Macroscopic Rate Constants Involved in the Formation and Interconversion of the Two Central Enzyme–Substrate Complexes of the Lactate Dehydrogenase Turnover

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The preceding paper (Südi, 1974) reports partial success in describing the conversion of E^{NADH} plus pyruvate into E^{NAD^+} plus lactate in terms of a simple Haldane-type scheme which involves two intermediates (E_{Pyr}^{NADH} and $E_{Lac}^{NAD^+}$), where E represents lactate dehydrogenase. This information is completed here by reporting kinetic results obtained by carrying out the same reaction in the opposite direction. The combined results of these two papers confirm the findings of Holbrook & Gutfreund (1973) that the observed spectral changes do take place at the level of resolution of this simple two-intermediate scheme. The following numerical values for the rate (and equilibrium) constants involved in their formation and decomposition are reported:

It is shown that although the precision of estimation of some of these numerical values is subject to some experimental uncertainty, their derivation from direct experimental observations only involves the principle of microscopic reversibility. This paper describes stopped-flow kinetic observations made with E^{NAD^+} and lactate as the two reactants. It is shown that fluorescence and u.v.-absorption measurements yield the same experimental rate constant for the last reaction step in which E^{NADH} is generated. On the other hand, the generation of E_{Pyr}^{NADH} can only be indirectly observed, as a less than stoichiometric ‘burst’, and by u.v.-absorption measurements only. It is shown that the stoichiometry of this partial ‘burst reaction’, and a pre-equilibrium factor in the directly observed rate of E^{NADH} -production, yield equivalent information about the reversible oxidation–reduction step. It is further shown that the pre-equilibrium factor that is involved in the generation of E^{NADH} can be determined because $k_{+4} = 222\text{ s}^{-1}$ is already known (Südi, 1974). Since the fluorescence measurements yield much more precise estimations, and their interpretation is considered by the author to be free of ambiguity, the presented quantitative analysis is based on the fluorescence observations.

Stopped-flow kinetic studies of the ‘reverse’ lactate dehydrogenase (EC 1.1.1.27) reaction have demonstrated (Südi, 1974) that NADH bound to lactate dehydrogenase is only fluorescent in the binary complex E^{NADH} † but not in the ternary complex

E_{Pyr}^{NADH} . It follows from this observation that the

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† Abbreviation: E, enzyme (lactate dehydrogenase).

‘forward’ reaction, in which NAD^+ and lactate are the two substrates, can also involve only one fluorescent intermediate, i.e. E^{NADH} . Further, since at substrate saturation the dissociation of NADH can be shown to be the rate-limiting step of the overall reaction, E^{NADH} must be the main steady-state form of the enzyme which accumulates in the pre-steady-state phase of the forward reaction.

In addition to the intermediate E^{NADH} , free NADH, which is one of the end products of the reaction, is also fluorescent. However, the excitation

Scheme 1.

spectrum of NADH bound to lactate dehydrogenase is markedly different from that of free NADH, in that the fluorescence of E^{NADH} is excited at 280–300 nm with a yield about 30 times higher. Fluorescence excited at such low wavelengths may (Kosower, 1962) or may not be due (on the basis of X-ray-crystallographic results of Rossmann and co-workers; see Adams *et al.*, 1973) to the formation of a charge-transfer complex. In any case, I find (see also Südi, 1974) that at such relatively low wavelengths of excitation the production of E^{NADH} can be followed without significant interference by the accumulation of free NADH.

The overall equilibrium of the lactate dehydrogenase reaction is unfavourable with respect to the production of NADH and pyruvate, and this has two consequences. (1) The overall reaction approaches equilibrium and therefore single-turnover experiments are not practicable. (2) If the initial concentrations of NAD^+ and lactate are not widely different, both concentrations will remain practically constant during the total reaction in which the final equilibrium is approached.

All reactions described in this paper were carried out with saturating concentrations of NAD^+ . This meant that the concentration of free enzyme was negligible, i.e. that E^{NAD^+} and lactate were the two reactants actually mixed, and free NAD^+ was present in great excess. The experimental conditions were identical with those in our parallel studies of the reverse reaction (Südi, 1974).

It follows from the foregoing considerations that the kinetic scheme which describes the pre-steady-state generation of E^{NADH} (solid arrows in Scheme 1) is analogous to that describing the corresponding reverse reaction in single-turnover experiments. Of the five macroscopic rate constants which are indicated by solid arrows in Scheme 1, k_{+4} and k_{-2} have been already determined from the reverse reaction (222s^{-1} and 1200s^{-1} respectively; Südi, 1974). The observations described here are not only consistent with these numerical values (see the Appendix), but also allow for the calculation of the numerical values of k_{+2} , k_{+3} and k_{-3} . Since the value of k_{-4} ($8.33 \times 10^5 \text{M}^{-1} \cdot \text{s}^{-1}$) is also known from the preceding paper (Südi, 1974), this yields an internally consistent set of six rate constants. These six macroscopic rate constants yield a Haldane-type description of the so far unresolved central part of the lactate dehydrogenase turnover reaction (Scheme 1).

Experimental

Experimental conditions, materials and apparatus have been described by Südi (1974). There is a well-known and marked difference in the fluorescence excitation spectra of free NADH and NADH bound in the binary complex E^{NADH} . A distinction between these two species is an important feature of the studies described in this paper. Therefore the resolving power of our instrumentation is shown in Table 1.

Table 1. *Difference in the fluorescence excitation spectra of free NADH and NADH bound in the binary E^{NADH} complex*

Measurements were made in the stopped-flow fluorimeter at 6.3°C, with emission filter KV 408 (Jenaer Glaswerk). Values were corrected for dark current. Because of internal filter effects, the numerical values only hold strictly if the only solutes present are those specified in column 1. The solute was dissolved in 0.1M-Tris-HCl, pH8.4. Photomultiplier voltage is uncorrected. Results were obtained with identical optical and electronic settings.

Solute Wavelength of excitation (nm) ...	Photomultiplier voltage (mV)			
	280	300	330	370
10 μM-NADH	1.24	1.60	5.00	3.86
10 μM-E ^{NADH} complex*	41.0	28.0	14.7	7.90
Ratio E ^{NADH} /NADH	33.1	17.5	2.94	2.04

* Enzyme in 1.1-fold molar excess. Dissociation constant of the complex 4.5×10^{-7} M (Fig. 2). Values are corrected for enzyme.

Fig. 1. *Equilibrium reduction of 0.5 mM-NAD⁺ by 40 mM-lactate in the presence of 50 μM-enzyme; oscillograms of the increase in fluorescence excited at 280 nm (a) and 370 nm (b)*
See the Experimental section for details. The zero point on the abscissa indicates the moment when flow has been stopped; the zero point on the ordinate corresponds to the final equilibrium.

Results

Fig. 1 shows the time-course of the production of fluorescent material after the rapid mixing of (2×)40mM-lactate with (2×)0.5mM-NAD⁺ plus (2×)

50 μM-enzyme. The two traces illustrate the marked difference that is observed when fluorescence excited at 280 nm (Fig. 1a) is compared with fluorescence excited at 370 nm (Fig. 1b). At 280 nm a single exponential process is observed, which is characterized by a half-life of 5.2ms, corresponding to a first-order experimental rate constant of 133 s⁻¹ (Fig. 1a). The same reaction step is only part of the total fluorescence change observed at 370 nm (Fig. 1b). Graphical resolution of Fig. 1(b) into two consecutive reaction steps yields a second 'slow step' ($k_{obs.} = 5.3 s^{-1}$), which describes about half of the total observed fluorescence change.

This second slow process in Fig. 1(b) corresponds to the approach to equilibrium in free NADH. The evidence that supports this statement is summarized as follows. (1) The amplitude of the slow fluorescence change is hardly affected by enzyme concentration. (2) To a first approximation, the amplitude is linearly proportional to the square root of the concentrations of both lactate and NAD⁺. (3) Conversely, the half-time of the slow relaxation process is also roughly proportional to the square root of the concentrations of both lactate and NAD⁺. (4) Absence of the slow process at 280nm (Fig. 1a) is accounted for by the observation (Table 1) that the ratio of the relative

fluorescence yields of E^{NADH} and NADH are about 33 (280nm) and 2 (370nm) respectively. Indeed, the slow process observed at 370nm can be also detected at 280nm, with the same half-time but with a much lower relative amplitude, if the concentration of enzyme is decreased and the concentrations of NAD⁺ and/or lactate are increased.

The observations outlined above can be summarized by the conclusion that, at lactate saturation (40mM, Fig. 1), only a single process is observed in the pre-steady-state phase of the formation of fluorescent material. Our previous studies in the reverse direction (Südi, 1974) have demonstrated that there is only one fluorescent intermediate in the

minimal mechanism of the lactate dehydrogenase turnover (Scheme 1), and that this is the E^{NADH} complex. Accordingly, the single observed reaction step in the pre-steady-state phase of the forward reaction (Fig. 1) must correspond to the building-up of the steady-state concentration of E^{NADH} .

The turnover cycle (Scheme 1) indicates that it might be possible to observe three phases in the accumulation of E^{NADH} . The first of these would be a lag phase, corresponding to the period of time required for attaining a transient (and quasi-stationary) pre-equilibrium among the enzyme-substrate complexes $E^{NAD^+}_{Lac}$ and $E^{NAD^+}_{Lac}$, and the enzyme-product complex E^{NADH}_{Pyr} . During the second phase, the steady-state concentration of E^{NADH} would be built up. After these two pre-steady-state reaction steps, the final equilibrium concentration of E^{NADH} would be established. The half-time of this third 'steady-state process' should be identical with the half-time in which free NADH is accumulated.

We have seen above that at lactate saturation the slowest process cannot be detected. It has been shown that, if the optical signal that is used for following the progress of reaction is hardly sensitive to free NADH (excitation at 280 nm; Fig. 1a), no reaction step can be observed that is as slow as the accumulation of free NADH (excitation at 370 nm; Fig. 1b). On the other hand, Fig. 1(a) does not show a lag phase in the formation of E^{NADH} . This latter observation could be accounted for either by a very low steady-state concentration of E^{NADH}_{Pyr} , or by the instrumental dead time, which is 2.5 ms. In the latter case Fig. 1(a) would indicate that the lag phase is completed within the first 2.5 ms after the mixing of reactants. Whether or not one of these alternative explanations holds depends on the numerical values of k_{+3} , k_{-3} and k_{+4} (Scheme 1). It is shown in the next section that these numerical values can be determined from the observed reaction step (Fig. 1) and that a quasi-stationary pre-equilibration among the non-fluorescent enzyme-substrate and -product complexes must indeed be completed within 2.5 ms.

Derivation of k_F and determination of k_{+3} and k_{-3}

For the determination of the values of k_{+3} and k_{-3} the rate equation for the observed reaction step in the formation of E^{NADH} (Fig. 1a) is required. Scheme 1 shows that at lactate saturation this equation would take a very simple form, if the dissociation of NADH were very slow compared

with the dissociation of pyruvate. Both dissociation constants are known from independent measurements. Results obtained in the reverse direction (Südi, 1974) have demonstrated that $k_{+4} = 222 \text{ s}^{-1}$ and Fig. 2 shows that $k_{+5} = 16 \text{ s}^{-1}$. The difference between these two values is sufficiently large to derive the required rate equation from Scheme 2, which is the mirror image of the scheme that refers to the maximal velocity of the absorption change in the reverse direction, in the single-turnover experiments described by Südi (1974). As shown there, the corresponding experimental rate constant (k_R) can be expressed as

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

Therefore the experimental rate constant which is obtained from Fig. 1 ($k_F = 133 \text{ s}^{-1}$; a refinement of this statement is found below) can be expressed as

$$k_F = 133 \text{ s}^{-1} = \frac{k_{+3}k_{+4}}{k_{+3} + k_{+4} + k_{-3}} \quad (2)$$

Eq. (1) and (2) contain two unknowns (k_{+3} and k_{-3}), since the values of k_{-2} (1200 s^{-1}), and k_{+4} (222 s^{-1}) have been determined in the preceding paper

Fig. 2. Rate of binding of NADH to 20 μM -enzyme

Fluorescence was excited at 280 nm. Because of (great) excess of NADH, the reversible binding reaction is first-order in time. The plot yields the association constant as the slope, and the dissociation constant as the ordinate intercept.

Scheme 2.

(cf. Appendix). By solving eqns. (1) and (2) for k_{+3} and k_{-3} we obtain $k_{+3} = 636 \text{ s}^{-1}$ and $k_{-3} = 204 \text{ s}^{-1}$.

It follows from Scheme 2 and these numerical values that quasi-stationary equilibrium between $\text{E}_{\text{Pyr}}^{\text{NADH}}$ and $\text{E}_{\text{Lac}}^{\text{NAD}^+}$ is only reached, in the forward direction, when about 60% of the sum of the concentrations of these two complexes is present in the form of $\text{E}_{\text{Pyr}}^{\text{NADH}}$ since

$$\frac{[\text{E}_{\text{Pyr}}^{\text{NADH}}]}{[\text{E}_{\text{Pyr}}^{\text{NADH}}] + [\text{E}_{\text{Lac}}^{\text{NAD}^+}]} = \frac{k_{+3}}{k_{+3} + k_{+4} + k_{-3}} = 0.60 \quad (3)$$

The experimentally observed rate constant k_F (Fig. 1, eqn. 2) refers to this quasi-stationary pre-equilibrium (cf. the algebraic expressions of eqns. 2 and 3). It is also noteworthy that the numerical values of k_{+3} and k_{-3} which are obtained from eqns. (1) and (2) are consistent with the notion that the attainment of this transient (and quasi-stationary) pre-equilibrium is too fast to be detected kinetically as a lag phase in the formation of fluorescent products.

Implicit in the foregoing considerations are three assumptions which can be easily tested. They are (a) that $k_F = 133 \text{ s}^{-1}$ (Fig. 1, eqn. 2) is a true saturation value obtained at infinitely high lactate concentration; (b) that the numerical value of k_F is independent of enzyme concentration; (c) that the amplitude of the fluorescence change described by k_F is linearly proportional to enzyme concentration.

Experiments specially designed to test point (c) have shown no detectable deviation. As required by Scheme 2, it was further found that the fluorescent material produced by the pre-steady-state reaction corresponded within experimental error to a stoichiometric conversion of enzyme into E^{NADH} . On the other hand, the experimental evidence (Fig. 3) did not seem to support point (b). Fig. 3 shows that there is a relatively small but significant increase in the value of ' k_F ' (observed with 0.1M-lactate), as enzyme concentration is increased over a 16-fold range. However, it should be noted that the highest enzyme concentrations in Fig. 3 are comparable in magnitude with the dissociation constant of pyruvate ($K_{d4} = 2.67 \times 10^{-4} \text{ M}$; Südi, 1974), and this appears to account for the observed drift (Fig. 3). Eqn. (2) has been derived from Scheme 2 on the assumption that the dissociation of pyruvate is irreversible, whereas it must be detectably reversible if the concentration of enzyme is higher than about 20–30 μM (cf. Fig. 3).

With respect to point (a), it is obvious that a true saturation value can only be obtained by extrapolation to infinitely high lactate concentration. Indeed,

the value used in eqn. (2) ($k_F = 133 \text{ s}^{-1}$) has actually been obtained by this procedure, from Fig. 4. As seen from Fig. 4, extrapolation is further justified by the finding that, at lactate concentrations higher than about 30 mM, a relatively small but significant decrease is observed in the experimental value of k_F . Fig. 4 is discussed in detail in the next section.

Half-saturating lactate concentration (K_{mF}), and determination of k_{+2}

It has been pointed out during discussion of Fig. 1 that, according to Scheme 1, three phases might be distinguishable in the accumulation of E^{NADH} . The numerical values obtained for k_{+3} and k_{-3} exclude the possibility of directly observing the first of these three phases, which is a transient equilibration between the two central complexes, and this is independent of lactate concentration. With respect to the slowest phase the case is different. We have seen (Fig. 1) that it cannot be detected at lactate saturation, but also that the reason for this is a practically complete conversion of enzyme into E^{NADH} in the pre-steady-state phase of the reaction.

It is obvious that, as the concentration of lactate is progressively lowered below the saturation value, the difference between the final and steady-state concentrations of E^{NADH} must increase. As a consequence, the generation of E^{NADH} must become a consecutive reaction, with different half-saturating values for each of the two processes. The half-saturating concentration for the pre-steady-state reaction step refers to the scheme defined by the solid arrows in Scheme 1, whereas that for the steady-state reaction refers to the complete turnover cycle (Scheme 1). The pre-steady-state reaction (Scheme 1, solid arrows) is the complementation of Scheme 2 with a

Fig. 3. Dependence of the observed rate constant of the pre-steady-state reaction on enzyme concentration

Lactate concn. = 0.1 M, NAD^+ concn. = 0.5 mM. The reaction was followed by fluorescence excited at 280 nm.

Fig. 4. Double-reciprocal plot of k'_F as a function of the concentration of lactate

Enzyme concn. = $20\ \mu\text{M}$, NAD^+ concn. = 0.5 mM . The reaction was followed by fluorescence excited at 280 nm . The plot yields the (reciprocal) constants $k_F = 133\text{ s}^{-1}$ (ordinate intercept), and $K_{mF} = 3.80\text{ mM}$ (slope $\times$ ordinate intercept).

(quasi-stationary) pre-equilibrium step in which lactate is reversibly bound. Therefore it is the mirror image of the kinetic scheme by which the single-turnover experiments in the reverse direction have been described in the preceding paper (Südi, 1974).

It can be easily shown that the half-saturating concentration of lactate that refers to the steady-state reaction is lower than the half-saturating concentration that refers to the pre-steady-state reaction. Therefore, there must be a (relatively high) range of sub-saturating concentrations of lactate, over which the enzyme is still completely converted into E^{NADH} in the pre-steady-state phase. As long as this can be proved, the generation of E^{NADH} can be described by the pre-steady-state kinetic scheme (Scheme 1, solid arrows).

Fig. 4 shows the double-reciprocal plot of some representative results obtained under such conditions. The concentration of enzyme was $20\ \mu\text{M}$ (cf. Fig. 3) and, over the whole range of lactate concentrations considered in Fig. 4, the observed fluorescence change corresponded within experimental error to the production of $20\ \mu\text{M-E}^{\text{NADH}}$. Fluorescence was

excited at 280 nm , and even with the lowest concentration of lactate included (i.e. 2 mM) a simple exponential time-course was observed.

As discussed in the preceding section, the ordinate intercept of Fig. 4 has been used for determining the numerical value of k_F at infinite lactate concentration. Fig. 4 also yields a half-saturating concentration of lactate, and this value ($K_{mF} = 3.8 \times 10^{-3}\text{ M}$) has been used for calculating k_{+2} . For this purpose, K_{mF} was expressed by analogy with K_{mR} , the derivation of which is given in the preceding paper (Südi, 1974). By applying this analogy (cf. eqns. 1 and 2) we obtain

$$3.80 \times 10^{-3}\text{ M} = K_{mF} = \frac{k_{-2}(k_{-3} + k_{+4})}{k_{+2}(k_{+3} + k_{+4} + k_{-3})} \quad (4)$$

The only unknown in eqn. (4) is the value of k_{+2} . By solving the equation we obtain $k_{+2} = 1.27 \times 10^5\text{ M}^{-1} \cdot \text{s}^{-1}$.

U.v.-absorption measurements

The kinetic evidence presented by Südi (1974) demonstrated that the change in u.v. absorption was not simultaneous with the change in fluorescence. It was shown that, most probably, the change in u.v. absorption occurred in the catalytic step proper, but the possibility could not be excluded that even $\text{E}^{\text{NAD}^+}_{\text{Lac}}$ complex had a high molar absorption at 330 nm .

Obviously, in the case where the molar absorptions of the $\text{E}^{\text{NAD}^+}_{\text{Lac}}$ and $\text{E}^{\text{NADH}}_{\text{Pyr}}$ complexes were very similar, the total pre-steady-state absorption change must be completed within the dead time of the instrument if a high concentration of lactate is mixed with E^{NAD^+} . In the alternative case the pre-steady-state absorption change would be coincident with the formation of $\text{E}^{\text{NADH}}_{\text{Pyr}}$. In the latter case the pre-steady-state change in u.v. absorption should be a biphasic process in which transient and quasi-stationary equilibration between $\text{E}^{\text{NAD}^+}_{\text{Lac}}$ and $\text{E}^{\text{NADH}}_{\text{Pyr}}$ would occur in the first step, whereas in the second reaction step this 'equilibrium' would be extended to include E^{NADH} . Quantitative consistency with the fluorescence observations would require that about 0.6 of the total absorption change were completed, as a 'burst reaction', within the dead time of the instrument, and that about 0.4 of the pre-steady-state absorption change took place at the same rate ($k_F = 133\text{ s}^{-1}$) as the pre-steady-state fluorescence change.

The results of my u.v.-absorption measurements can be summarily discussed with reference to Fig. 5. Fig. 5 shows with two different time axes, and also

Fig. 5. Equilibrium reduction of 0.5mM-NAD^+ by 0.25M-lactate in the presence of $50\text{ }\mu\text{M-enzyme}$; oscillograms of the total increase in u.v. absorption (a), and of the pre-steady-state reaction (b)

(b) has 2 times higher sensitivity, and 100 times smaller time scale, than (a). The zero point on the abscissa indicates the moment when flow has been stopped; the zero point on the ordinate corresponds to the final equilibrium. U.v. absorption was measured at 330nm .

two different sensitivities, the time-course of increasing u.v. absorption with 0.25M-lactate . The same reaction is shown in Figs. 5(a) and 5(b), except that less than one-hundredth of the total reaction time is shown in Fig. 5(b). Fig. 5(b) shows that a pre-steady-state change in u.v. absorption can be observed even with 0.25M-lactate (cf. $k_{+2} = 1.27 \times 10^5 \text{M}^{-1} \cdot \text{s}^{-1}$), and also that the experimental rate constant which characterizes this reaction step is equal within experimental error to the rate constant calculated from the change of fluorescence (Fig. 1). Fig. 5(a) shows that the pre-steady-state change in u.v. absorption is about one-sixth of the change that is due to the formation of free NADH. On the other hand, if the observable pre-steady-state change in u.v. absorption is corrected for the instrumental dead time (2.5ms), the corrected value corresponds to $15 \pm 5\text{ }\mu\text{M-NADH}$, which is $30 \pm 10\%$ of the total enzyme concentration (cf. eqn. 3). The result of the

latter calculation can also be formulated by stating that 0.7 ± 0.1 mol of NADH/mol has been produced by a partial 'burst reaction', which is even faster than the observed pre-steady-state reaction step (Fig. 5), and which has been completed within the instrumental dead time.

These results are in very good agreement with the numerical values summarized in Scheme 3. At the same time, they provide unequivocal evidence that, at least the predominant part of the observable absorption change is simultaneous with the reversible interconversion of the two central complexes $\text{E}_{\text{Lac}}^{\text{NAD}^+}$ and $\text{E}_{\text{Pyr}}^{\text{NADH}}$. The results of some further control measurements which support this claim are summarized in the following section.

Stoichiometry

Determination of the kinetic order of the observable reaction steps, and of the numerical values of the corresponding rate constants, is only one part of the experimental information presented in the foregoing sections. Stoichiometry of the separable reaction steps is an equally important part of the results and owing to some special problems involved in these determinations, a separate consideration of this aspect of the results seems appropriate.

With respect to stoichiometry at lactate saturation, the following main statements were made in the foregoing sections. (1) The kinetically observed fluorescence change corresponds to a stoichiometric conversion of E^{NAD^+} into E^{NADH} . (2) The kinetically observed absorption change corresponds to a stoichiometric conversion of $0.3(\pm 0.1) \times [\text{E}^{\text{NAD}^+}]_0$ into the same product; therefore $0.7(\pm 0.1) \times [\text{E}^{\text{NAD}^+}]_0$ must be transformed, in a kinetically unobserved 'burst reaction', into the liganded intermediate $\text{E}_{\text{Pyr}}^{\text{NADH}}$. These formulations implicitly involve the assumptions that, of all enzyme-bound forms of coenzyme, only the E^{NADH} complex is fluorescent, whereas (at 330nm) the molar absorbancies of NADH and NAD^+ do not change detectably on the formation of binary and ternary enzyme-substrate complexes. Both assumptions are satisfactorily borne out by the results presented in this and the preceding paper (Südi, 1974) (see also below).

Each estimation of stoichiometry involves two independent measurements. One of these is the amplitude of the kinetically observed spectral change and the other is the amplitude of the spectral change that corresponds to the complete conversion of E^{NAD^+} into E^{NADH} . Amplitudes are directly

Scheme 3.

obtained as mV readings of oscillograph voltage, and it has been shown (Fig. 2 of Südi, 1974) that these readings can be easily corrected for the instrumental dead time. Such corrected mV readings were compared in each experiment, under identical optical and electronic conditions, with the difference in oscillograph voltage [i.e. ΔE and/or Δ (fluorescence) values] observed at equal concentrations of E^{NAD^+}

and E^{NADH} by substituting plain buffer for a solution of lactate in the second mixing syringe. E^{NADH} , in a concentration equivalent to the total concentration of enzyme with which the experimental series was run, was measured both as the first and as the last 'treatment'. These two measurements also served as control for checking the optical and electronic constancy of the system, which was necessary because most experiments covered a period of 6–12 h. Most experiments involved simultaneous or parallel measurements of both u.v. absorption and fluorescence. As seen from a comparison of Figs. 1 and 5, the latter measurements have yielded much more precise determinations of 'kinetic amplitudes' (Fig. 1a; excitation at 280 nm), since no consecutive reaction step (corresponding to the accumulation of free NADH; Figs. 5a and 5b) interfered with the determination. It has also been pointed out in connection with Fig. 4 that the kinetic fluorescence amplitude was constant over a wide range of concentration of lactate.

The fluorescence yields of NADH and E^{NADH} are compared in Table 1, and Fig. 2 shows that the observable fluorescence perturbation provides a convenient way for determining the kinetics of binding of NADH to the enzyme. The u.v. absorption (at 330 nm) of $\text{E} + \text{NADH}$ and the fluorescence and u.v. absorption of $\text{E} + \text{NAD}^+$ were found to be additive in conventional spectrophotometric assays. In addition, no reaction was detectable by mixing enzyme with NAD^+ in the stopped-flow instrument, either by fluorescence or by u.v.-absorption measurements. In further control experiments with the stopped-flow instrument it was found that the amplitude of the kinetically detectable reaction ($k_F = 133 \text{ s}^{-1}$) yielded 1:1 stoichiometry in fluore-

scence measurements and $0.3 \pm 0.1:1$ stoichiometry in u.v.-absorption measurements, which was independent of the order of mixing of enzyme with NAD^+ and lactate. The combinations tested were $(\text{E} + \text{NAD}^+)/\text{lactate}$, $\text{E}/(\text{NAD}^+ + \text{lactate})$ and $(\text{E} + \text{lactate})/\text{NAD}^+$.

Discussion

The results of this and the preceding paper (Südi, 1974) are summarized in Scheme 3, which describes the central part of the minimal mechanism of the enzymic turnover, which consists of two straightforward binding steps, and of the catalytic step proper. Even if the detailed course of the reaction is more sophisticated and, correspondingly, there were more than two kinetically important intermediates in this partial reaction, Scheme 3 would still yield an exact description at a lower level of resolution. In support of this claim I wish to re-formulate the essentials of Haldane's (1930) considerations, and apply it to Scheme 3 in the following way. There is no upper limit for the number of possible intermediates in the reversible oxidation of enzyme-bound NADH to enzyme-bound NAD^+ . However, a lower limit of two is provided by the criterion that, on dissociation, all such intermediates must yield either pyruvate or lactate. Scheme 3 is a Haldane-type minimal mechanism in the above sense, that is, it operates with the collective species $\text{E}_{\text{Lac}}^{\text{NAD}^+}$ and $\text{E}_{\text{Pyr}}^{\text{NADH}}$. This is equivalent to an unambiguous definition of all three equilibrium constants in the scheme. The presented kinetic observations provide a similarly unambiguous spectral definition of $\text{E}_{\text{Pyr}}^{\text{NADH}}$, which is one of the two collective species in Scheme 3. According to this operational definition, $\text{E}_{\text{Pyr}}^{\text{NADH}}$ is a (macroscopic) liganded state in which NADH (as characterized by its u.v. absorption) is present, but its fluorescence is quenched. The results summarized in Scheme 3 have been obtained by studying the kinetics of both spectral changes. This justifies, in the author's opinion, the claim that the reaction was actually

observed at the same level of resolution as that of the Haldane-type minimal mechanism.

Scheme 3 describes a symmetrical reversible system, the main characteristics of which are as follows. (a) The forward and reverse reactions are mirror images of each other. Both consist of three consecutive reaction steps. The first of these is a reversible association reaction. The second step (i.e. the catalytic step proper) is a monomolecular and reversible isomerization reaction. Finally, both reactions can be carried out under conditions where the third step is a practically irreversible dissociation reaction. (b) The numerical values of the four first-order rate constants involved in the reaction are all within the same order of magnitude. This means that the kinetic equations for each of the consecutive reaction steps are complex expressions, which also contain the rate constants of the other two coupled reaction steps. (c) The experimental handling of three coupled consecutive reaction steps presents considerable difficulties under the conditions specified in (a) and (b).

The system considered (Scheme 3) has some favourable characteristics, which have enabled us to carry out a meaningful kinetic analysis. These are the following. (d) The reaction can be followed by monitoring both the u.v. absorption, and the fluorescence of NADH. These two spectral changes do not occur simultaneously. (e) By increasing the concentration of substrate, the reversible association reaction can be made practically irreversible and instantaneous. Thereby the observable number of coupled reaction steps can be decreased to two. It is in this way that we have succeeded in locating unambiguously the absorption change by studying the forward reaction, and the fluorescence change by studying the reverse reaction (Scheme 3). (f) The fluorescence change occurs in the first reaction step in the reverse direction. This has enabled us (in the preceding paper; Südi, 1974) to determine more or less directly both rate constants involved in the reversible binding of pyruvate. Thereby the number of unknowns in Scheme 3 was decreased to four. (g) I should have been able to determine the values of the four remaining rate constants directly, had I been able to follow the kinetics of two reaction steps, in either the forward or the reverse direction. Unfortunately, this was not feasible because quasi-stationary equilibrium was reached between the two central complexes so fast, in both directions, that it was completed within the dead time of the instrument. This was directly shown by the observation that the total absorption change was markedly greater than the kinetically observed absorption change, corrected for the instrumental dead time. (h) The four additional numerical values shown in Scheme 3 (cf. section f) were obtained by the explicit solution of the rate equations for the final reaction step in both directions.

The corresponding experimental rate constants (k_F and k_R) could be determined under very favourable experimental conditions, namely from the pre-steady-state change in fluorescence excited at 280nm in the forward direction, and from the absorption change observed in single-turnover experiments in the reverse direction (cf. the Appendix).

The precision of estimation of the numerical values (Scheme 3) appears to be quite good, especially if one considers (see the Appendix) that these values are consistent with a great number of independent kinetic observations reported in this and the preceding paper (Südi, 1974). The observed pre-steady-state changes (Scheme 3) can be correlated with, and controlled by, the steady-state kinetics of the reaction if the numerical values in Scheme 3 are complemented with kinetic information about two further reaction steps, in which coenzyme is bound to the enzyme (Scheme 1). The kinetics of coenzyme binding can be determined in isolation from the turnover cycle in simple two-component systems (see Fig. 2; or, e.g., Heck, 1969).

In addition to steady-state kinetics, a number of further kinetic (and equilibrium) methods are obviously suited not only to controlling the numerical values shown in Scheme 3, but possibly also to describing the reaction in more detail. More detailed information is certainly required before one should attempt to relate the reported quantitative findings to the catalytic mechanism of the lactate dehydrogenase reaction. The most obvious way in which further progress might be made in this direction is an extension of these studies to both lower and higher pH values, especially since a proton is involved as a fifth reactant in the oxidation-reduction reaction catalysed by lactate dehydrogenase. In fact, the present studies form the first part of an investigation which is aimed at obtaining the pH profiles of the six rate constants in Scheme 3.

After the pioneering work of Criddle *et al.* (1968), a number of papers were published from Bristol on the pre-steady-state kinetics of the lactate dehydrogenase reaction. The more recent work (Holbrook & Gutfreund, 1973; Bennett & Gutfreund, 1973) has led to conclusions that agree quite well with the findings summarized in Scheme 3. Unfortunately, a quantitative comparison is not yet possible. One apparent disagreement between my two papers and the most recent publications from Bristol (Holbrook & Gutfreund, 1973; Bennett & Gutfreund, 1973) concerns the question whether the mechanism of the lactate dehydrogenase reaction can be discussed in terms of such simple schemes as Schemes 1 and 3. I suggest that the results presented here do not allow the application of more complex mechanisms such as, for instance, Scheme 3 of Holbrook & Gutfreund (1973). The main arguments that support this view

can be recapitulated in the following two points. First, the dissociation (rate and equilibrium) constants for pyruvate and lactate that could be determined in the present studies refer to the weighted average of all complexes that yield pyruvate and lactate, respectively, by dissociation. Secondly, no additional information has been obtained in these studies which would reveal further details of the mechanism, such as, for instance, the kinetic isotope effects observed, and accounted for, by Gutfreund and co-workers (Holbrook & Gutfreund, 1973).

I report that, under the standard conditions of these experiments, it is possible to describe the lactate dehydrogenase reaction in terms of a Haldane-type minimal mechanism. The demonstration of this possibility appears to deserve some brief comments. Some 40 years ago Haldane (1930) pointed out that, even though the kinetic characteristics of particular enzymic reactions might allow some further simplifications in a kinetic description, the actual number of intermediates can never be less than the minimal number of liganded complexes that would yield different products on complete dissociation. Accordingly, the reversible binding of the last substrate and of the first product are 'inseparably' coupled through the reversible catalytic step in all enzymic reactions. I should like to refer to the latter formulation as the Haldane problem, and should like to remark that this problem is generally recognized in all currently used algebraic treatments of enzymic reactions. However, there appear to be no reports in the literature that an explicit numerical solution of the Haldane problem is experimentally feasible. No doubt this is partly due to the fact that, even though the Haldane problem concerns three coupled equilibria, it can only be solved in such kinetic systems in which production and decomposition of the two central complexes can be directly observed (cf. the first part of this Discussion section). Considering the high rates of reaction of enzymes with their specific substrates, such kinetic

studies can be carried out most conveniently by application of the stopped-flow principle. However, for some reason the present work appears to be the first serious attempt at interpreting stopped-flow kinetic results in terms of a Haldane-type, as opposed to a Michaelis-Menten-type minimal mechanism. By demonstrating the feasibility of solving a particular Haldane problem through stopped-flow kinetic studies, it is hoped that my findings will stimulate an extension of this experimental approach to further enzymic reactions. Some arguments for the importance of such information have been published by Plapp (1973).

I am greatly obliged to the Deutsche Forschungsgemeinschaft for a grant that has enabled me to carry out the studies described in this and the preceding paper. A number of stimulating discussions have also played an essential role in developing the presented line of argument. I am especially indebted to the late Dr. Malcolm Riley (Cambridge), visiting scientist at Stöckheim during the autumn of 1972, for his sharp criticism and helpful suggestions.

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APPENDIX

The observations reported in this and the preceding paper (Südi, 1974) yield more than sufficient information for calculating the rate constants shown in Scheme 3. Accordingly, the numerical value of each of the six constants can be derived in alternative ways. A survey of these alternatives is given here, together with a list of the directly obtained experimental constants, and the error limits with which the latter have been determined.

By studying the 'reverse' reaction (Südi, 1974), the following experimental values were obtained (spectral signal and type of experiment are indicated after the semicolon):

- (i) $k_{-4} = 8.33 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ ($\pm 5\%$); fluorescence, Fig. 4.
 - (ii) $k_{+4\text{app.}} = 200 \text{ s}^{-1}$ ($\pm 5\%$); fluorescence, Fig. 4.
 - (iii) Fractional amplitude of the kinetically observed fluorescence change (at pyruvate saturation) = 0.0; Fig. 4 ('burst size' = 1-0).
 - (iv) $k_R = 120 \text{ s}^{-1}$ ($\pm 5\%$); u.v. absorption*, Fig. 3.
 - (v) $K_{mR} = 2.4 \times 10^{-4} \text{ M}$ ($\pm 5\%$); u.v. absorption*, Fig. 3.
 - (vi) Fractional amplitude of the kinetically observed u.v.-absorption change (at pyruvate saturation) = 0.9 (± 0.1); Fig. 2 ('burst size' = 1-0.9).
- (N.B.: *also observed by measuring fluorescence.)

By studying the 'forward' reaction (present paper), the following experimental values were obtained (spectral signal and type of experiment are indicated after the semicolon):

(vii) $k_F = 133 \text{ s}^{-1}$ ($\pm 5\%$); fluorescence†, Fig. 4.

(viii) $K_{mF} = 3.80 \times 10^{-3} \text{ mM}$ ($\pm 5\%$); fluorescence†, Fig. 4.

(ix) Fractional amplitude of the kinetically observed fluorescence change (at lactate saturation) = $1.0 (\pm 0.1)$; Fig. 1 ('burst size' = $1-1.0$).

(x) Fractional amplitude of the kinetically observed u.v.-absorption change (at lactate saturation) = $0.3 (\pm 0.1)$; Fig. 5(b) ('burst size' = $1-0.3$).

(N.B.: † also observed by measuring u.v. absorption.)

From the above ten experimental values and the equations given in the text, the six rate constants in Scheme 3 can be obtained in a number of alternative ways, of which the most direct ones are as follows:

k_{-4} : directly obtained.

k_{+4} : (a)*† = (ii)/(vi); i.e. obtained by applying a (relatively small) correction factor to an experimental value. (b) = (vii)/(1 - x).

k_{-3} and k_{+3} , simultaneously: (a) from (1 - vi), (iv), (1 - x), and (vii); i.e. from four eqns. with four

unknowns (k_{-2} , k_{-3} , k_{+3} , k_{+4}). (b)* from k_{-4} , k_{+4} , (iv), (v), and (vii); i.e. from three eqns. with three unknowns, by using the numerical values of k_{-4} and k_{+4} . (c) † from k_{+4} , (iv), k_{-2} , and (vii); i.e. from two eqns. with two unknowns, by using the numerical values of k_{+4} and k_{-2} .

k_{-2} : (a)† = (iv)/(1 - vi). (b)* from k_{-4} , k_{+4} , (iv), (v), and (vii); as above.

k_{+2} : (a) = $\frac{(\text{iv})/(1 - \text{vi})}{(\text{viii})}$. (b)*† from (viii) and the numerical values of k_{-4} , k_{+4} , k_{-3} , k_{+3} , and k_{-2} .

(N.B. 1: *procedure actually used.)

(N.B. 2: † procedure indicated in the text.)

It should be noted that, of the four 'burst size' estimations [(iii), (vi), (ix) and (x)] the numerical value of only (vi) has been used in the procedure actually followed. The reason for this is the low error limits with which this fractional amplitude could be obtained (0.9 ± 0.1). Further, it is also important to note that the overall equilibrium constant for the partial reaction shown in Scheme 3 is not involved in the derivation of any of the six numerical values.

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Südi, J. (1974) *Biochem. J.* **139**, 251-259