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The Kinetics of the Reversible Inhibition of Heart Lactate Dehydrogenase through the Formation of the Enzyme–Oxidized Nicotinamide–Adenine Dinucleotide–Pyruvate Compound

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The inhibition of lactate dehydrogenase at high pyruvate concentration was studied in three ways. First, a rapid decrease in the rate of the enzyme reaction was observed; secondly, the rate of formation of a pyruvate–NAD^+ compound was followed by the change in E_{325}; thirdly, the rate of quenching of the protein fluorescence was measured. The data obtained at pH 6·0 at different temperatures and ionic strengths as functions of pyruvate, NAD^+ and enzyme concentrations show that the extent of inhibition can be correlated with the reversible formation of a compound between pyruvate and enzyme-bound NAD^+. It is suggested that the detailed kinetic analysis of the formation of this abortive ternary compound will give pertinent information about properties of the enzyme–NAD^+ compound involved in the normal catalytic process.

The phenomenon of substrate inhibition of pyruvate reduction by lactate dehydrogenase at high pyruvate concentrations has been studied by a number of authors. The contradictory reports reviewed by Vesell & Pool (1966) are likely to be due to the sensitivity of the relation between pyruvate concentration and the extent of inhibition to various conditions of pH, temperature, ionic strength and the proportion of different isoenzymes. In connexion with our study of the kinetics and mechanism of the individual steps of the reactions catalysed by lactate dehydrogenase, we wished to obtain kinetic data for the formation of the inhibited compound of this enzyme with NAD^+ and pyruvate under precisely defined conditions. The formation of an abortive ternary complex between LDH,* NAD^+ and pyruvate with a distinctive absorption spectrum had been suggested by Fromm (1961). In the present study we correlate changes in the spectrum of the nucleotide and the fluorescence of the protein with the extent of inhibition and the rate at which the inhibited steady state is reached at various pyruvate concentrations.

METHODS AND RESULTS

Enzyme solutions. All the experiments reported here were carried out with pig heart LDH (batch LDH-H15352) obtained as a suspension in $(NH_4)_2SO_4$ solution from Boehringer Corp. (London) Ltd., London, W. 5. This preparation was chosen because it contains less than 5% of isoenzymes other than isoenzyme H4 (isoenzyme 1), and its response to pyruvate inhibition was indistinguishable from that of the pure isoenzyme 1 prepared from bovine heart LDH by Hathaway & Criddle (1966) or the pure pig heart isoenzyme 1 supplied by Boehringer Corp. (London) Ltd. It was pointed out by Hathaway & Criddle (1966) that the contamination of the heart enzyme with isoenzyme 5 from skeletal muscle causes considerable changes in a number of physical properties of lactate dehydrogenase.

Enzyme stock solutions were prepared by removal of $(NH_4)_2SO_4$ by passing the dissolved protein through a Sephadex G-25 column equilibrated with 0·2M-sodium phosphate buffer, pH 6·0, containing 0·08M-NaCl. The protein concentration was calculated from measurements of E_{280} by using the value 1·45 for E_{280} of a solution containing 1 mg. of LDH/ml. All experiments were performed on solutions in 0·2M-phosphate buffer, pH 6·0, containing 0·08M-NaCl.

Reagents. Sodium pyruvate, NAD^+ and NADH were obtained from Boehringer Corp. (London) Ltd. We did not study in any detail the possibility that the inhibition of LDH at high pyruvate concentration may be due to some impurity or product of pyruvate in aqueous solution. The proton-magnetic-resonance spectra, studied in collaboration with Dr D. R. Trentham, gave information about the structure and purity of sodium pyruvate in solution. At 38° in D_2O the pyruvate anion existed 93% in the keto form and 7% in the hydrated *gem*-diol form. The enthalpy change associated with the hydration reaction in D_2O is about −9 kcal./mole. A peak in the spectrum representing 1% of the hydrogen of the solute was removed by recrystallization of the pyruvate from water. This recrystallization

* Abbreviation: LDH, lactate dehydrogenase.

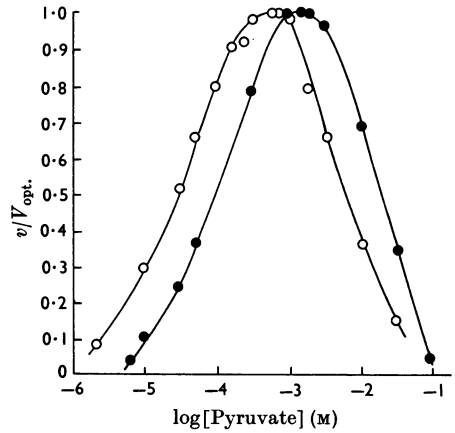


Fig. 1. Effect of pyruvate concentration on the steady-state rates (v) of NADH oxidation catalysed by LDH relative to the optimum rates ($V_{opt.}$) at 24° (○) and 40° (●). The reaction mixture contained phosphate buffer, pH 6·0 (0·2 M), NaCl (0·08 M), NADH (0·15 mM) and enzyme (1–10 μg./ml.).

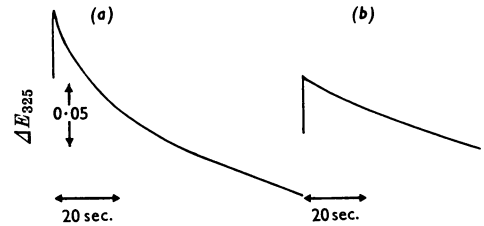


Fig. 2. Records of the change in E_{325} at 12·5° on addition of: (*a*) LDH (1·3 μg./ml.) to a reaction mixture containing NADH (0·15 mM), pyruvate (30 mM), phosphate buffer, pH 6·0 (0·2 M), and NaCl (0·08 M); (*b*) LDH (1·3 μg./ml.), inhibited by incubation in the presence of 0·33 mM-NAD^+ and 30 mM-pyruvate, to a reaction mixture as in (*a*).

had no effect on our enzyme experiments. The stock solutions of pyruvate kept for 1 day gave consistent results in all measurements and showed no change in their proton-magnetic-resonance spectrum. It should be pointed out, however, that nuclear-magnetic-resonance measurements would not normally detect an impurity at a concentration of up to 1%.

Rate of pyruvate reduction as a function of pyruvate concentration. The rate of NADH oxidation linked to pyruvate reduction was measured at 340 mμ in the 1 cm. thermostatically controlled cuvette of a Uvispek spectrophotometer (Hilger and Watts Ltd.) provided with a Gilford recording attachment.

Fig. 1 shows the dependence of the rate of NADH oxidation catalysed by LDH in the presence of pyruvate at concentrations in the range 0·03–30 mM at 24° and 40°. When the rate of NADH oxidation was followed in the presence of 30 mM-pyruvate, an initial decrease in the rate occurred before any appreciable depletion of NADH or pyruvate. This phenomenon was observed more clearly at lower temperatures. The record of the experiment at 12·5° (Fig. 2*a*) shows that the initial velocity with 30 mM-pyruvate approached that observed near the optimum pyruvate concentration (0·3 mM). The deceleration to the inhibited steady state had a half-time of approx. 15 sec. at 12·5°. The initial velocity of the reduction of pyruvate during the first 100 msec. was measured in a stopped-flow apparatus employing the equipment described by Barman & Gutfreund (1966), and it was found that over such periods rates were identical in the presence of 0·3 mM- and of 30 mM-pyruvate. Higher enzyme concentrations (0·1–1 mg./ml.) were used for the stopped-flow experiments compared with 1·3 μg./ml. used for the experiments recorded in Fig. 2. Enzyme concentration had some effect on the rate of inhibition, but formation of the inhibited enzyme still took place at high enzyme concentration. Fig. 2(*b*) shows an experiment in which an enzyme solution (0·1 mg./ml.) was incubated for 2 min. with 0·33 mM-NAD^+ and 30 mM-pyruvate and then a sample was added to a cuvette containing NADH and 30 mM-pyruvate. Incubation of the enzyme with high concentrations of pyruvate inhibited the enzyme only in the presence of NAD^+. Incubation with pyruvate (30 mM) alone before addition to the cuvette resulted in records of the type shown in Fig. 2(*a*). The addition of inhibited enzyme to a cuvette containing NADH, resulting in dilution of pyruvate to its optimum concentration, caused a rapid acceleration of NADH oxidation.

Effect of temperature on the rate of pyruvate reduction. Vesell & Pool (1966) reported that the substrate inhibition of reactions catalysed by LDH decreases as the temperature is raised. This is also shown in Fig. 1. Measurements of the rate of NADH oxidation at various temperatures in the presence of 0·03 mM-, 0·3 mM- and 30 mM-pyruvate were used for the calculation of the Arrhenius activation energies of 6·0, 13·3 and 18·6 kcal./mole respectively.

Rate of formation of an LDH–NAD^+–pyruvate compound. During the incubation of LDH with NAD^+ and pyruvate there occurred an increase in E_{325} and a decrease in the fluorescence of the protein. Fluorescence measurements were carried out in 3 ml. cuvettes at 90° angle in a Farrand Optical Co. (Mount Vernon, N.Y., U.S.A.) fluorimeter. Rates of fluorescence quenching were observed by recording changes in emission at 345 mμ on excitation at 285 mμ.

The rate of formation of the ternary compound was measured both as a function of NAD^+ concentration and as a function of pyruvate concentration by following the change in E_{325}. No difference could be found by changing the order in which the three components were added to the buffer in the cuvette. Variation of the NAD^+ concentration at constant pyruvate concentration (30 mM) showed that the reaction was independent of NAD^+ concentration above 0·2 mM with a half-time of 50 sec. (maximum error 5 sec.) at 24°. The rate decreased at lower NAD^+ concentrations and was approximately halved at 0·02 mM.

The dependence of the rate of increase in E_{325} on the pyruvate concentration at constant NAD^+ (0·5 mM) did not result in a saturation; the rate doubled when pyruvate concentration was increased from 0·03 M to 0·1 M.

The changes in E_{325} were measured on solutions containing 0·6–0·9 mg. of enzyme/ml., but fluorescence changes were measured on much more dilute enzyme solutions (see Fig. 3). When pyruvate was added to a solution of LDH

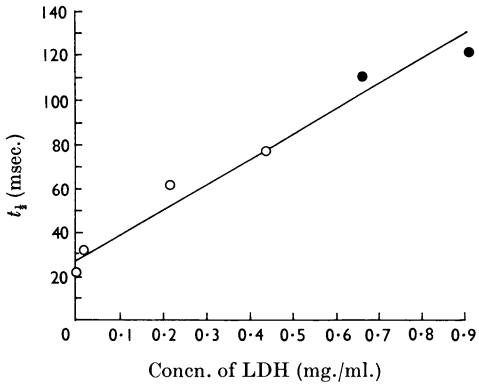


Fig. 3. Relation between the half-time ($t_{\frac{1}{2}}$) of the increase in E_{325} (●), the half-time ($t_{\frac{1}{2}}$) of the fluorescence quenching (○) and the enzyme concentration in a reaction mixture containing phosphate buffer, pH 6·0 (0·2 M), NaCl (0·08 M), pyruvate (10 mM) and NAD^+ (0·1 mM).

(0·01 mg./ml.) and NAD^+ (0·1 mM) the time-course of the decrease in protein fluorescence could be observed. The relation between ternary-compound formation and fluorescence quenching could be obtained if the time-dependent fluorescence change was separated from the instantaneous change due to the light-absorption of the added pyruvate. The titration of enzyme (10 μg./ml.) and NAD^+ (0·1 mM) with pyruvate gave half the maximal time-dependent change when pyruvate had been added to give a concentration of 4 mM. In a similar experiment with a solution containing 0·7 mg. of LDH/ml., the increase in the time-dependent change in E_{325} being measured, the half-maximum of the change in E_{325} occurred when pyruvate had been added to give a concentration of 2 mM.

The reversibility of the reaction involved in fluorescence quenching was studied in an experiment where the rate of fluorescence quenching on addition of 10 mM-pyruvate to a solution containing 0·022 mg. of LDH/ml. and 0·1 mM-NAD^+ was compared with the rate of reappearance of fluorescence when a solution of 0·66 mg. of LDH/ml., 0·1 mM-NAD^+ and 10 mM-pyruvate, incubated for 5 min., was diluted 30-fold into the cuvette of the fluorimeter with 0·1 mM-NAD^+. The half-time of the fluorescence quenching was 30 sec., whereas the half-time of the reappearance of fluorescence on diluting the pyruvate was 50 sec. At this protein concentration one can calculate the equilibrium between the inhibited and the fully active enzyme from the ratio of the forward and reverse reaction rates and the known pyruvate concentration. As the rate of formation of the inhibited form of the enzyme is approximately proportional to the pyruvate concentration when this is less than 30 mM, the equilibrium constant K is given by:

$$K = [\text{Pyruvate}] \times \frac{\text{Rate of formation}}{\text{Rate of decomposition}} = 6 \times 10^{-3}\,\text{M}.$$

This value is in reasonable agreement with the value of 4×10^{-3} M obtained from the mid-point of the fluorescence titration curve under similar conditions, especially when the approximate nature of the dependence on pyruvate concentration is taken into consideration.

The rate of formation of the inhibited complex was also dependent on the enzyme concentration; diluting the enzyme enhanced the rate of the quenching reaction. This was also true of the rate of appearance of the spectral peak at 325 mμ. Though experimental difficulties prevented measurement of the appearance of the spectral maximum and the decrease of fluorescence over identical regions of protein concentration, the rate constants for these two processes measured over the protein concentrations experimentally available indicated that the rate constants for the two are identical, as shown in Fig. 3.

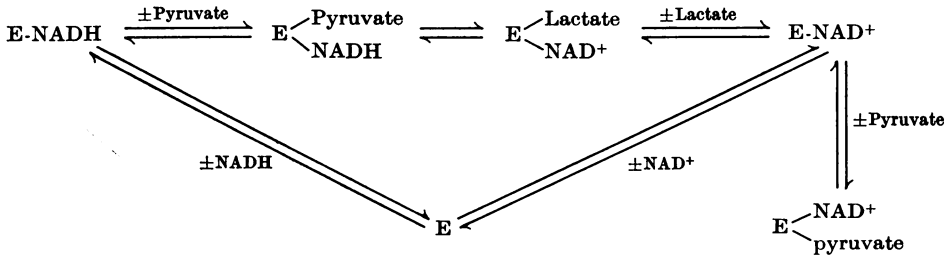
Oxamate is a competitive inhibitor for pyruvate reduction with an affinity for the enzyme that is approximately the same as that of pyruvate. It was found that incubation of enzyme with 0·3 mM-NAD^+ and up to 0·1 M-oxamate had no effect on the rate at which the inhibited ternary compound was formed on subsequent addition of pyruvate to give a concentration 10 mM. This was tested both by observation of fluorescence quenching and by the rate of inhibition of the enzymic steady-state reduction of pyruvate.

DISCUSSION

The experiments reported in the present paper were carried out to obtain further information about the mechanism of the substrate inhibition of lactate dehydrogenase by pyruvate. The suggestion by Fromm (1961) that this phenomenon is due to the formation of an abortive ternary compound between enzyme, NAD^+ and pyruvate made it attractive to study the kinetics of the formation of this compound. Information obtained from such rate studies should prove useful for further investigations of the mechanism of the reaction catalysed by this enzyme.

Direct studies of the initial phase of the reduction of pyruvate as well as spectrophotometric and fluorescence observations show that the rate of inhibition is slow compared with the turnover of the enzyme and that it is concomitant with the formation of a pyruvate–NAD^+ compound. This compound has an absorption band with a peak at 325 mμ and quenches the protein fluorescence of the enzyme. The final E_{325} value at fixed NAD^+ and pyruvate concentrations is proportional to the enzyme concentration. This suggests that the compound only exists in an enzyme-bound form and permits an estimate of $\epsilon_{325}^{1\text{cm.}} = 6 \times 10^3$ for the molar extinction coefficient/active site. This approximate value is based on the assumption of 1 mole of active sites/35 000 g. of LDH.

The concomitance of the three phenomena observed is of course established only circumstantially. The three types of measurements were made at different enzyme concentrations and the rate of formation of the compound was shown to be inversely related to enzyme concentration. Fig. 3 shows that the E_{325} measurements fall on the same



Scheme 1. E, Enzyme.

line as the fluorescence measurements. The time-course of inhibition (Fig. 2*a*) has so far only been measured at LDH concentrations of 1·3 μg./ml. at 12·5° in the presence of 30 mM-pyruvate, and it can be concluded, from the mechanism suggested below, that its time constant need not be identical with that of the formation of the compound from LDH, NAD^+ and pyruvate.

The reversibility of the formation of the inhibited ternary compound of LDH was established by the rapid acceleration when enzyme incubated with inhibitory concentrations of NAD^+ and pyruvate was diluted into a reaction mixture to give optimum concentrations of all components for the reduction of pyruvate. Similarly we were able to study the reversal of the fluorescence quenching.

Though the K_m for pyruvate for enzyme-catalysed reduction is 3×10^{-5} M, no dissociation or steady-state constant could be determined for pyruvate for the reaction involved in the formation of the ternary compound with enzyme and NAD^+. At pyruvate concentrations above 30 mM the rate is not directly proportional to the pyruvate concentration, but still doubles when the pyruvate concentration is increased from 30 mM to 0·1 M. This, together with the fact that oxamate does not inhibit formation of the compound, indicates that the pyruvate-binding site for the enzymic reduction of this substrate is not involved in formation of the ternary compound. The effect of changing NAD^+ concentration on the formation of the compound from LDH, NAD^+ and pyruvate indicates a dissociation constant of approx. 2×10^{-5} M, which is similar to the K_m for the enzymic reduction of NAD^+.

It is suggested that Scheme 1 can be used to interpret the results so far obtained, as well as for planning further experiments.

The inhibition of the steady-state enzymic reduction of pyruvate is studied under conditions when the NADH concentration is large compared with its enzyme dissociation constant, and no free enzyme need be considered. The degree of inhibition depends on the competition between the cyclic process of interconversion of enzyme–NADH and enzyme–NAD^+ and the equilibrium between enzyme–NAD^+ and abortive ternary compound enzyme$<^{NAD^+}_{pyruvate}$. Our findings that the rate of inhibition of the enzyme is approximately the same as the rate of formation of the ternary compound from enzyme saturated with NAD^+ at equivalent pyruvate concentration, and that the percentage inhibition follows closely the percentage of fluorescence quenching at equivalent pyruvate concentration, suggest that during steady-state pyruvate reduction most of the enzyme is in the form enzyme–NAD^+. The conclusion that the dissociation of the enzyme–NAD^+ compound is rate-determining for the enzymic oxidation of NADH to NAD^+ is compatible with the fact that the dissociation constant for NADH ($3{\cdot}9 \times 10^{-7}$ M) determined by Anderson & Weber (1965) is much smaller than the K_m (1×10^{-5} M). This suggests that the K_m is a steady-state constant and that the interconversion of enzyme$<^{pyruvate}_{NADH}$ and enzyme$<^{lactate}_{NAD^+}$ is very fast compared with the dissociation of enzyme–NADH. The rate of dissociation of enzyme–NADH has been determined by stopped-flow techniques (Gutfreund, 1967) to be 40 $sec.^{-1}$; this is rate-determining for the reverse reaction, the oxidation of lactate. It would be premature to go further with the numerical analysis of the individual rate constants before several of the interactions between the two members of each pair of substrates have been studied under a wider range of conditions. Some technical difficulties in measuring the rate of inhibition and the formation of the ternary compound under identical conditions have also to be overcome.

At this stage of the work it is not possible to draw any conclusions about the nature of the compound formed between pyruvate and enzyme-bound NAD^+. It is clear, however, that a study of this reaction offers a useful tool for the exploration of the mechanism of this enzyme.

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REFERENCES

Anderson, S. R. & Weber, G. (1965). *Biochemistry*, **4**, 1948.

Barman, T. E. & Gutfreund, H. (1966). *Biochem. J.* **101**, 411.

Fromm, J. H. (1961). *Biochim. biophys. Acta*, **52**, 199.

Gutfreund, H. (1967). *Proc. 5th Nobel Symp.*, p. 429. Uppsala: Almqvist and Wiksell.

Hathaway, G. & Criddle, R. S. (1966). *Proc. nat. acad. Sci., Wash.*, **56**, 680.

Vesell, E. S. & Pool, P. E. (1966). *Proc. nat. acad. Sci., Wash.*, **55**, 756.