NADH were all measured. The fluorescence of totally liganded enzyme was calculated from these measurements by the method described under 'Observation of nucleotide fluorescence during lactate oxidation'. The fluorescence of unliganded enzyme was recorded within 10 ms of mixing pig heart lactate dehydrogenase with NAD^+ at the concentrations being studied in the experiment.

The interpretation of protein fluorescence and its changes in the presence of ligands is inherently more complex than that of nucleotide extinction and nucleotide fluorescence. It is therefore necessary to establish that the kinetics of observed protein-fluorescence changes agree with the kinetics of the changes observed by nucleotide extinction and nucleotide fluorescence. Therefore, the changes observed when enzyme was mixed with NAD^+ and lactate at pH 6 in stopped-flow machines were monitored by the three methods. A typical set of experiments is shown in Fig. 9, where both the original traces and the derivative plots are given. The derivative plots for all three traces show first-order processes with similar rates. In the examples shown, the transient rates are 109, 95.0 and 87.0 s^{-1} by nucleotide extinction, nucleotide fluorescence and protein fluorescence respectively. Data from other experiments at different pH values are given in Table 1. Because of differences in sensitivity of the methods, $15 \mu\text{M}$ -enzyme, 148 mM-lactate (a large excess of substrate was added to inhibit the steady-state rate) and 2 mM- NAD^+ were used for nucleotide extinction and $1 \mu\text{M}$ -enzyme, 50 mM-lactate and 0.65 mM- NAD^+ were used for nucleotide fluorescence and protein fluorescence. The differences in reactant concentrations had no effect on the transient rate.

Whereas the transient rates are probably not significantly different, there are two differences in the traces obtained by the three methods. With nucleotide extinction and protein fluorescence,

Fig. 9. Reaction of lactate dehydrogenase with NAD^+ and lactate at pH6 monitored by nucleotide extinction, nucleotide fluorescence and protein fluorescence

For the extinction trace (a), the reaction contained $15 \mu\text{M}$ -pig heart lactate dehydrogenase (final subunit concentration) from one syringe and 2 mM - NAD^+ and 148 mM -lithium lactate from another in 0.1 M -phosphate buffer. For the two fluorescence traces (b, c) the reaction contained $1 \mu\text{M}$ -pig heart lactate dehydrogenase from one syringe and 0.65 mM - NAD^+ and 50 mM -lithium lactate from another in 0.1 M -phosphate buffer. All reactions were performed at 21°C . The derivative plots (a', b', c') represent the initial transient approach to a steady-state rate of NADH production for nucleotide extinction and fluorescence and are calculated as described in the legends to Fig. 1 and 4. To obtain the derivative plot for protein fluorescence (c'), the fluorescence was measured as a fraction of the fluorescence of unliganded enzyme at each time t , and the fractional fluorescence was then converted into fractional saturation of the enzyme with ligand by using the equation $F^{1/n} = 1 - \alpha(1 - x)$ as described in the text. The logarithm of the change in fractional saturation, $\log(\alpha' - \alpha)$, is then plotted against time. (a) Nucleotide extinction (k 109 s^{-1} , steady-state rate 4.80 s^{-1}); (b) nucleotide fluorescence (k 95.0 s^{-1} , steady-state rate 6.55 s^{-1}); (c) protein fluorescence (k 87.0 s^{-1}).

extrapolation of the derivative plots back to zero time, to include the dead-time of the instrument, indicates that there is an instantaneous transient 'burst' (phase 1) of NADH followed by a slower transient phase (phase 2), but no instantaneous 'burst' phase is seen with nucleotide fluorescence.

There is a second difference in the data given by the three methods. In traces monitoring both nucleotide extinction and nucleotide fluorescence, the transient phase is followed by an observable steady-state phase whose rate eventually decreases as equilibrium of the reaction is approached. When

Fig. 10. Reaction of lactate dehydrogenase with NAD^+ and lactate at pH 6, 7 and 8 monitored by protein fluorescence

The reaction conditions for these traces (a, b and c) are identical with those used for nucleotide fluorescence traces in Fig. 4. The derivative plots (a', b' and c') are calculated by the method described in the legend to Fig. 9. On each trace the baseline and end point are shown. pH 6, $k = 94.0\text{s}^{-1}$; pH 7, $k = 194\text{s}^{-1}$; pH 8, $k = 258\text{s}^{-1}$.

protein fluorescence is observed in the presence of saturating concentrations of lactate and NAD^+ , only the transient phase is seen.

Typical results are shown in Fig. 10 of experiments where enzyme is mixed with NAD^+ and lactate and the reaction is monitored by protein fluorescence at pH 6, 7 and 8. Derivative plots are included which allow determination of the rates of the observed transients, and reference to Fig. 4 shows that the transient rates determined under identical conditions by nucleotide and protein fluorescence are in good agreement. When these traces are extrapolated back

1 ms, the amplitude of the observed transient can be measured and it is evident that an instantaneous quench of protein fluorescence occurs, which represents an instantaneous 'burst' of NADH production. The amplitude of this 'burst' is equivalent to 0.08, 0.20 and 0.29 NADH equiv. at pH 6, 7 and 8 respectively. These values are in good agreement with the rapid 'burst' of NADH observed in experiments monitoring nucleotide extinction.

Having established that the phase 2 transient observed by protein fluorescence has the same rate as that observed by nucleotide fluorescence, we then

Fig. 11. Reaction of lactate dehydrogenase with NAD^+ and lactate studied by measuring the rate of the initial transient as a function of lactate concentration at pH 6, 7 and 8 monitored by protein fluorescence

Reaction conditions were similar to those used for Fig. 4 with various lactate concentrations. The rate of the initial transient was measured by the method described in the legend to Fig. 9, and the reciprocal rate is plotted against reciprocal lactate concentration. Δ , pH 6 (k_0 91.7 s $^{-1}$, K_m 1.61 mM); $\circ$, pH 7 (k_0 167 s $^{-1}$, K_m 0.80 mM); $\square$, pH 8 (k_0 263 s $^{-1}$, K_m = 0.58 mM).

Fig. 12. Reaction of lactate dehydrogenase with NAD^+ and lactate studied by measuring the amplitude of the initial transient as a function of lactate concentration at pH 6, 7 and 8, monitored by protein fluorescence

used this sensitive method to study the effect of various concentrations of substrates and other ligands on the reaction. The effect of various lactate concentrations at a fixed NAD^+ concentration is shown in Fig. 11 for pH 6, 7 and 8. The transient rate increases until saturating concentrations of lactate are used. From the data, plotted as $1/(\text{transient rate})$ against $1/[\text{lactate}]$, the maximum transient rates and K_m for lactate were calculated. These values, given in Tables 1 and 2, are in excellent agreement with those determined from nucleotide-fluorescence changes.

For reasons to be stated in the Discussion section, there should be a direct relationship between the amplitude and rate of the slower transient (phase 2) after the instant 'burst' phase at less than saturating concentrations of substrates. In Fig. 12, the relationship between this transient amplitude and lactate concentration is shown as a reciprocal plot. From this plot, analogous to that in Fig. 11, the intercept gives the maximum amplitude of the transient and the ratio slope/intercept gives the K_m for lactate. These values are given in Tables 1 and 2.

These experiments monitoring protein fluorescence demonstrate that the rate and amplitude of the phase 2 transient is dependent on lactate concentration. The amplitude of the instant 'burst' of NADH production extrapolated to infinite lactate concentration can be measured to a high degree of accuracy.

Reaction conditions were identical with those in Fig. 11. The change in fractional saturation of the enzyme with NADH during the transient is measured and the reciprocal amplitude in units of enzyme equivalents is plotted against reciprocal lactate concentration. The fractional saturation of the enzyme is calculated from the amplitude of phase 1 + the amplitude of phase 2. At infinite lactate concentration the sum of the two phases is equal to unity, and phase 1 corresponds to 1 - maximum transient amplitude. (a), pH 6 (maximum transient amplitude 0.92, K_m 1.46 mM); (b), pH 7 (maximum transient amplitude 0.78, K_m 0.41 mM); (c), pH 8 (maximum transient amplitude 0.70 K_m 0.34 mM).

Oxidation of lactate in the presence of oxamate, monitored by protein fluorescence

Inhibition of lactate dehydrogenase turnover by oxamate has been discussed briefly under 'Spectrophotometric observation of the production of H^+ '. The addition of oxamate to enzyme and lactate was thought to affect primarily the steady-state rate by forming an abortive $\text{E}^{\text{NADH}}_{\text{Oxamate}}$ complex (Winer & Schwert, 1959), although there was some indication of an effect on the transient rate as well. Protein fluorescence monitors only the transient rate, and

Fig. 13. Reaction of lactate dehydrogenase with NAD^+ and lactate studied by measuring the rate of the transient as a function of oxamate concentration monitored by protein fluorescence

The reaction contained $1\text{ }\mu\text{M}$ -pig heart lactate dehydrogenase (final subunit concentration) from one syringe and 0.65 mM - NAD^+ , 20 mM -lithium lactate and various oxamate concentrations from another in 0.1 M -phosphate buffer, pH 6, at 21°C . Transient rates were calculated as described in the legend to Fig. 9. The insets are plotted as described by Dixon (1953) for a competitive inhibitor.

oxamate does not affect the quenching of protein fluorescence by NADH , so this is an ideal technique for studying the effect of oxamate on the transient. Fig. 13 shows the effect of adding increasing concentrations of oxamate to enzyme and NAD^+ at pH 6. The data, plotted as transient rate against oxamate concentration, give a biphasic curve indicating that oxamate is bound to enzyme or some liganded complex with two different binding constants. The same data were replotted by the method of Dixon (1953) according to the relationship

$$\frac{1}{k} = \frac{1}{k_0} + \frac{K_m^{\text{lactate}}}{k_0[\text{lactate}]} + \frac{K_i^{\text{lactate}} \cdot [\text{oxamate}]}{K_i^{\text{oxamate}} \cdot k_0[\text{lactate}]}$$

so as to determine k_0 , the maximum transient rate and K_i^{oxamate} . This is shown in the inset of Fig. 13 with separate plots for ranges of high and low oxamate concentration. Similar data were obtained from experiments at pH 7 and 8 and values for K_i^{oxamate} are given in Table 4 for all pH values.

In the above treatment it was assumed that oxamate is competitive with lactate in its effect on

Fig. 14. Reaction of lactate dehydrogenase with NAD^+ and lactate studied by measuring the rate of the transient as a function of lactate concentration in the presence of fixed concentrations of oxamate

The reactions are monitored by protein fluorescence. The reaction mixture contained $1\text{ }\mu\text{M}$ -pig heart lactate dehydrogenase (final subunit concentration) from one syringe and 0.65 mM - NAD^+ , various concentrations of lithium lactate and zero oxamate ($\square$), $25\text{ }\mu\text{M}$ -oxamate ($\circ$) or 1 mM -oxamate ($\triangle$) from another in 0.1 M -phosphate buffer, pH 6, at 21°C . The transient rates were calculated as described in the legend to Fig. 9, and plotted as reciprocal rate against reciprocal lactate concentration.

the transient rate. This assumption was justified by further experiments, which determined the transient rate as a function of lactate concentration at 0, $25\text{ }\mu\text{M}$ - and 1 mM -oxamate concentrations. The concentrations of oxamate were chosen to permit measurement of the two binding constants of oxamate separately. As shown by the data in Fig. 14, oxamate is competitive with lactate in both cases. This indicates that it competes with lactate in binding to the binary complex E^{NAD^+} . To support further our treatment of the data in Fig. 13, the values of K_i^{oxamate} obtained from the data of Fig. 14 were 6.3 and $69\text{ }\mu\text{M}$. Data at pH 7 and 8 on the effect of oxamate as a function of lactate concentration also indicated oxamate to be a competitive inhibitor with respect to lactate at these pH values. The K_i^{oxamate} values are given in Table 4. Also given in Table 4 are values for K_i^{oxamate} determined from the effect of oxamate on transient

Table 4. K_i for oxamate on the oxidation of lactate by pig heart lactate dehydrogenase

The reaction mixtures contained 1 μM pig heart lactate dehydrogenase from one syringe (15 μM for nucleotide extinction), 0.65 mM-NAD⁺ and various concentrations of either lithium lactate or oxamate from another in 0.1 M-phosphate buffer at 21°C.

Method	K_i (μM)		
	pH ... 6	7	8
Protein fluorescence			
From effect on transient rate			
Varying oxamate*	8.0 and 48	2.2 and 81	5.0 and 124
Varying lactate†	6.3 and 69	— and 58	15.2‡ and 127
From effect on transient amplitude			
Varying lactate§		— and 119	
Nucleotide fluorescence			
From effect on steady-state rate		57	
From effect on transient amplitude		27¶	
Nucleotide extinction			
By varying lactate§			
From effect on transient rate		— and 65	
From effect on transient amplitude		— and 94	

* At 20 mM-lithium lactate (e.g. see Fig. 13).

† At 25 μM - and 1 mM-oxamate at pH 6 and 7 (e.g. see Fig. 14).

‡ At 50 μM -oxamate concentration, which was a little too high for measuring tight binding constant only.

§ At 1 mM-oxamate.

|| At 0–50 μM -oxamate and 20 mM-lactate; treated as non-competitive inhibitor with respect to lactate.

¶ At 0–50 μM -oxamate and 20 mM-lactate; treated as non-competitive inhibitor with respect to lactate. The difference between this K_i value and that determined from the effect on steady-state rate is that the transient amplitude is affected by added oxamate not only by formation of $\text{E}^{\text{NADH}}_{\text{Oxamate}}$ which does not fluoresce, but by preventing formation of $\text{E}^{\text{NADH}}_{\text{Lactate}}$ which fluoresces.

amplitude by protein-fluorescence changes, on transient rate and amplitude by nucleotide-extinction changes and on steady-state rate and transient amplitude by nucleotide fluorescence. Oxamate was treated as a non-competitive inhibitor in evaluating its effect on the nucleotide fluorescence parameters. All methods gave substantially the same value(s) for K^{oxamate} .

If, as indicated above, oxamate is competitive with lactate because both bind to E^{NAD^+} , then oxamate should be an uncompetitive inhibitor with respect to NAD⁺. This is the case, as shown by the data in Fig. 15, demonstrating the effect of oxamate at 0, 25 μM and 1 mM on the transient rate of NADH formation as a function of NAD⁺. At 1 mM-oxamate and 50 mM-lactate, oxamate is strictly uncompetitive with NAD⁺. At 25 μM -oxamate and 6 mM-lactate, the data do not show strict uncompetitive behaviour between oxamate and NAD⁺. It was necessary to use less than a saturating concentration of lactate [$K_m = 1.61$ mM at pH 6 in absence of oxamate, and 6.6 mM (calculated) in presence of 25 μM -oxamate] to show clearly the degree of inhibition desired in these studies, and this undoubtedly contributed to the observed results.

From the data shown in Fig. 15, K_m for NAD⁺ at pH 6 and saturating lactate concentration was 0.22 mM. At pH 7 and 8, K_m for NAD⁺ was 0.12 ± 0.05 and less than 0.2 mM respectively.

These experiments measuring the effect of oxamate on the transient rate of NADH production during lactate oxidation show that oxamate competes with lactate for binding to lactate dehydrogenase and shows two separate binding constants, but does not compete with NAD⁺.

Kinetics of enzyme–NADH complex-formation

Protein-fluorescence changes were used for determining the rate of formation and dissociation of the complex of pig heart lactate dehydrogenase with NADH. The evaluation of a second-order rate constant of a reversible association is easier and more accurate under conditions where the reverse rate is negligible compared with the rate of association (Gutfreund, 1972, p. 122). For determining the rate of complex-formation a solution containing 0.4 μM -enzyme in 0.1 M-phosphate buffer, pH 7, was mixed with a solution of 2.4 μM -NADH and 10 mM-oxamate in the same buffer. In the presence of oxamate and with excess of NADH the rate of dissociation of

Fig. 15. Reaction of lactate dehydrogenase with NAD^+ and lactate studied by measuring the rate of the transient as a function of $[\text{NAD}^+]$ in the presence and absence of oxamate monitored by protein fluorescence

(a) The reaction mixture contained $1 \mu\text{M}$ -pig heart lactate dehydrogenase from one syringe and 50 mM -lithium lactate, various concentrations of NAD^+ and 1 mM -oxamate [Δ (apparent K_m 0.089 mM , apparent maximum transient rate 41.5 s^{-1}), or zero oxamate [$\circ$ (apparent K_m 0.22 mM , apparent maximum transient rate 105 s^{-1})] from another syringe in 0.1 M -phosphate buffer, pH 6, at 21°C . (b) The reaction mixture contained $1 \mu\text{M}$ -pig heart lactate dehydrogenase from one syringe and 6 mM -lithium lactate, various concentrations of NAD^+ and $25 \mu\text{M}$ -oxamate [$\square$ (apparent K_m 0.15 mM , apparent maximum transient rate 53.2 s^{-1})] or zero oxamate [$\bullet$ (apparent K_m 0.13 mM , apparent maximum transient rate 75.9 s^{-1})] from another in 0.1 M -phosphate buffer, pH 6, at 21°C . Transient rates for both sets of data were calculated by the method described in the legend to Fig. 9 and plotted as reciprocal rate against reciprocal NAD^+ concentration.

NADH is slowed down and the reaction goes to complete saturation of enzyme with NADH in a single exponential step as shown in Fig. 16. The association constant, k_1 , was determined to be $2.77 (\pm 0.14) \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ at pH 7 and $3.08 (\pm 0.23) \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ at pH 6.

As described by Gutfreund (1972, p. 205) the dissociation constant for

Fig. 16. Rate of association of lactate dehydrogenase with NADH at pH 7 monitored by protein fluorescence

(a) The reaction mixture contained $0.2 \mu\text{M}$ -pig heart lactate dehydrogenase (final subunit concentration) from one syringe and $1.2 \mu\text{M}$ - NADH and 5 mM -oxamate from another in 0.1 M -phosphate buffer at 21°C . The reaction follows pseudo-first-order kinetics because of the excess of NADH , and the derivative plot (b) is calculated by the method given in the legend to Fig. 9. The observed rate constant is 33.4 s^{-1} and the second-order rate constant for the reaction is $2.78 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$.

can be studied by relaxation experiments in which the concentration of the complex is suddenly changed by dilution with buffer or with buffer containing NaCl . [NaCl is more effective than other salts in dissociating the complex (Stinson & Holbrook, 1973).] A typical example of this type of experiment is shown in Fig. 17(a). Values for k_{-1} at pH 6 and 7 obtained by dilution with buffer and with buffer containing NaCl are given in Table 5. These values agree well with that of $50 \pm 10 \text{ s}^{-1}$ reported by Heck *et al.* (1968).

The rate of dissociation of NADH from enzyme complex can also be determined by use of an excess of a displacing agent (Gutfreund, 1974). Two displacing agents were used in this work, adenosine

Fig. 17. Rate of dissociation of NADH from pig heart lactate dehydrogenase at pH 7 monitored by protein fluorescence

In the relaxation experiment (a) the mixture contained $1\text{ }\mu\text{M}$ each of pig heart lactate dehydrogenase (final subunit concentration) and NADH from one syringe and an equal volume of buffer from another, in 0.1 M -phosphate buffer at 21°C . The change in enzyme liganding is small (approx. 20%), so that the fluorescence change observed is approximately equal to change in E-NADH concentration. A Guggenheim plot (a') is calculated (Guggenheim, 1926) giving a reciprocal relaxation time of 65.5 s^{-1} and k_{-1} calculated as described in Table 5 is 48.3 s^{-1} . In the displacement experiment (b) the mixture contained $2\text{ }\mu\text{M}$ -pig heart lactate dehydrogenase (final subunit concentration) and $1.8\text{ }\mu\text{M}$ -NADH from one syringe and 1.5 mM -ADP-ribose from another in 0.1 M -phosphate buffer at 21°C . The derivative plot (b') was calculated by the method in the legend to Fig. 9, and gave a dissociation constant of 53.4 s^{-1} . Photograph (b) contains two superimposed traces.

diphosphoribose (ADP-ribose) and NAD^+ . A typical trace for the displacement of NADH from enzyme by excess of ADP-ribose is shown in Fig. 17(b). The reaction, monitored by change in protein fluorescence, is described by a single exponential. Typical values for k_{-1} determined by ADP-ribose and NAD^+ displacement are shown in Table 5. The k_{-1} determined by NAD^+ displacement is slightly higher than the values determined by ADP-ribose displacement or by dilution. The difference may be due to slow turnover in the presence of NAD^+ because of 'endogenous lactate', or it may be due to some secondary effect of NAD^+ (Heck *et al.*, 1968). The same rate for displacement of NADH by NAD^+ was determined by both protein fluorescence and nucleotide fluorescence.

Competition between two enzymes for a common ligand can also be used as a method for determining k_{-1} . Horse liver alcohol dehydrogenase and acetaldehyde, both in large excess, were used to remove NADH from pig heart lactate dehydrogenase. The

reaction was monitored by nucleotide fluorescence. The value of $45 \pm 5\text{ s}^{-1}$ (Table 5) for k_{-1} is in agreement with that determined by the other methods.

Discussion

A record, on a millisecond time scale, of changes in extinction at 340 nm , when pig heart lactate dehydrogenase is mixed with saturating concentrations of NAD^+ and lactate in a stopped-flow spectrophotometer, can be interpreted in terms of the time-course of the formation of the product, NADH, in all its bound and free forms. From such observations it was concluded that some enzyme-bound NADH is formed with a half-time of less than 1 ms . This essentially instant reaction (phase 1) is followed by a slower one, which results in almost complete occupation of enzyme sites by NADH (phase 2) before a third phase, the steady-state rate of NADH formation. A record of the formation of free protons, monitored spectrophotometrically in the presence of

The relative fluorescence of the five forms of enzyme during progressive ligand binding is 1, 0.59, 0.35, 0.20 and 0.12 (Holbrook, 1972). Well-purified NAD^+ quenches the fluorescence of the enzyme by less than 5% when observed within a few milliseconds after mixing in the stopped-flow apparatus. Some slow quenching of the protein fluorescence occurs after NAD^+ binding is completed, and this can be attributed to NADH formation by substrate present as an impurity in the enzyme- NAD^+ system. This is borne out by the appearance of nucleotide fluorescence and extinction at 340 nm during the time-course of the decrease in protein fluorescence.

Observations of protein-fluorescence changes on mixing enzyme with NAD^+ and lactate in the stopped-flow fluorimeter give accurate information about phases 1 and 2 of the reaction. The amplitude of phase 1 and the rate and amplitude of phase 2 have to be calculated after converting protein fluorescence into a record of fractional occupation of sites as a function of time by the method of Holbrook (1972). The first-order plot obtained from the latter derivative data are extrapolated by 1.0 ms (the dead-time of the fluorescence stopped-flow machine) to obtain the amplitude of phase 2. The amplitude of the instant reaction (phase 1) is obtained in terms of sites occupied by NADH by subtracting the amplitude of phase 2 from the total fluorescence change when unliganded enzyme is saturated with NADH. At saturating lactate concentrations, the sum of the amplitudes of phases 1 and 2 corresponds to full quenching of the enzyme with NADH, and no subsequent turnover is seen. This suggests that the rate determining step in the steady-state turnover involves $\text{E}^{\text{NADH}}_{\text{Pyruvate}}$, $\text{E}^{\text{NADH}}_{\text{Lactate}}$ or some intermediate step between the two. Oxamate prevents dissociation of NADH from the enzyme, but the amplitude of the transient seen in the presence of 1 mM-oxamate and 20 mM-lactate is unchanged, lending further support to the above suggestion. The different values for the amplitudes of phase 1 obtained at pH 6, 7 and 8 are significant, but they are small when one considers that there is only a threefold change over a hundred-fold change in H^+ concentration. This finding is further evidence for the proposal that the proton produced is not in equilibrium between the ternary complex and the medium. It is a very interesting finding that the equilibrium constant for the reaction:

at pH 7 is 0.25, whereas the equilibrium constant for the reaction:

at the same pH is approx. 4×10^{-5} . This phenomenon of the effect on reaction equilibria of binding reac-

tants to the active site of a protein has also been observed in this laboratory by Bagshaw & Trentham (1973) for the equilibrium:

when all reactants are bound to the active site of myosin. When the kinetics of phase 2 are considered in conjunction with the amplitude of the pre-equilibration of $\text{E}^{\text{NADH}}_{\text{Pyruvate}}$ in phase 1, it turns out that the true rate constant for dissociation of pyruvate from its complex with enzyme is pH-independent ($k = 1200 \text{ s}^{-1}$):

$$k = k_{\text{obs}}(1 + K)/K,$$

$$\text{where } K = [\text{H}^+ \text{E}^{\text{NADH}}_{\text{Pyruvate}}] / [\text{E}^{\text{NAD}^+}_{\text{Lactate}}]$$

It is of interest that the pH-dependences of the equilibrium $[\text{H}^+ \text{E}^{\text{NADH}}_{\text{Pyruvate}}] / [\text{E}^{\text{NAD}^+}_{\text{Lactate}}]$ and the steady-state rate are similar and change only approximately threefold for a 100-fold change in proton concentration. The pH-dependence of the steady-state rate is probably a combination of several factors including the effect of pH on formation of the abortive ternary complex $\text{E}^{\text{NADH}}_{\text{Lactate}}$ and on the rate of the conformation change $\text{E}^{\text{NADH}}_{\text{Lactate}}$ to $\text{E}^{\text{NADH}}_{\text{Pyruvate}}$. The rate of dissociation of NADH from E^{NADH} appears to be constant at $50 \pm 10 \text{ s}^{-1}$ at pH 6-8.

The investigation of the kinetics of the formation of the enzyme-NADH complex revealed a number of interesting features, especially if taken in conjunction with Holbrook's (1972) studies of the equilibrium binding and its fluorescence characteristics. The reaction can be followed either by the 3.5-fold enhancement of NADH fluorescence or the 88% quenching of protein fluorescence on binding the reduced nucleotide to all four sites of the enzyme molecule. The latter procedure was found to be more suitable for the measurements of association rates under pseudo-first-order conditions (concentration of NADH in six-fold excess of enzyme sites). A series of measurements at pH 6 and 7 in the presence and absence of oxamate gave data for the association rate constant within the range $4 \times 10^7 \pm 1 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$. The report of Everse & Kaplan (1973) that two separate steps are observed in the association of NADH with chicken heart lactate dehydrogenase is the result of misinterpretation of their experimental data. It has been shown by Gutfreund (1972, p. 122) that a second-order reaction, under conditions of concentrations which result in 80% or less completion on equilibrium being reached, gives a first-order-reaction profile,

This is the reason for the first-order process observed by nucleotide fluorescence enhancement. Under identical conditions an apparently second-order profile was obtained from the record of protein-fluorescence quenching. This is due to the non-linear effects of successive site occupation by NADH on the protein fluorescence (Holbrook, 1972). There is no evidence of more than one step being involved in the association of the pig heart lactate dehydrogenase with NADH.

Further information and proof of the above statements are obtained from the fact that essentially the same value for the dissociation-rate constant was obtained by three different methods: displacement, relaxation and removal of free NADH. The values for the dissociation rate constant fall within the range $50 \pm 10 \text{ s}^{-1}$. Also the equilibrium dissociation constant (Holbrook, 1972) is in good agreement with the kinetic ratio k_{-1}/k_1 . The fact that dissociation from the fully liganded enzyme (displacement method) and sequential dissociation (NADH-removal method) give the same rate constant indicates that the rates of dissociation from the four sites of the enzyme molecule are identical. All these results indicate that NADH binds in a single-step process to four identical non-interacting sites of pig heart lactate dehydrogenase.

Investigation of the reaction of the enzyme-NADH complex with excess of pyruvate gave very similar results with H_4 lactate dehydrogenase, when compared with those previously obtained with M_4 lactate dehydrogenase (Stinson & Gutfreund, 1971) and horse liver alcohol dehydrogenase (Shore & Gutfreund, 1970). The evidence presented above shows clearly that the four E^{NADH} units within a tetramer react at identical rates independently of each other. For this conclusion the kinetic evidence has to be taken in conjunction with the evidence of Holbrook (1972) that the four NADH-binding sites are identical and independent. The absence of a kinetic deuterium-isotope effect in the single turnover

suggests that an isomerization rather than the hydride-transfer process is rate limiting. Further investigation of the nature of this isomerization will have to be carried out together with the elucidation of the role of the proton uptake in this process.

There is some evidence that, during lactate oxidation, an isomerization occurs after pyruvate and the proton have dissociated from the ternary complex and before the dissociation of NADH. Holbrook & Gutfreund (1973) have summarized the evidence that the dissociation rate:

is not limiting for lactate oxidation. The experiments reported here also indicate that below pH 7 the rate constant k_{-1} is larger than k_{cat} . Yet in the steady state all the enzyme sites occur in some form of E^{NADH} complex. The situation is complicated by the fact that at high lactate concentration some abortive ternary complex $\text{E}^{\text{NADH}}_{\text{Lactate}}$ is formed.

Bennett & Gutfreund (1973) showed that non-competitive inhibitors, such as oxamate for lactate oxidation, can serve as useful tools for kinetic studies of enzyme intermediates. When the rate of dissociation of NADH from the enzyme complex is slowed down by the inhibitor, the rate of formation of E^{NADH} complexes can be studied more conveniently. With M_4 lactate dehydrogenase, trapping of the intermediate in the form $\text{H}^+\text{E}^{\text{NADH}}_{\text{Oxamate}}$ was essential to obtain transient kinetic data (Bennett & Gutfreund, 1973). With H_4 lactate dehydrogenase, transient kinetic investigations could be carried out in the presence and absence of oxamate. This provided interesting information about properties of ternary complexes (the retention of H^+ , fluorescence characteristics), but it also exposed some as yet unexplained properties of the enzyme. Oxamate acted as a competitive inhibitor for lactate in the transient phenomena and there appeared to be two distinct binding constants for oxamate to E^{NAD^+} . These values are approx. $10 \mu\text{M}$ and $125 \mu\text{M}$ at pH 8, and about 30% of transient rate is lost on saturating the first binding site. The fact that oxamate competition slows down the rate of phase 2 of the trace is further evidence that $\text{E}^{\text{NAD}^+}_{\text{Lactate}}$ and $\text{H}^+\text{E}^{\text{NADH}}_{\text{Pyruvate}}$ are in free equilibrium. If this inter-conversion were partly rate limiting for phase 2, then oxamate binding to E^{NAD^+} would diminish the amplitude of phase 2 and not the rate. No satisfactory explanation can yet be put forward for the two binding constants of oxamate to E^{NAD^+} , and this phenomenon requires further study.

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