The Lactate Dehydrogenase–Reduced Nicotinamide–Adenine Dinucleotide–Pyruvate Complex

KINETICS OF PYRUVATE BINDING AND QUENCHING OF COENZYME FLUORESCENCE

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The stopped-flow kinetic studies described in this and the following paper (Südi, 1974) demonstrate that a Haldane-type description of the reversible lactate dehydrogenase reaction presents an experimentally feasible task. Combined results of these two papers yield numerical values for the six rate constants defined by the following equilibrium scheme, where E represents lactate dehydrogenase:

The experiments were carried out at pH 8.4 at a relatively low temperature (6.3°C) with the pig heart enzyme. Identification of the above two intermediates and determination of the corresponding rate constants actually involve four series of independent observations in these studies, since (a) the reaction can be followed in both directions, and (b) both the u.v. absorption and the fluorescence of the coenzymes are altered in the reaction, and it is shown that these two spectral changes do not occur simultaneously. Kinetic observations made in the reverse direction are reported in this paper. It is demonstrated that the fluorescence of NADH can no longer be observed in the ternary complex E_{Pyr}^{NADH} . Even though the oxidation–reduction reaction rapidly follows the formation of this complex, the numerical values of k_{-4} ($8.33 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$) and k_{+4} (222 s^{-1}) are easily obtained from a directly observed second-order reaction step in which fluorescent but not u.v.-absorbing material is disappearing. U.v.-absorption measurements do not clearly resolve the subsequent oxidation–reduction step from the dissociation of lactate. It is shown that this must be due partly to the instrumental dead time, and partly to a low transient concentration of $E_{Lac}^{NAD^+}$ in the two-step sequential reaction in which the detectable disappearance of u.v.-absorbing material takes place. It is estimated that about one-tenth of the total change in u.v. absorption is due to a ‘burst reaction’ in which $E_{Lac}^{NAD^+}$ is produced, and this estimation yields, from $k_{obs.} = 120 \text{ s}^{-1}$, $k_{-2} = 1200 \text{ s}^{-1}$.

Owing to the favourable position of the overall equilibrium, NADH bound to lactate dehydrogenase (EC 1.1.1.27) can be completely oxidized even with a stoichiometric amount of pyruvate. This reaction can be carried out with an excess of enzyme over NADH, in which case a single enzymic turnover will be operating (Criddle *et al.*, 1968). Moreover, if the reaction is initiated by rapidly mixing enzyme + NADH with pyruvate, and the concentrations of enzyme and NADH are of the order of $10 \mu\text{M}$, one of

the reactants will be the binary complex E^{NADH} because of the strong binding of reduced coenzyme ($K_d = \text{approx. } 4.5 \times 10^{-7} \text{ M}$; see Südi, 1974). With reference to a Haldane (1930)-type minimal mechanism, one can describe such single-turnover reactions by Scheme 1. Three equilibrium constants can be derived from Scheme 1 and these are defined as $K_{d4} = k_{+4}/k_{-4}$; $K_3 = k_{+3}/k_{-3}$; and $K_{d2} = k_{-2}/k_{+2}$. The time-course of this reaction can be studied with a stopped-flow spectrophotometer (fluorimeter), by recording the disappearance of the characteristic u.v. absorption and/or fluorescence of E^{NADH} . The results of such studies carried out at a relatively low temperature (6.3°C) are reported below.

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Scheme 1.

Scheme 1 defines two intermediates and five rate constants. In order to obtain a kinetic description of the overall reaction at this level of resolution, one should be able to observe the formation and decomposition of these two intermediates. The feasibility of this obviously depends not only on the kinetic characteristics of the system, but also on the spectral characteristics of E_{Pyr}^{NADH} and $E_{Lac}^{NAD^+}$, and this additional information must be derived from the kinetic observations themselves. An apparently satisfactory solution to this quite complex kinetic puzzle is presented in this and the following paper (Südi, 1974). The latter paper deals with the same reaction in the opposite direction and yields complementary information, since it follows from the principle of microscopic reversibility that the forward and reverse reactions involve the same two intermediates and the same four first-order rate constants (see Scheme 1). A preliminary account of this work has been presented (Südi, 1972).

The kinetic problem outlined in Scheme 1 and in the foregoing discussion is not a special characteristic of the lactate dehydrogenase reaction. Indeed, it is generally recognized (e.g. Reiner, 1964; Plowman, 1972) that the reversible binding of the 'last substrate' and the 'first product' of all reversible enzyme-catalysed reactions are coupled through a reversible reaction step in which the reaction proper takes place. The recognition of this fact is attributed to Haldane (1930), and kinetic descriptions of the type of Scheme 1 are generally referred to as Haldane-type, as opposed to Michaelis-Menten-type schemes. It follows from the foregoing considerations that the experimental approach that is presented in this and the following papers (Südi, 1974) might prove to be of more general interest, since similar studies with the stopped-flow method have not yet been carried out.

Experimental

Materials

Pig heart lactate dehydrogenase was obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. The crystalline precipitate of these preparations was directly dissolved in 0.1 M-Tris-HCl buffer, pH 8.0 (at 22°C), which was the buffer used in all experiments. The pH of this buffer at the temperature of the kinetic experiments (6.3°C) was 8.4. Gel filtration for the removal of residual $(NH_4)_2SO_4$ was

found not to affect reproducibility of the first results and was later omitted. Enzyme concentration was determined from an absorbance of 1.29 at 280 nm for a 1 mg/ml solution. The number of active sites was checked by the method of Pfeleiderer *et al.* (1957) with the enzyme- NAD^+ -sulphite complex. Enzyme concentrations refer to the monomer, mol.wt. 35000. NAD^+ , NADH and sodium pyruvate were obtained from Boehringer and sodium L-lactate was purchased from Th. Schuchardt, München, Germany.

Apparatus

The stopped-flow instrument used was a sensitive workshop construction designed by Dr. D. Riesner, for both absorption and fluorescence measurements over a wide range of temperature. Throughout these studies, both the optical cell and the mixing syringes were thermostatically controlled at 6.3°C, which was the value determined for the reaction mixture itself. The optical cell had an inner diameter of 2 × 2 mm. A 75 W xenon lamp was used as the light-source. A Bausch and Lomb (Rochester, N.Y., U.S.A.) monochromator was inserted between the lamp and the optical cell and there was a narrow optical slit and silica lenses both before and after the monochromator. In a number of experiments the change in both u.v. absorption and fluorescence was determined with the same filling of the mixing syringes. In this case, two photomultipliers were attached to the optical cell, one at 180° (absorption) and the other at 90° (fluorescence) to the light-path. For fluorescence measurements a KV-408 filter (50% transmission at 408 nm, sharp cut-off of light of lower wavelengths; Jenaer Glaswerk, Schott and Gen., Mainz, Germany) was mounted between optical cell and photomultiplier. For recording the optical change after mixing of the two solutions, storage oscilloscope(s) were connected to the photomultipliers. It was of great advantage to record individual 'shots' with two different time axes or rise times and possibly even sensitivities. For this purpose, either two single-beam oscilloscopes, or a double-beam oscilloscope (Tetronix Types 549 and 5103) were connected to the photomultiplier. The oscillograms were recorded photographically. Equal volumes were mixed in all experiments. The total mixed volume was about 0.3 ml. All concentrations specified in the text refer to those initially present in the optical cell. The experimental dead time of the instrument was 2.5 ms at the temperature of these experiments.

* Abbreviation: E, enzyme (lactate dehydrogenase).

Presentation of results

The reported progress curves could be all evaluated as simple exponentials (Fig. 2), as two consecutive first-order reactions (Fig. 5), or as simple second-order reactions (Fig. 1). The observations made are determined by two characteristics of these reactions: (a) the experimental rate constant(s), and (b) the amplitudes of the experimentally separable reaction steps.

In nearly all experiments, fluorescence was excited at four different wavelengths; 280, 300, 330 and 370 nm. All observations reported were independent of the wavelength of excitation, with the exception of the signal-to-noise ratio, which was related to the internal filter effects of the reaction mixture.

Results and Discussion

Fig. 1 shows the time-course of the oxidation of $5 \mu\text{M-E}^{\text{NADH}}$ by $5 \mu\text{M}$ -pyruvate. Under such conditions a simple second-order reaction is observed ($k_{\text{obs}} = 5.0 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$; Fig. 1a) and the kinetics obtained by monitoring the u.v. absorption or the fluorescence of enzyme-bound NADH cannot be distinguished (Fig. 1b).

As the concentration of pyruvate is increased to above a threshold value of about $20 \mu\text{M}$, u.v.-absorption (Figs. 2 and 3) and fluorescence (Figs. 4 and 5) kinetics of the reaction become distinctly different. Fig. 2 shows the time-course of the reaction with $50 \mu\text{M-E}^{\text{NADH}}$ and 0.1 M -pyruvate, as indicated by the rate of disappearance of the u.v. absorption of enzyme-bound NADH. Analysis of the time-course shown in Fig. 2(a) yields a single first-order rate constant, $k_R = 120 \text{ s}^{-1}$ (Fig. 2b). One can correct the observed amplitude of change (Fig. 2a) for the dead time of the stopped-flow device (2.5 ms) by simple graphical extrapolation (Fig. 2b). The amount of u.v.-absorbing material that disappears with $k_R = 120 \text{ s}^{-1}$ (at pyruvate saturation, Fig. 2) cannot at present be given with any greater precision than $90 \pm 10\%$ of the total oxidizable material at low pyruvate concentration (Fig. 1). As seen from Fig. 2(b) estimation of the amplitude of a kinetically observed reaction, which is as fast as k_R , requires much more care than a determination of the numerical value of the corresponding rate constant (see the section on stoichiometry in Südi, 1974). In addition, the reproducibility of amplitude determinations between experiments shows a variation of about $\pm 10\%$ of the average. This high variation appears to be due, at least partly, to some not quite clearly identified mechanical properties of the stopped-flow instrument. A third point which is relevant to this question is the fact that the molar absorptions of $\text{E}^{\text{NADH}}_{\text{Pyr}}$ and $\text{E}^{\text{NAD}^+}_{\text{Lac}}$ cannot be precisely estimated (see below). For these reasons, the

amplitude of the k_R reaction (Figs. 2 and 3) will be considered below in conjunction with further relevant observations as if obtained with no higher precision than 0.9 ± 0.1 . However, it should be noted that the values obtained in three independently controlled experiments, each of which involved more than 20 determinations, were 0.87, 0.89 and 0.94.

It could be easily demonstrated that the absorption change shown in Fig. 2 was completed with the rate constant k_R , i.e. that it was not followed by a (slower) second reaction step. However, because of the dead time of the instrument, no kinetic observation could be made during the first 2.5 ms after mixing. Therefore the observed reaction (k_R , Fig. 2) could have been preceded by a faster reaction step which was completed within the dead time and this would account for the

Fig. 1. Oxidation of $5 \mu\text{M-E}^{\text{NADH}}$ by $5 \mu\text{M}$ -pyruvate

The decrease in u.v. absorption ($330 \mu\text{m}$) was measured. (a) Oscillogram of the time-course of the reaction; photomultiplier voltage (10 mV/cm) against time (0.5 s/cm). (b) Evaluation of the same time-course as a second-order reaction (O); $y/(a-y)$ against time (s), where a = total amplitude of the observed reaction. (●) In (b) are points obtained from another time-course of the same reaction followed by monitoring fluorescence excited at 330 nm (and observed simultaneously with u.v. absorption; see the Experimental section for a description of the apparatus).

Fig. 2. Oxidation of $50\text{ }\mu\text{M-E}^{\text{NADH}}$ by 0.1 M-pyruvate

The decrease in u.v. absorption ($330\text{ }\mu\text{m}$) was measured. (a) Oscillogram of the time-course of the reaction; photomultiplier voltage (100 mV/cm) against time (5 ms/cm). (b) Evaluation of the same time-course as a single exponential reaction; $\log y$ against time (ms). Ordinate intercepts at $t = 0$ and $t = -2.5\text{ ms}$ give the logarithms of the amplitude of the observed reaction and of the total reaction corrected for the dead time of the instrument respectively.

observation that the total amplitude of the k_R process was less than stoichiometric.

Scheme 1 shows the importance of the question of whether the single first-order reaction step observed at high pyruvate concentration (Fig. 2) is preceded by a faster reaction step. If the disappearance of the 330 nm absorption is coincident with the reversible oxidation-reduction step (Scheme 1), the time-course of the overall reaction should, in principle, be biphasic, i.e. the reversible formation of the reaction intermediate $\text{E}_{\text{Lac}}^{\text{NAD}^+}$ should precede the practically irreversible step of lactate dissociation (Scheme 1). At saturating pyruvate concentrations one obtains the maximal transient concentration of $\text{E}_{\text{Lac}}^{\text{NAD}^+}$, and this can be expressed as the product

$$[\text{E}^{\text{NADH}}]_0 \times \frac{\text{amplitude of the fast reaction step}}{\text{total amplitude}}$$

Therefore, our experimental finding that $90 \pm 10\%$ of the total reaction takes place with a single first-order rate constant of 120 s^{-1} sets the limits of $0\text{--}20\%$ of $[\text{E}^{\text{NADH}}]_0$ as the maximal permissible transient concentration of $\text{E}_{\text{Lac}}^{\text{NAD}^+}$.

Fig. 3 shows the pyruvate concentration-dependence of the single first-order rate constant (k'_R) which is observed if pyruvate is present in great excess over E^{NADH} . It is seen that k_R (120 s^{-1}), discussed above in connection with Fig. 2, is indeed a saturation value which is observed over the range of pyruvate concentrations of about $2\text{ mM--}0.5\text{ M}$. Fig. 3(b) further shows that k'_R is a hyperbolic function of pyruvate concentration, and that the half-saturating concentration of pyruvate (K_{mR}) appears to be $2.4 \times 10^{-4}\text{ M}$.

It follows that k_R (Fig. 2) and k'_R (Fig. 3) represent the overall rate constant with which the first product, lactate, dissociates (Scheme 1) and also that these overall rate constants refer to the total concentration of coenzyme present as $\text{E}^{\text{NADH}} + \text{E}_{\text{Pyr}}^{\text{NADH}} + \text{E}_{\text{Lac}}^{\text{NAD}^+}$. Accordingly (see the Appendix), Scheme 1 yields the following expressions for k_R , K_{mR} , and the 'second-order catalytic rate constant' k_R/K_{mR} :

$$k_R = 120\text{ s}^{-1} = \frac{k_{-3}k_{-2}}{k_{-2} + k_{-3} + k_{+3}} \quad (1)$$

$$K_{mR} = 2.4 \times 10^{-4}\text{ M} = \frac{k_{+4}(k_{+3} + k_{-2})}{k_{-4}(k_{-2} + k_{-3} + k_{+3})} \quad (2)$$

$$\frac{k_R}{K_{mR}} = 5 \times 10^5\text{ M}^{-1}\cdot\text{s}^{-1} = \frac{k_{-4}k_{-3}k_{-2}}{k_{+4}(k_{+3} + k_{-2})} \quad (3)$$

The second-order catalytic rate constant derived from k_R and K_{mR} (eqn. 3; Fig. 3) must be equal to the apparent second-order rate constant with which E^{NADH} is completely oxidized at low reactant concentrations (e.g. Fig. 1). Since the ratio k_R/K_{mR} yields the same numerical value as Fig. 1, the numerical values of the two parameters calculated from the saturation curve (Fig. 3b) are controlled through an independent determination (Fig. 1). However, since it has been observed that, at pyruvate saturation,

$$(0.9 \pm 0.1) \times \left([\text{E}_{\text{Pyr}}^{\text{NADH}}] + [\text{E}_{\text{Lac}}^{\text{NAD}^+}] \right) = [\text{E}_{\text{Pyr}}^{\text{NADH}}] \quad (\text{Figs. 2 and 3}),$$

this relationship between the rapidly adjusted steady-state concentrations of $\text{E}_{\text{Pyr}}^{\text{NADH}}$ and $\text{E}_{\text{Lac}}^{\text{NAD}^+}$ must also hold under the experimental conditions of Fig. 1.

Fig. 1(b) shows that, if the transient concentrations of both $\text{E}_{\text{Pyr}}^{\text{NADH}}$ and $\text{E}_{\text{Lac}}^{\text{NAD}^+}$ are very low, the time-

Fig. 3. Dependence of the observed single first-order rate constant (k'_R) on pyruvate concentration

The decrease in u.v. absorption at $330 \mu\text{m}$ was measured. (a) Semilogarithmic plot of k'_R against pyruvate concentration. (b) Double-reciprocal plot of k'_R against pyruvate concentration ranging from 0.1 to 50 mM. Concentration of $\text{E}^{\text{NADH}} = 10 \mu\text{M}$.

Fig. 4. Two-step oxidation of E^{NADH} by pyruvate, observed by monitoring the decrease in fluorescence intensity

(a) Dependence of the two rate constants $k'_{R\text{fast}}$ (upper curve) and $k'_{R\text{slow}}$ (lower curve) on pyruvate concentration. (b) Dependence of the corresponding amplitudes on pyruvate concentration. Upper curve (∇): total amplitude, both reaction steps corrected for dead time. Lower curves: amplitude of the fast reaction step with ($\bullet$) and without ($\circ$) dead-time correction.

course of the observable reaction is identical, whether one follows the progress of the reaction by monitoring u.v. absorption or fluorescence. A comparison of Figs. 3 and 4 shows that this is no longer true at higher

pyruvate concentrations, where the transient concentration of $\text{E}^{\text{NADH}}_{\text{Pyr}}$ is no longer negligible. Fig. 4(b) also shows that at substrate saturation, where the total

Fig. 5. Oxidation of $10 \mu\text{M-E}^{\text{NADH}}$ by 0.1 mM-pyruvate

The decrease in fluorescence was measured. (a) Two oscillograms of the time-course of the same reaction, observed with a double-beam oscillograph with identical sensitivity settings (1 mV/cm), but different time axes [5 ms/cm (lower scan), and 20 ms/cm (upper scan)]. (b) Evaluation of the lower scan as a consecutive reaction consisting of two first-order reaction steps; $\log y$ against time (ms).

amount of E^{NADH} must be very rapidly transformed into $\text{E}^{\text{NADH}}_{\text{Pyr}}$ (and $\text{E}^{\text{NAD}^+}_{\text{Lac}}$), the disappearance of the total amount of fluorescent material is completed within the dead time of the instrument. The highest concentration of pyruvate shown in Fig. 4 is 0.5 mM . This is because at still higher concentrations of pyruvate no fluorescence change could be observed at all. The time-course of the disappearance of fluorescence material is biphasic over an intermediate range of pyruvate concentrations, from about 0.04 mM to 0.25 mM (Fig. 4). A representative oscilloscope tracing of such a biphasic time-course is shown in Fig. 5.

The lower of the two experimental rate constants in Figs. 4(a) and 5(b) has a value equal to that which describes the disappearance of u.v.-absorbing material under identical conditions (k'_{R} , Fig. 3a). We have

already seen that the fast step in the disappearance of fluorescence (Figs. 4 and 5) must represent the reversible formation of the two ternary complexes in Scheme 1, of which $\text{E}^{\text{NADH}}_{\text{Pyr}}$ is the major component.

This obviously means that the binding of pyruvate results in a complete quenching of the fluorescence of enzyme-bound NADH.

The experimental points in Fig. 4(a) (upper curve) clearly demonstrate that there is a linear relationship between pyruvate concentration and the pseudo-first-order rate constant that describes the reversible binding of pyruvate. The slope of this linear function should directly yield the second-order rate constant k_{-4} (Scheme 1), and there is a simple relationship between the dissociation rate constant k_{+4} (Scheme 1) and the ordinate intercept in Fig. 4(a) ($k_{+4 \text{ app.}}$). The line drawn in Fig. 4(a) (upper curve) was fitted not only to the experimental points, but also to this relationship between k_{+4} and $k_{+4 \text{ app.}}$.

The numerical value of $k_{+4 \text{ app.}}$, as obtained from Fig. 4(a), is 200 s^{-1} . We have seen that the apparent rate constant for the overall reaction has a maximal value of 120 s^{-1} (Fig. 3a). This value is comparable in magnitude with that of $k_{+4 \text{ app.}}$. We have further seen that establishment of a constant ratio between the transient concentrations of $\text{E}^{\text{NADH}}_{\text{Pyr}}$ and $\text{E}^{\text{NAD}^+}_{\text{Lac}}$ (derived from eqn. 9 of the Appendix) must occur with a pseudo-first-order rate constant which is much higher than k_{R} . Accordingly, transient equilibration between $\text{E}^{\text{NADH}}_{\text{Pyr}}$ and $\text{E}^{\text{NAD}^+}_{\text{Lac}}$ is kinetically inseparable from the equilibration between $\text{E}^{\text{NADH}}_{\text{pyruvate}}$ and $\text{E}^{\text{NADH}}_{\text{Pyr}}$. Thirdly, we have concluded from Fig. 2 that the transient concentration of $\text{E}^{\text{NADH}}_{\text{Pyr}}$ is 0.9 ± 0.1 of the total of the transient concentrations of $\text{E}^{\text{NADH}}_{\text{Pyr}}$ and $\text{E}^{\text{NAD}^+}_{\text{Lac}}$.

The above considerations yield the following relationships between $k_{+4 \text{ app.}}$ (Fig. 4a), k_{+4} (Scheme 1), and the ratio between the transient concentrations of the two 'ternary complexes':

$$k_{+4 \text{ app.}} = k_{+4} \left(\frac{[\text{E}^{\text{NADH}}_{\text{Pyr}}]}{[\text{E}^{\text{NADH}}_{\text{Pyr}}] + [\text{E}^{\text{NAD}^+}_{\text{Lac}}]} \right) = (0.9 \pm 0.1)k_{+4} \quad (4)$$

whereas Fig. 4(a) (upper curve) and eqn. (4) yield the following numerical values: $k_{+4} = 222 \text{ s}^{-1}$, $k_{-4} = 8.33 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$, $K_{d4} = 2.67 \times 10^{-4} \text{ M}$. It can be seen from eqn. (2) that the value of K_{d4} ($2.67 \times 10^{-4} \text{ M}$) is consistent with the value of K_{mR} ($2.4 \times 10^{-4} \text{ M}$)

obtained by monitoring the change in u.v. absorption, since

$$\frac{[E_{\text{Pyr}}^{\text{NADH}}]}{[E_{\text{Pyr}}^{\text{NADH}}] + [E_{\text{Lac}}^{\text{NAD}^+}]} = \frac{k_{+3} + k_{-2}}{k_{-2} + k_{-3} + k_{+3}} = \frac{K_{\text{mR}}}{K_{\text{d4}}} \quad (5)$$

and the ratio

$$\frac{K_{\text{mR}}}{K_{\text{d4}}} = \frac{2.4 \times 10^{-4} \text{ M}}{2.67 \times 10^{-4} \text{ M}} = 0.9$$

is equal to the numerical value used in eqn. (4).

These results yield the numerical values of k_{+4} and k_{-4} of the Haldane-type minimal mechanism (Scheme 1). Internal consistency is obtained between the suggested values and the time-courses of the observed change in u.v. absorption and fluorescence by assigning a value of 0.9 to the relationship defined by eqn. (5). However, this consistency, obtained through eqn. (5), only proves the precision of the estimation of k_{-4} , but not that of k_{+4} (compare eqns. 4 and 5). Therefore the precision with which k_{+4} (and K_{d4}) can be estimated from Fig. 4 (fluorescence change) depends to a marked extent on the evidence obtained from Fig. 2 (change in u.v. absorption).

The present estimations of k_{+4} and K_{d4} include an uncertainty of $\pm 10\%$. This, in itself, still appears to be highly satisfactory. However, in the case of the upper limit (i.e. if the total initial u.v. absorption of E^{NADH} should disappear with a single first-order rate constant) the relationship defined by eqn. (5) would not necessarily be equal to unity. Alternatively, this might also indicate that the observed absorption change is due to the dissociation of lactate, and not to the oxidation of NADH to NAD^+ , i.e. that the molar absorption coefficients of $E_{\text{Lac}}^{\text{NAD}^+}$ and $E_{\text{Pyr}}^{\text{NADH}}$ are comparable at $330 \mu\text{m}$. Ternary complexes of lactate dehydrogenase and NAD^+ (e.g. with sulphite; Pfeleiderer *et al.*, 1957) are known to have an absorption peak in this region, and no independent evidence is available on the spectral properties of $E_{\text{Lac}}^{\text{NAD}^+}$. As shown in the foregoing discussion, the present studies do not provide fully conclusive evidence that would justify the exclusion of this possibility. However, the kinetic observations described by Südi (1974) demonstrate that formation of the $E_{\text{Lac}}^{\text{NAD}^+}$ complex is not accompanied by any easily detectable change in u.v. absorption. I should like to point out that only with this additional information (Südi, 1974) can we get an unambiguous determination of k_{+4} and K_{d4} from Fig. 4(a).

The $E_{\text{Pyr}}^{\text{NADH}}$ complex is one of the two central enzyme-substrate complexes that have to be formed in

the turnover cycle (Scheme 1). The separation of the equilibrium constant K_{d4} from K_{d2} and K_3 has not yet been achieved. It is not simply the rapidity of the observation method that has enabled us to achieve this, but rather our observation that the fluorescence of enzyme-bound NADH is fully quenched on the binding of pyruvate. The present results provide the first evidence that demonstrates this fluorescence quenching, whereas our statement, that the binding of pyruvate completely abolishes the fluorescence of NADH, is naturally subject to the experimental limitations of these studies. It appears that fluorescence

quenching in the $E_{\text{Pyr}}^{\text{NADH}}$ complex is indicative of a specific mode of binding and that this property of pyruvate is shared by some analogous compounds, independently of their behaviour in the coupled oxidation-reduction reaction. Fluorescence quenching was first demonstrated in the non-reactive complex lactate dehydrogenase-NADH-oxamate (Winer & Schwert, 1959); oxamate is known (Novoa *et al.* 1959) as a uniquely specific inhibitor which is competitive with pyruvate. After the present paper was first submitted, fluorescence quenching has also been reported in the ternary complex lactate dehydrogenase-NADH-*o*-nitrophenylpyruvate (Holbrook & Stinson, 1973). This latter pyruvate analogue is a very poor substrate, which is reduced to the corresponding lactate derivative at least 1000 times more slowly than pyruvate.

Although fluorescence disappears in the first reversible step of the three-step reaction sequence (Scheme 1), the second reversible step is found to be characterized by the disappearance of u.v. absorption (see above). Since the overall reaction is only completed by a third, practically irreversible step, and the transient equilibration in the second reaction step appears to be too fast to be followed kinetically, eqns. (3) and (5) can be solved for k_{-2} , but not for k_{-3} and k_{+3} . The calculated value of the dissociation rate constant for lactate is $k_{-2} = 1200 \text{ s}^{-1}$. The values of k_{-3} , k_{+3} and k_{+2} (Scheme 1) can only be obtained if the present results are combined with those obtained in the 'forward direction', where E^{NAD^+} and lactate are the two reactants (Südi, 1974).

Calculating the value of k_{-2} by solving eqns. (3) and (5) actually amounts to deriving it directly from the experimental values of k_{R} (120 s^{-1}) and the size of the 'burst reaction' (one-tenth of the total change in u.v. absorption; Figs. 2 and 3). As discussed above, the estimation of the size of such a small partial 'burst reaction' necessarily involves a very substantial experimental error. When calculating k_{-2} from eqns. (3) and (5) this experimental error is directly introduced into the estimation of k_{-2} , which means that its numerical value cannot be obtained with satisfactory precision this way. Fortunately, the experimental

information presented in this and the following paper (Südi, 1974) provides an alternative way of determining the numerical value of k_{-2} . This latter derivation of k_{-2} is presented in the Appendix of Südi (1974). The numerical value of 1200 s^{-1} has been actually calculated in the present studies, as indicated in this Appendix (Südi, 1974).

In this context I wish to point out that it is a general feature of the closely related studies presented in this and the following paper (Südi, 1974) that they provide a surplus of experimental information for calculating the numerical values of the six rate constants defined by Scheme 1. The quantitative results presented are based in the case of each rate constant on a choice of those experimental observations that could be made with the highest degree of precision. A survey of these decisions together with an estimation of error limits is given in the Appendix of Südi (1974).

The key experiment in these quantitative studies is given in Fig. 4, which demonstrates the second-order nature of fluorescence quenching when E^{NADH} is mixed with pyruvate. As shown above, Fig. 4 yields more or less directly the numerical values of k_{-4} and k_{+4} , thereby decreasing the number of unknowns of the Haldane problem from six to four. The experiments described in the present paper and in Südi (1974) were carried out at a relatively low temperature (6.3°C) and this was done after the completion of a very similar series of unpublished experiments (Südi, 1972) at 22°C . Half-saturating concentrations of lactate (K_{mF}) and pyruvate (K_{mR}), and the corresponding specific rates at substrate saturation (k_F and k_R) were also obtained at the higher temperature, all four values being markedly higher at 22° than at 6.3°C . However, this information could only be completed in the present studies, i.e. at the lower temperature, by directly measuring the second-order rate constant with which pyruvate is bound to the enzyme-coenzyme complex (Fig. 4). The reason for this appears to be as follows: Fig. 4 illustrates that k_{obs} for the reversible binding of pyruvate must be higher than the apparent rate constant with which pyruvate dissociates and that this value is 200 s^{-1} at 6.3°C . It seems safe to assume that k_{obs} must be at least 600 s^{-1} at 22°C and a numerical value of this magnitude would account for our finding that, in experiments similar to those in Fig. 4 but at 22°C , the 'fast' second-order

reaction step is almost completed within the dead time of the instrument and is independent of the concentration of pyruvate.

The small partial 'burst reaction' reported here (about 10%; Figs. 2 and 3) has not been observed in previous studies of the 'reverse' lactate dehydrogenase reaction (for reviews see Gutfreund, 1971; Holbrook & Gutfreund, 1973). I suggest that at least one of the reasons for this is the special attention with which the possibility of detecting such a small 'burst reaction' has been investigated in the present studies. Scheme 1 only requires the obligatory occurrence of $\text{E}^{\text{NAD}^+}_{\text{Lac}}$ as a reaction intermediate without any prediction about its concentration (and even less about its detectability). As discussed in some detail above, studies of the 'forward' reaction (Südi, 1974) yield the final piece of evidence that is needed for demonstrating that the small partial burst in the 'reverse' reaction must be mainly due to the oxidation-reduction reaction proper, i.e. to the transient equilibration between $\text{E}^{\text{NADH}}_{\text{Pyr}}$ and $\text{E}^{\text{NAD}^+}_{\text{Lac}}$ which is completed within the experimental dead time.

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APPENDIX

Eqns. (1)–(3) in the main paper refer to the reciprocal form of the hyperbolic function:

$$k'_R = \frac{v}{[N_T]} = \frac{k_{-2} [E_{Lac}^{NAD^+}]}{[N_T]} = \frac{k_{-4} k_{-3} k_{-2} [P]}{(k_{+3} + k_{-3} + k_{-2}) k_{-4} [P] + k_{+4} (k_{+3} + k_{-2})} \quad (6)$$

where N_T is the total residual concentration of those enzyme-substrate complexes in which NADH has not yet been 'irreversibly' oxidized, i.e.:

$$[N_T] = [E^{NADH}] + [E_{Pyr}^{NADH}] + [E_{Lac}^{NAD^+}] \quad (7)$$

$[P]$ is the constant concentration of pyruvate (present in great excess over NADH and enzyme); k'_R is the observed pseudo-first-order rate constant which is obtained for the 'irreversible' overall reaction. The indexing of all other rate constants refers to Scheme 1 of the main paper.

Eqn. (6) refers to the final reaction step in Scheme 1 of the main paper. Inspection of Scheme 1 reveals that it defines a final stage ('final reaction step') of the overall reaction, which is subsequent to the transient equilibration among the three concentrations on the right of eqn. (7). It is evident from Scheme 1 that, once this transient equilibration has been realized, the subsequent time-course of the overall reaction should be described by a single pseudo-first-order rate constant (k'_R , eqn. 6). It is seen from eqn. (6) that k'_R can be expressed with the rate constants in Scheme 1, if one can express the concentration ratio $[E_{Lac}^{NAD^+}]/[N_T]$ with these rate constants. For this purpose the following differential equations can be used:

$$\frac{d([E_{Pyr}^{NADH}]/[E_{Pyr}^{NADH}])}{dt} = 0 = k_{+4} [E_{Pyr}^{NADH}] - k_{-4} [P] [E^{NADH}] \quad (8)$$

$$\frac{d([E_{Pyr}^{NADH}]/[E_{Lac}^{NAD^+}])}{dt} = 0 = k_{-3} [E_{Pyr}^{NADH}] - (k_{+3} + k_{-2}) [E_{Lac}^{NAD^+}] \quad (9)$$

In conclusion, eqn. (6) is obtained if one expresses, from eqns. (8) and (9), $[E^{NADH}]$ and $[E_{Pyr}^{NADH}]$ with $[E_{Lac}^{NAD^+}]$, then substitutes these expressions into eqn. (7) and finally substitute $[E_{Lac}^{NAD^+}]/[N_T]$ from eqn. (7) into eqn. (6).

The two 'steady-state concentration ratios' defined by eqns. (8) and (9) hold independent of the concentration ratio between reactant (E^{NADH}) and intermediates ($E_{Pyr}^{NADH} + E_{Lac}^{NAD^+}$). The outlined procedure refers to a non-cyclic reaction (Scheme 1) and therefore it is fundamentally different from the steady-state analysis of a cyclic turnover reaction. Although the relative amplitude of this final reaction step is dependent on the numerical values of the rate constants in Scheme 1, eqns. (6)–(9) hold for the final reaction step whatever these numerical values are.