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STEADY-STATE KINETICS OF LOW MOLECULAR WEIGHT (TYPE-II) NADH DEHYDROGENASE

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SUMMARY

(1) The steady-state kinetics of the NADH dehydrogenase activity of Type-II (low molecular weight) NADH dehydrogenase with the acceptors ferricyanide, cytochrome *c* and 2,6-dichloroindophenol are consistent with the simultaneous operation of an ordered and a ping-pong mechanism. Thus, depending on the acceptor concentration, the reduced enzyme is preferentially oxidized before or after NAD^+ dissociates from it.

(2) The acceptors are able to oxidize the reduced enzyme and its NAD^+ complex equally well. In contrast to the kinetics of the Type-I (high molecular weight) enzyme, double substrate inhibition is not found, implying that the site of oxidation of the reduced enzyme by acceptors and the NADH-binding site are remote.

(3) With the indophenol, in the concentration range measured, the ordered mechanism is mainly operative. At infinite NADH and acceptor concentrations the rate constant of the reduction of enzyme by bound NADH is measured.

(4) With ferricyanide and cytochrome *c*, in the concentration range measured, erroneous conclusions may be drawn from extrapolations owing to the fact that extrapolated lines in double-reciprocal plots of turnover number against acceptor concentration, at different NADH concentrations, intersect in the third quadrant. A method is described that allows the extrapolation of these data to zero acceptor concentrations.

(5) The relation between activity and NADH concentration is sigmoidal ($h = 2.0$) with ferricyanide or cytochrome *c* as acceptor, but hyperbolic with 2,6-dichloroindophenol. The latter is also an inhibitor, competitive with respect to NADH. It is concluded that this two-electron acceptor, like ubiquinone, acts as an allosteric effector.

(6) Type II is isolated from Type I without gross changes in tertiary structure, as judged by the unaltered rate constants of dissociation of NADH (k_{-1}) and NAD^+ (k_4) and association of NADH (k_1).

(7) Type II differs from Type I in two respects. (a) The accessibility of the acceptors is greater by at least two orders of magnitude (k_3). (b) The redox potential of the prosthetic group FMN is 120 mV less, as judged by a drop in the value of k_2 by four orders of magnitude. It is suggested that one or more of the iron-sulphur

proteins present in Type-I but lacking in Type-II dehydrogenase functions as an effector, regulating the redox potential of the FMN.

INTRODUCTION

Two types of preparation of NADH dehydrogenase have been isolated from mitochondria or submitochondrial particles, one with a high molecular weight (Type I) and one with a low molecular weight (Type II) [1–6]. The differences between the two types are discussed in the accompanying paper on Type-I dehydrogenase [7].

This paper deals with Type-II dehydrogenase. Its enzymic properties are characterized by a series of diaphorase activities, in which electrons are transferred from reduced nicotinamide nucleotide to ferric complexes, cytochrome *c* or quinoid compounds. The K_m and V values with respect to NADH and acceptor and the degree of substrate inhibition depend critically on the nature of the acceptor [5, 8, 9]. In this study an attempt is made to resolve these differences by comparing the steady-state kinetics with the different acceptors. The implications of the results of this and the accompanying paper [7] with respect to the question whether or not Type-II dehydrogenase should be considered as an artefact are discussed. Some of this work has been presented elsewhere [10].

RESULTS

Figs. 1A and 1B describe the effect of varying the concentration of NADH and ferricyanide, respectively, at fixed concentration of the other reactant, on the initial rate of the reaction, measured at 420 nm. The reaction is about 10 times slower than with Type-I dehydrogenase. The curves relating activity to NADH concentration are sigmoidal without the inhibition by high concentrations of substrate that is characteristic for the Type-I preparation. Inhibition is seen at higher ferricyanide concentrations, but this is due to an irreversible inactivation, which is so rapid with concentrations above 1 mM that it is not possible to measure the initial rate with the equipment used (see Fig. 2). Pre-incubation of the enzyme with 1 mM ferricyanide, in the absence of NADH, rapidly inactivated the enzyme (half time about 3 min).

Below 0.5 mM, a linear relation is found between activity and ferricyanide concentration, at different NADH concentrations, the straight lines intersecting on the abscissa at −0.1 mM (see Fig. 3). As expected from Fig. 3, the double-reciprocal plot shown in Fig. 4 yields a set of hyperbolae concave to the abscissa, when the points below 1.5 mM^{-1} are ignored (owing to inactivation). These hyperbolae converge in the third quadrant at $-1.5\ mM^{-1}$ and $-7.8 \cdot 10^{-3}$ s. Thus, ferricyanide, besides acting as electron acceptor, appears to activate the reaction. This behaviour is to be expected if both ping-pong and ordered mechanisms are operative (see ref. 11). At low acceptor concentrations, NAD^+ dissociates from the NAD^+-reduced enzyme complex before the oxidation by acceptor (i.e. a ping-pong mechanism), whereas at high acceptor concentrations the oxidation of the NAD^+-reduced enzyme complex is more rapid than the dissociation of the NAD^+, resulting in an ordered mechanism.

The double-reciprocal plot for the other substrate, shown in Fig. 5, reveals that

the sigmoidal NADH saturation curve is present at all concentrations of ferricyanide investigated, including the values obtained in Fig. 3 by extrapolating to zero ferricyanide concentration. The points corresponding to low NADH concentration ($[NADH]^{-1} > 30\ mM^{-1}$) tend to lie on lines converging at the abscissa at about $-10\ mM^{-1}$, corresponding to a K_m(NADH) of about 100 μM. At higher concentrations, the points lie on lines converging at about $-40\ mM^{-1}$ corresponding to a K_m (NADH) of about 25 μM. The data at all ferricyanide concentrations fit on the same Hill plot (Fig. 6) with $h = 2.0$. The K_m calculated from the lines with slope equal to 1 at low and high NADH concentrations are 85 μM and 25 μM, respectively, in good agreement with those calculated from Fig. 5.

Type-II dehydrogenase reacts with ferricytochrome *c* in an antimycin-insensitive reaction about 10 times more rapidly than the antimycin-insensitive reaction of the Type-I enzyme. The dependence on NADH (Fig. 7) and ferricytochrome *c* (Fig. 8)

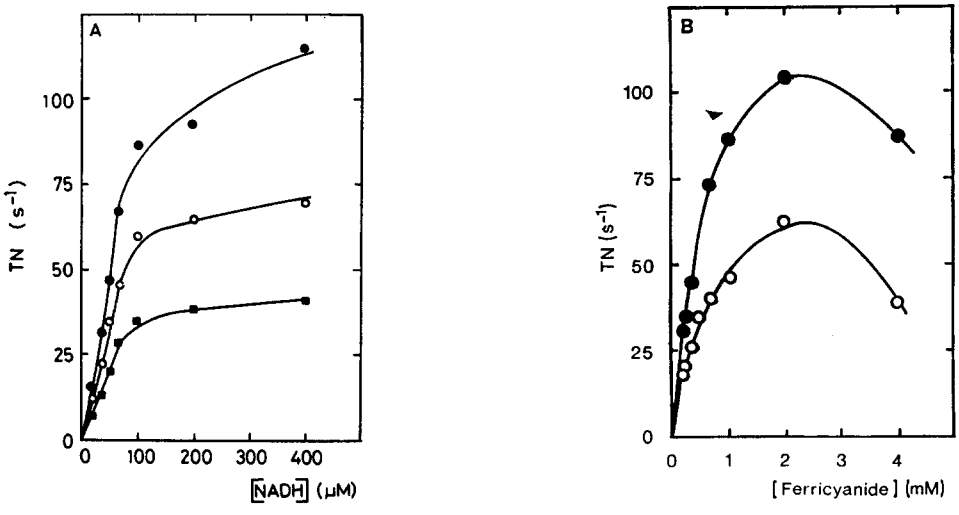


Fig. 1. The initial rate of the NADH-ferricyanide activity as function of NADH (A) and ferricyanide (B) concentration at fixed concentration of the other reactant. (A) Ferricyanide (■) 0.25 mM, (○) 0.5 mM and (●) 1 mM. (B) NADH (○) 50 μM and (●) 100 μM. The initial rate is expressed as turnover number (TN) in mol NADH oxidized/mol Type-II dehydrogenase (FMN basis) per s. Enzyme concentration, 21 nM; pH 8.0.

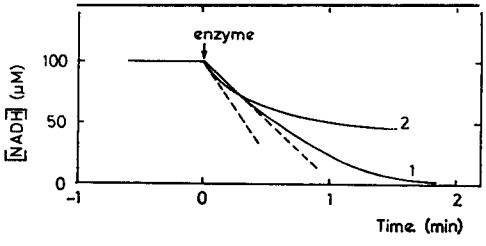


Fig. 2. Oxidation of 100 μM NADH by enzyme at 1 mM (trace 1) and 2 mM (trace 2) ferricyanide. Conditions as described in Fig. 1.

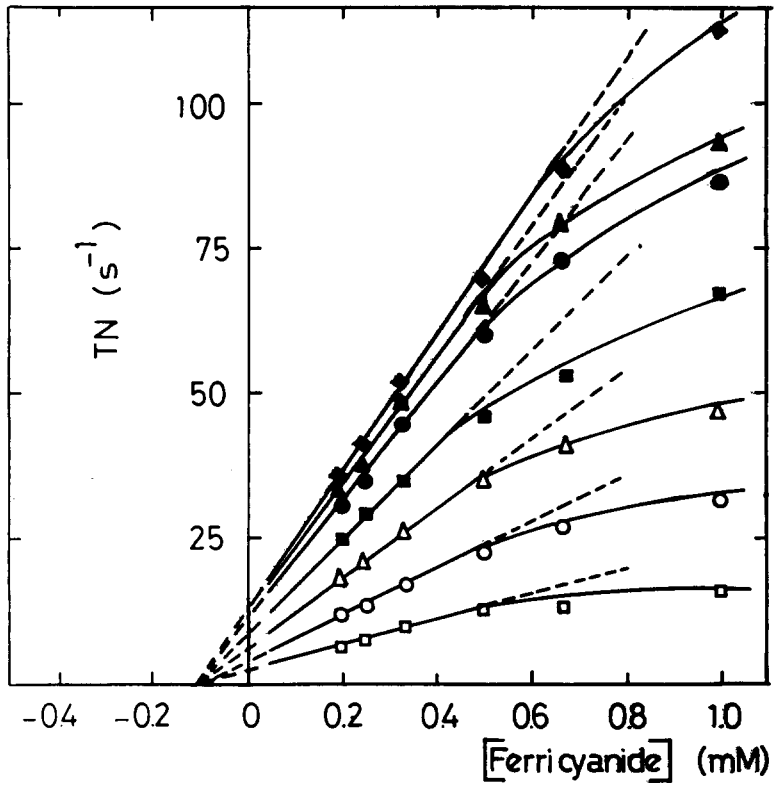


Fig. 3. The initial rate of the NADH-ferricyanide activity as function of ferricyanide concentration at fixed concentration of NADH of (□) 16 μM, (○) 33 μM, (△) 50 μM, (■) 67 μM, (●) 100 μM, (▲) 200 μM and (◆) 400 μM. Conditions as described in Fig. 1.

concentrations is similar to that already shown for the NADH-ferricyanide reductase activity in Figs. 5 and 3, respectively, but ferricytochrome *c* has a much greater affinity.

The rate at infinite NADH and acceptor concentrations is greater with ferricyanide, twice as much at pH 8.5 (optimum for ferricytochrome *c*) and 10 times as much at pH 8.0 (optimum for ferricyanide). Table I shows the same ratios for the rate at infinite NADH and zero acceptor concentration, obtained from the intercept on the ordinate in plots corresponding to Figs. 5 and 7. The intersection points on the abscissa in plots corresponding to Figs. 3 and 8 are independent of pH between 8 and 9 with cytochrome *c* as acceptor.

2,6-Dichloroindophenol behaves quite differently from ferricytochrome *c* and ferricyanide, straight lines being obtained in a double-reciprocal plot (Fig. 9). No inhibition by excess NADH is found. The lines obtained at concentrations of 2,6-dichloroindophenol of 300 μM or higher intersect on the ordinate. Lower concentrations intersect on the abscissa, as with ferricyanide and ferricytochrome *c*. It seems, then, that dichloroindophenol acts as an inhibitor, competitive with respect to NADH, at higher concentrations.

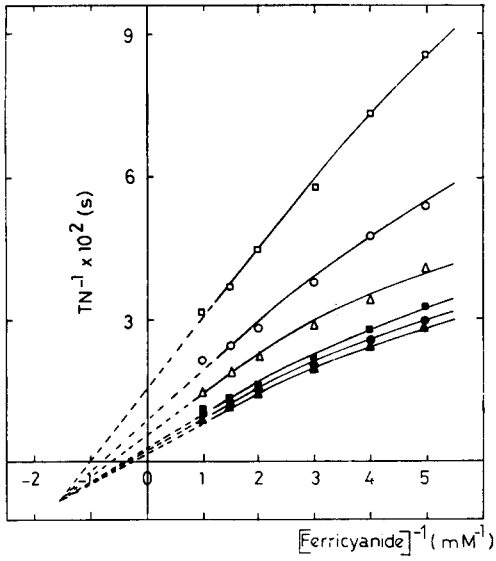


Fig. 4. Lineweaver-Burk plot of data in Fig. 3. NADH concentrations were (□) 33 μM, (○) 50 μM, (△) 67 μM, (■) 100 μM, (●) 200 μM and (▲) 400 μM.

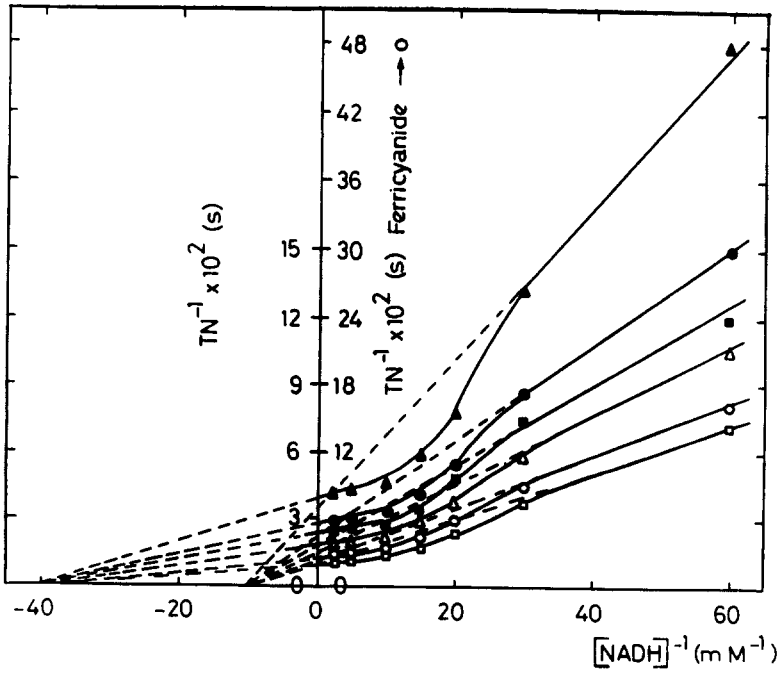


Fig. 5. Lineweaver-Burk plot of initial rate of NADH-ferricyanide activity as function of NADH concentration at fixed concentration of ferricyanide of (□) 0.67 mM, (○) 0.50 mM, (△) 0.33 mM, (■) 0.25 mM and (●) 0.20 mM, and (▲) data of Fig. 3 extrapolated to zero ferricyanide concentration. Conditions as described in Fig. 1.

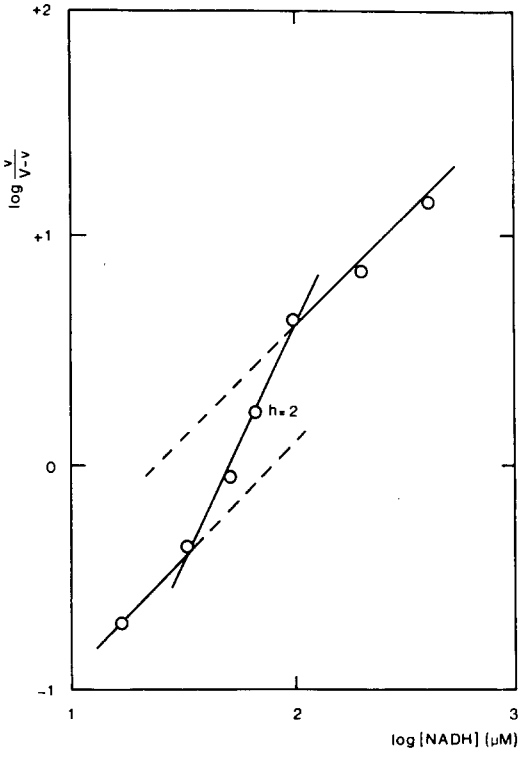


Fig. 6. Hill plot for the NADH-ferricyanide reductase activity of Type-II dehydrogenase as a function of NADH concentration. Conditions as described in Fig. 1. V refers to infinite NADH concentration, at a particular ferricyanide concentration, and v to a specified NADH concentration. The mean of the data at all ferricyanide concentrations in Fig. 5 is given.

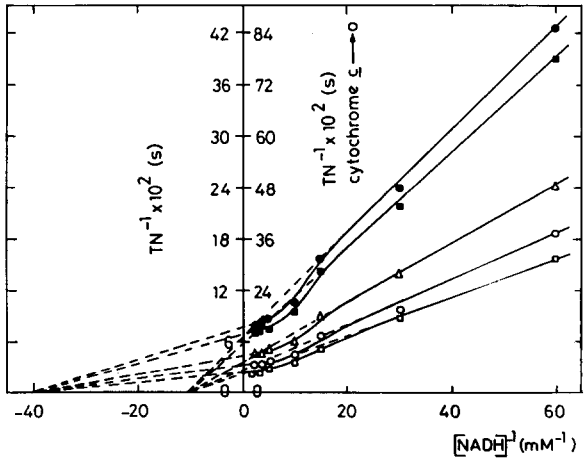


Fig. 7. Lineweaver-Burk plot of initial rate of NADH-ctyochrome c reductase activity as function of NADH concentration at fixed concentration of cytochrome c of (□) 15.1 μM, (○) 11.3 μM, (△) 7.5 μM and (■) 3.8 μM, and (●) extrapolated to zero cytochrome c concentration from data in Fig. 8. Enzyme concentration, 5.2 nM; pH 8.5.

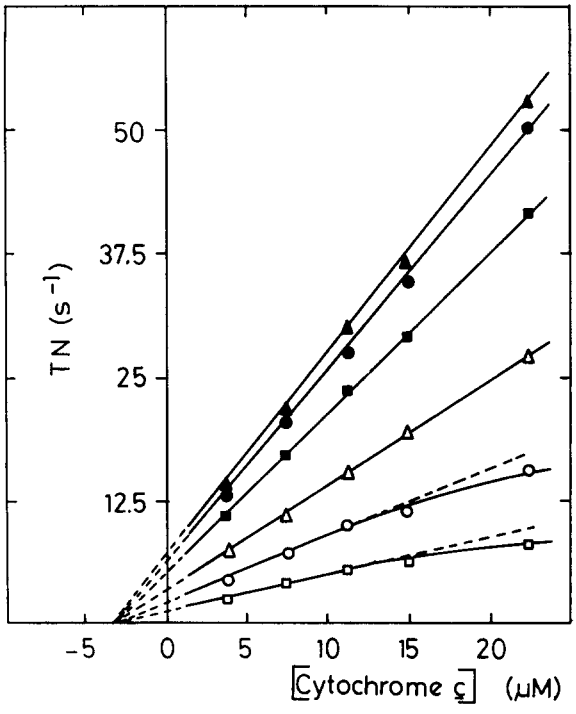


Fig. 8. The initial rate of the NADH-cytochrome *c* reductase activity, as function of the cytochrome *c* concentration at fixed concentration of NADH of (□) 16 μM, (○) 33 μM, (△) 67 μM, (■) 100 μM, (●) 200 μM and (▲) 300 μM. Conditions as described in Fig. 7.

TABLE I

COMPARISON OF RATE CONSTANTS FOR NADH-FERRICYANIDE AND NADH-CYTOCHROME *c* REDUCTASE ACTIVITY AT DIFFERENT pH

The intersection points on the abscissa are extrapolated from TN vs. acceptor-concentration plots. The values of V (acceptor $\rightarrow$ 0) are extrapolated from TN^{-1} (at zero acceptor concentration) vs. $[NADH]^{-1}$ plots.

	Intersection point (μM)	V (acceptor $\rightarrow$ 0) (s^{-1})
NADH-ferricyanide activity		
pH 8.0*	100	13
NADH-cytochrome *c* reductase activity		
pH 8.0*	3	1.3
pH 8.5**	3	6.7
pH 9.0**	3	4.8

* 20 mM phosphate buffer.
** 20 mM glycylglycine buffer.

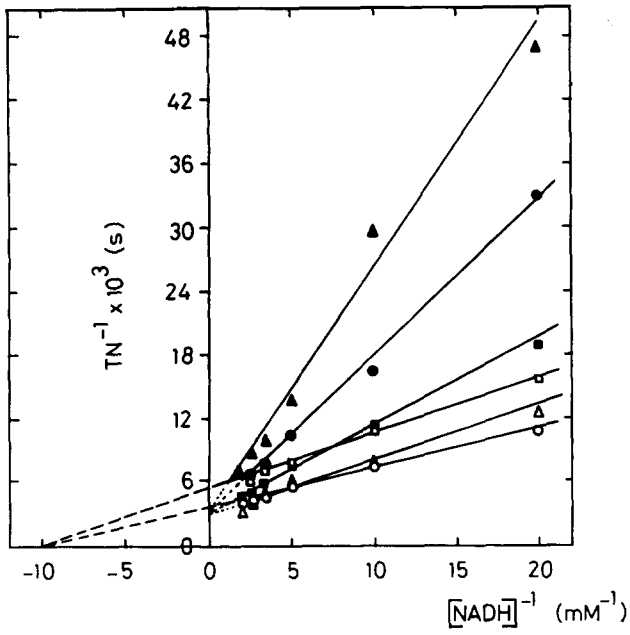
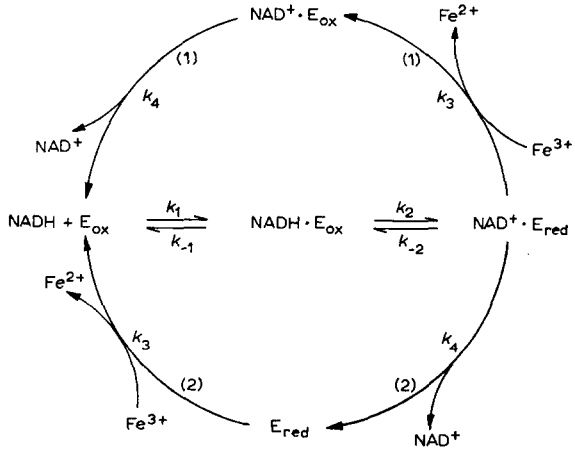


Fig. 9. Lineweaver-Burk plot of initial rate of NADH-2,6-dichloroindophenol activity as function of NADH concentration at fixed concentration of the indophenol of (□) 100 μM, (○) 200 μM, (△) 300 μM, (■) 400 μM, (●) 450 μM and (▲) 500 μM. Enzyme concentration, 5.2 nM; pH 8.0.

THEORETICAL

Minimum hypothesis

The data can be explained, if for each binding site for NADH both an ordered and a ping-pong mechanism are operative as formulated in Scheme A.



Scheme A

According to this scheme, NADH is capable of binding to the oxidized enzyme, forming an NADH-oxidized enzyme complex, in equilibrium with an NAD^+-reduced enzyme complex. The artificial acceptor ferricyanide is able to oxidize the fully reduced enzyme, whether or not NAD^+ is bound to it. Thus, depending on the acceptor concentration, the reduced enzyme is preferentially oxidized before (ordered mechanism; pathway 1) or after (ping-pong mechanism; pathway 2) NAD^+ dissociates from it. It is assumed that ferricyanide reacts equally well with both NAD^+-free reduced enzyme and its NAD^+ complex, as would be expected, for example, if it reacts with the enzyme at a site remote from the NADH-binding site. This is consistent with the absence of double substrate inhibition (see Figs. 1A and 1B). In other words, binding and subsequent oxidation of NADH by the oxidized enzyme and oxidation of reduced enzyme by ferricyanide are not interdependent, except insofar as NADH is the leading substrate.

If for initial rates a steady state in all enzyme forms may be assumed, the reciprocal rate equation may be written as

$$TN^{-1} = \underbrace{\frac{k_{-2}/k_2}{k_3 a + k_4}\left(1+\frac{K_s}{s}\right)}_{\text{main term}} + \frac{1}{k_2}\left(1+\frac{K}{s}\right) + \frac{1}{k_3 a} + \frac{1}{k_4} - \frac{1}{k_3 a + k_4} \tag{1}$$

in which TN = turnover rate, a = [ferricyanide], s = [NADH], $K_s = k_{-1}/k_1$ and $K = (k_{-1}+k_2)/k_1$.
If the first term predominates, the rate equation simplifies to

$$TN = \frac{k_3 a + k_4}{(k_{-2}/k_2)\left(1+\frac{K_s}{s}\right)} \tag{2}$$

which describes a linear relationship between rate and ferricyanide concentration. This appears to be so for ferricyanide concentrations between 0.2 and 0.5 mM (see Fig. 3). The intersection on the abscissa at −0.1 mM in Fig. 3 and the intercept on the ordinate at $7.8 \cdot 10^{-2}$ s for zero ferricyanide concentration in Fig. 5 represent $-k_4/k_3$ and $k_{-2}/k_2 \cdot k_4$, respectively. Thus, at 0.1 mM ferricyanide, $k_3 a = k_4$, that is half of the enzyme molecules are operative in a ping-pong mechanism and half in an ordered one.

The linear relationship between rate and ferricyanide concentration in the range 0.2–0.5 mM shows that the terms $(1/k_2)(1+K/s)$ and $1/k_4$ are small compared to the main term in Eqn. 1, say less than 10 %. From this consideration the following conclusions may be drawn.

(1) $k_{-2} > 10(k_3 a + k_4)$

From this condition and the steady-state assumptions

$k_2[\text{NADH} \cdot E_{ox}] = (k_{-2}+k_3 a+k_4)[NAD^+ \cdot E_{red}]$ and
$k_1 s[E_{ox}]+k_{-2}[NAD^+ \cdot E_{red}] = (k_{-1}+k_2)[\text{NADH} \cdot E_{ox}]$

it follows that

$k_2[\text{NADH} \cdot E_{ox}] \approx k_{-2}[NAD^+ \cdot E_{red}]$ and (3)

$k_1 s[E_{ox}] \approx k_{-1}[NADH \cdot E_{ox}]$

This means that, according to Scheme A and within this range of ferricyanide concentrations, $NAD^+ \cdot E_{red}$, $NADH \cdot E_{ox}$ and E_{ox} are in direct equilibrium with one another.

$$(2)\quad \frac{k_{-2}/k_2}{k_3 a + k_4} > 10\,\frac{1}{k_4} \qquad \text{at } s \gg K_s$$

which via $k_4/k_3 = 0.1$ mM and $a = 0.5$ mM (upper limit) results in $k_{-2}/k_2 > 60$, i.e. the equilibrium between $NADH \cdot E_{ox}$ and $NAD^+ \cdot E_{red}$ lies in the direction of the former.

(3) From Eqn. 3 and the steady-state equations

$k_3 a[NAD^+ \cdot E_{red}] = k_4[NAD^+ \cdot E_{ox}]$ and
$k_4[NAD^+ \cdot E_{red}] = k_3 a[E_{red}]$

it follows that

$$[NAD^+ \cdot E_{red}] \approx \frac{k_2}{k_{-2}}[NADH \cdot E_{ox}] < \frac{[NADH \cdot E_{ox}]}{60}$$

$$[NAD^+ \cdot E_{ox}] = \frac{k_3 a}{k_4}[NAD^+ \cdot E_{red}] < \frac{a[NADH \cdot E_{ox}]}{6}$$

$$[E_{red}] = \frac{k_4}{k_3 a}[NAD^+ \cdot E_{red}] < \frac{[NADH \cdot E_{ox}]}{600a}$$

i.e. between 0.2 and 0.5 mM ferricyanide the concentrations of $NAD^+ \cdot E_{red}$, $NAD^+ \cdot E_{ox}$ and E_{red} may be neglected with respect to $[NADH \cdot E_{ox}]$, so that the total enzyme concentration e equals $[E_{ox}] + [NADH \cdot E_{ox}]$.

At concentrations of ferricyanide above 0.5 mM, deviations from the straight lines in Fig. 3 occur, indicating that at least one other term in Eqn. 1 can no longer be neglected with respect to the main term. Since the deviations tend to be higher at the lower NADH concentrations, the term $(1/k_2)(1+K/s)$ is presumably responsible. If the term $1/k_4$ were responsible, the greatest deviations would be found at high values of s, when the main term in Eqn. 1 is minimal.

From Eqns. 1 and 2 it can be shown that the double-reciprocal plot in Fig. 4 should yield hyperbolae concave to the abscissa. When the ferricyanide concentration is so low that $k_3 a$ may be neglected with respect to k_4, Eqn. 1 simplifies to

$$TN_p^{-1} = \underbrace{\frac{k_{-2}}{k_2 k_4}\left(1 + \frac{K_s}{s}\right)}_{\text{main term}} + \frac{1}{k_2}\left(1 + \frac{K}{s}\right) + \frac{1}{k_3 a} \qquad (4)$$

which is the equation for the ping-pong mechanism. At high ferricyanide concentrations Eqn. 1 simplifies to

$$TN_o^{-1} = \underbrace{\frac{k_{-2}}{k_2 k_3 a}\left(1 + \frac{K_s}{s}\right)}_{\text{main term}} + \frac{1}{k_2}\left(1 + \frac{K}{s}\right) + \frac{1}{k_4} \qquad (5)$$

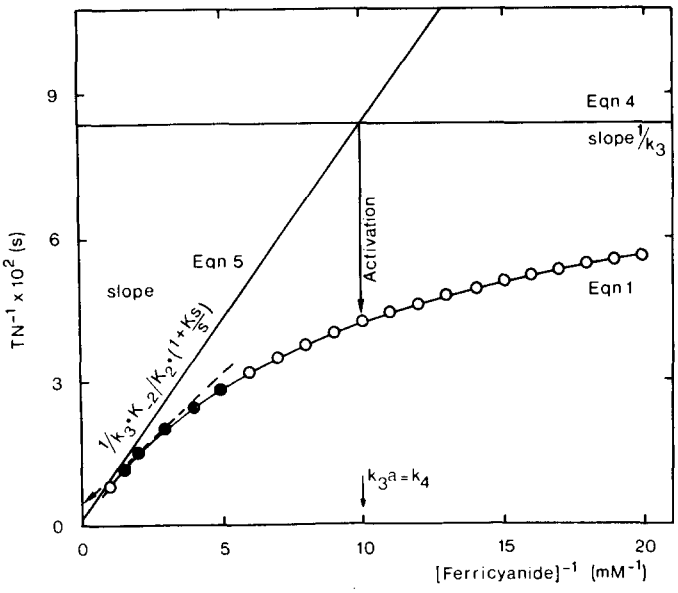


Fig. 10. Lineweaver-Burk plot of the initial rate of NADH-ferricyanide activity as function of ferricyanide concentration at 400 μM NADH, calculated from Eqn. 1 and the rate constants in Table III. The closed symbols correspond to those measured in Fig. 4. Eqn. 5: ordered mechanism. Eqn. 4: ping-pong mechanism.

the reciprocal-rate equation of the ordered mechanism. In Fig. 4 neither of these extreme situations is reached, k_3a being equal to k_4 at 0.1 mM ferricyanide. In the double-reciprocal plot shown in Fig. 10, Eqns. 1, 4 and 5 have been plotted on the basis of calculated rate constants (see later) over a much broader range of ferricyanide concentrations. Extrapolation to infinite ferricyanide concentration of the closed symbols between 1.5 and 5 mM^{-1}, as measured in Fig. 4, shows a marked deviation from Eqn. 5. This concentration range is, then, not suitable for an extrapolation to infinite ferricyanide concentration, but it is impossible to measure at ferricyanide concentrations above 1 mM, because of inactivation. Thus, the extrapolations shown in Fig. 4, although the lines intersect, give no quantitative information.

As illustrated in Fig. 10, the slope of the hyperbola varies between $(1/k_3)(k_{-2}/k_2)(1+(K_s/s))$ and $1/k_3$ (see Eqns. 5 and 4), the former being much greater, owing to the position of the rate-limiting equilibrium ($k_{-2}/k_2 = 780$.) Indeed, according to Scheme A an increase of ferricyanide concentration has no effect on this equilibrium so far as the ping-pong mechanism is concerned, whereas in the ordered mechanism it has an effect, pulling it to disequilibrium, directly coupled with an increase in overall reaction rate (compare also the main terms in Eqns. 4 and 5). Thus an increase in acceptor concentration, by changing the statistical distribution of the enzyme molecules over the two pathways in favour of the ordered mechanism, seems to activate the enzyme. It should be mentioned that the mechanism presented in Scheme A should not simply be considered as if the fraction $k_3a/(k_3a+k_4)$ of the enzyme molecules is operative in an ordered and $k_4/(k_3a+k_4)$ in a ping-pong mechanism. This "either-or" situation would result, via the main terms of Eqns. 4 and 5, in

$$TN_{mix} = \frac{k_3 a}{k_3 a + k_4} \cdot TN_o + \frac{k_4}{k_3 a + k_4} \cdot TN_p = \frac{(k_3 a)^2 + k_4^2}{(k_3 a + k_4)(k_{-2}/k_2)(1 + K_s/s)} \quad (6)$$

Since the two mechanisms are interconnected via common intermediates, as is expressed in Scheme B for the rate-limiting part of the reaction,

$$\overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} NAD^+ \cdot E_{red} \overset{k_{3a}}{\rightarrow} \quad (1) \text{ ordered}$$

$$\overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} NAD^+ \cdot E_{red} \overset{k_4}{\rightarrow} \quad (2) \text{ ping-pong}$$

Scheme B

the statistical weight of the $NAD^+ \cdot E_{red}$ concentration in both the ping-pong and the ordered mechanism is 1 and the overall turnover number is given by

$$TN = TN_o + TN_p = \frac{k_3 a + k_4}{(k_{-2}/k_2)\left(1 + \frac{K_s}{s}\right)} \quad (2)$$

From Eqns. 2 and 6 it follows that the "both-and" situation is a factor $(k_3a+k_4)^2/((k_3a)^2+k_4^2)$ more active than the "either-or". Thus the nonphysiological acceptor ferricyanide, being able to oxidize both $NAD^+ \cdot E_{red}$ and E_{red}, appears to activate as well as act as electron acceptor. As illustrated in Fig. 10 the maximal activation is a factor 2 if k_3a equals k_4.

The sigmoidal curves in Figs. 1A and 5 suggest the presence of more than one interacting NADH-binding site per enzyme molecule. Binding of NADH and oxidation by ferricyanide are independent, as shown by the fact that the data at all ferricyanide concentrations fit on the same Hill plot (Fig. 6). Moreover, the intercepts $-k_4/k_3$ in Fig. 3 and k_{-2}/k_2k_4 in Fig. 5 are independent of NADH concentration. Thus, as a minimum hypothesis, it is sufficient to assume that K_s is dependent on the NADH concentration. The K_s calculated from Eqn. 2 changes from about 100 μM at low substrate concentration to about 25 μM at high concentration.

From the Monod model of cooperativity for two interacting binding sites per enzyme molecule the relation $h = 2/(1+K_s^R/s_{h\rightarrow max})$ can be derived, where $s_{h\rightarrow max}$ is the substrate concentration when h is maximal, and K_s^R is the dissociation constant at high s. Since in Fig. 6 $s_{h\rightarrow max}$ and K_s^R amount to 50 and 25 μM, respectively, it follows that h should be 1.33. Thus, the Hill coefficient $h = 2.0$ found in Fig. 6 implies that more than two interacting NADH-binding sites per enzyme molecule are present in Type-II NADH dehydrogenase.

Evaluation of rate constants

It has been shown above that the deviations from the straight lines in Fig. 3 are due to the term $(1/k_2)(1+K/s)$. The deviation of the turnover number amounts to 6 and 14 s^{-1} at 200 μM NADH, 0.67 mM ferricyanide and 400 μM NADH, 1 mM ferricyanide, respectively. When converted to TN^{-1}, these correspond to 8.4 and

$9 \cdot 10^{-4}$ s. Since at these NADH concentrations $K_s = 25\ \mu M$, it follows that $k_2 = 1.1 \cdot 10^3\ s^{-1}$ if K can be equated with K_s. This is the only rate constant that can be calculated for the Type-II dehydrogenase according to the proposed mechanism. The calculated ratios between some of the rate constants are assembled in Table II.

Unitarian reaction mechanism of Type-I and Type-II NADH dehydrogenase; further evaluation of separate rate constants

Since Type-II dehydrogenase originates from Type I, a minimum hypothesis is to assume that as few as possible of the corresponding rate constants would be altered. It would not be unexpected that the accessibility of the reduced electron-accepting centre to artificial acceptors would be altered, so that we may confine our analysis to the sequence

$$NADH + E_{ox} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [NADH \cdot E_{ox}] \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} [NAD^+ \cdot E_{red}] \overset{k_4}{\rightarrow} NAD^+ + E_{red}$$

in which influences of the artificial acceptor are omitted. This yields the terms of the reciprocal rate equation

$$TN^{-1} = \frac{k_{-2}}{k_2 k_4}\left(1 + \frac{K_s}{s}\right) + \frac{1}{k_2}\left(1 + \frac{K}{s}\right) + \frac{1}{k_4} \text{ with}$$

$$V(NADH) = \frac{k_2 k_4}{k_2 + k_{-2} + k_4} \text{ and}$$

$$K_m(NADH) = \frac{(k_{-2} + k_4)k_{-1} + k_2 k_4}{(k_2 + k_{-2} + k_4)k_1}$$

where these terms now refer to the rate of reduction of the enzyme. Whether both intermediates or preferentially one is accumulated in the steady state cannot be deduced from the numerical values of V and K_m as such. Therefore, the following possibilities are distinguished.

(1) $k_2 \geqslant 10(k_{-2} + k_4)$, i.e. the $NAD^+ \cdot E_{red}$ complex is preferentially accumu-

TABLE II

COMPARISON OF KINETIC DATA AT pH 8.0 AVAILABLE FOR UNITARIAN MECHANISM

	Type I	Type II
K_m(NADH) (μM)	140*	—
K_s(NADH) (μM)	—	100–25**
V (s^{-1})	$\geqq 10^4$* (k_4)	$1.3 \cdot 10$ ($k_2 k_4 / k_{-2}$)
k_2 (s^{-1})	—	$1.1 \cdot 10^3$
k_3 ($M^{-1} \cdot s^{-1}$)	$1.8 \cdot 10^6$*	—
k_{-2}/k_4	—	85
k_4/k_3 (μM)	—	100
k_{-2}/k_2	—	$\geqq 60$
$k_2/(k_{-2}+k_4)$	$\geqq 10$	—

* Obtained from ref. 7.

** Depending on the NADH concentration.

lated in the steady state. It follows that $V = k_4$ and

$$K_m = \frac{\left(\frac{k_{-1}}{k_{-1}+k_2}\right) k_{-2}+k_4}{\left(\frac{k_2}{k_{-1}+k_2}\right) k_1}$$

(2) $10(k_{-2}+k_4) > k_2 > (k_{-2}+k_4)/10$, i.e. both intermediates are accumulated.

(3) $k_2 \leqslant (k_{-2}+k_4)/10$, i.e. the NADH · E_{ox} complex is preferentially accumulated. It follows that $V = k_2k_4/(k_{-2}+k_4)$ and

$$K_m = \frac{k_{-1}+\left(\frac{k_4}{k_{-2}+k_4}\right) k_2}{k_1}$$

The latter possibility can be subdivided into

(a) $k_{-2} \leqslant k_4/10$ with $V = k_2$ and $K_m = (k_{-1}+k_2)/k_1$

(b) $10k_4 > k_{-2} > k_4/10$

(c) $k_{-2} \geqslant 10k_4$, from which it follows that $k_{-2} \geqslant 10k_2$, $V = k_2k_4/k_{-2}$ and

$$K_m = \frac{k_{-1}+\frac{k_2k_4}{k_{-2}}}{k_1}$$

The data for Type-II dehydrogenase, $k_{-2}/k_2 > 60$ and $k_{-2}/k_4 = 85$, favour exclusively possibility 3c, the NADH · E_{ox} complex being preferentially accumulated. The data on Type-I dehydrogenase assembled in Table I of the preceding paper are as such compatible with all three possibilities. However, the assumption that the difference in mechanism of the two types is caused by the change of a single rate constant in the above sequence can be met only if possibility 1 applies to Type-I dehydrogenase, from which it follows that the difference lies in the value of k_2.

On the basis of this unitarian mechanism a further estimation of the separate rate constants can be made. Since k_4 should be equal in both types it follows from Table II that in Type-II dehydrogenase (1) $k_{-2} = k_2k_4/13\ s^{-1} \geqslant 7.8 \cdot 10^2 \cdot k_2$, consistent with $k_{-2} \geqslant 60\ k_2$, and (2) $k_3 = k_4/(100\ \mu M) \geqslant 10^8\ s^{-1} \cdot M^{-1}$, and that in both types (3) $k_{-2} = 85\ k_4 \geqslant 8.5 \cdot 10^5\ s^{-1}$. The condition $k_2 \geqslant 10(k_{-2}+k_4)$ for Type-I dehydrogenase simplifies via $k_{-2}/k_4 = 85$ to (4) $k_2 \geqslant 10\ k_{-2}$, which results in (5) $k_2 \geqslant 8.5 \cdot 10^6\ s^{-1}$. The expression for K_m(NADH) of Type-I dehydrogenase simplifies via $k_2 \geqslant 10\ k_{-2}$ and $k_{-2}/k_4 = 85$ to $K_m = (k_{-2}/k_2)(k_{-1}/k_1)+k_4/k_1 = 140\ \mu M$. Since $k_{-1}/k_1 = K_s$ lies between 25 and 100 μM and $k_{-2}/k_2 \leqslant 0.1$ it follows that $K_m = k_4/k_1 = 140\ \mu M$ and (6) $k_1 \geqslant 7.1 \cdot 10^7\ M^{-1} \cdot s^{-1}$ for Type-I dehydrogenase. Since $K_m = k_4/k_1$ is independent of NADH concentration, i.e. no cooperativity is observed, the intact enzyme complex in Type I, comprising flavoprotein and iron-sulphur proteins, is presumably in its active conformation. This implies that $K_s = 25\ \mu M$ and (7) $k_{-1} \geqslant 1.8 \cdot 10^3\ s^{-1}$.

TABLE III

ESTIMATED RATE CONSTANTS AT pH 8.0 BASED ON A UNITARIAN MECHANISM

	Type I	Type II
K_s(NADH) (μM)	25	100–25*
k_1 ($M^{-1} \cdot s^{-1}$)	$\geqq 7.1 \cdot 10^7$	$\geqq 7.1 \cdot 10^7$
k_{-1} (s^{-1})	$\geqq 1.8 \cdot 10$	$\geqq 1.8 \cdot 10^3$
K_m(NADH) (μM)	140 (k_4/k_1)	100–25* (k_{-1}/k_1)
V (s^{-1})	$\geqq 10^4$ (k_4)	$1.3 \cdot 10$ ($k_2 k_4/k_{-2}$)
k_2 (s^{-1})	$\geqq 8.5 \cdot 10^6$	$1.1 \cdot 10^3$
k_{-2}/k_2	$\leqq 0.1$	$\geqq 780$
k_4 (s^{-1})	$\geqq 10^4$	$\geqq 10^4$
k_3($M^{-1} \cdot s^{-1}$)	$1.8 \cdot 10^6$	$\geqq 10^8$

* Depending on the NADH concentration.

The estimated rate constants are summarized in Table III.

DISCUSSION

NADH-ferricyanide activity of Type-II dehydrogenase

On the basis of the data in Table III the differences in properties between Type-I and Type-II NADH dehydrogenase may now be resolved.

The association rate constant of the reaction between the physiological substrate NADH and the enzyme, k_1, is the same with both preparations, at least with NADH concentrations above 100 μM. This second-order rate constant is high, indicating that even in the complex (Type I) the enzyme is readily accessible to its substrate. The rate constant for the dissociation of NADH from the oxidized enzyme, k_{-1}, and the five times higher rate constant for dissociation of the product NAD^+ from the reduced enzyme, k_4, are also unchanged by isolation of the smaller protein from the complex. The higher k_4 is consistent with the weak competitive inhibition by the product NAD^+ ($K_i(NAD^+) = 1.1$ mM [12], cf. ref. 9) of the reaction between oxidized enzyme and NADH (K_s(NADH) = 25–100 μM). So far as these reactions are concerned it seems, then, that the flavoprotein subunit has been isolated without gross changes in tertiary structure.

The sigmoidal NADH-effect curve with Type-II dehydrogenase indicates that this preparation has not been isolated in its most active conformation, and that NADH may induce this. Since the intact enzyme complex is isolated in its active conformation (see above), one may speculate that one or more of the factors missing in Type-II dehydrogenase, i.e. iron-sulphur proteins, lipids or ubiquinone, operate as an effector for this flavoprotein. The finding in the preceding paper [7] that after extraction of ubiquinone from Complex I with pentane the binding of NADH changes from hyperbolic to sigmoidal, supports this conclusion, at least so far as ubiquinone is concerned.

The main differences between the two types lie in the values of k_2, the rate constant for electron flow from the enzyme-bound NADH to the enzyme, which is at least four orders of magnitude lower in Type II, and in k_3, which is nearly two orders of magnitude higher. The increase in k_3 presumably represents an increased accessi-

bility of ferricyanide to the reduced enzyme. It is suggested that, in the intact enzyme, this accessibility is impaired by iron-sulphur proteins and structural protein, so that ferricyanide reaches the active centre(s) via the same cleft as NADH, resulting in double substrate inhibition.

The reason for the concomitant decrease in k_2 is, however, not so obvious, since the unaltered association and dissociation rate constants of NADH and NAD^+ to the flavoprotein subunit suggest that its active centre has not been damaged. The rather high value of k_2 in Type-I NADH dehydrogenase implies that the equilibrium between the NADH-oxidized enzyme complex and the NAD^+-reduced enzyme complex lies in the direction of the latter. This is consistent with the results of others [13, 14] showing that all iron-sulphur centres are reduced by an excess of NADH within 6 ms, the mixing time of the freeze-quench method. The much lower value of k_2 in the flavoprotein subunit implies that, in this case, the equilibrium lies in the direction of the NADH-oxidized enzyme complex. This difference was confirmed by anaerobic titrations (not shown), in which the percentage of reduction of the flavin by NADH was measured spectrophotometrically at 460–510 nm. It was found that 100 μM NADH is able to reduce 2 μM (FMN) of Type-I dehydrogenase almost completely, whereas even 1 mM NADH reduced the same amount of Type-II dehydrogenase only to the extent of 70 % of that obtained with $Na_2S_2O_4$.

The changed position of the equilibrium corresponds to a 120 mV decrease in the redox potential of the prosthetic group FMN bound to the flavoprotein subunit. Since Type-II dehydrogenase is probably an intact subunit of Type-I dehydrogenase, this lowering of the redox potential, corresponding to an energy of 5.6 kcal/mol enzyme, presumably has its origin in the loss in quaternary structure resulting from the release of the flavoprotein subunit from the many iron-sulphur protein subunits.

It is tempting to speculate that the function of one or more of these iron-sulphur proteins is that of an effector, regulating the redox potential of the flavoprotein subunit by inter-subunit interactions. Such a possible control function of electron-accepting centres in the respiratory chain has been suggested by Slater [15]. Lee and Slater [16] have correlated the rate of transfer of electrons between cytochromes *b* and c_1 with the redox state of one of the iron-sulphur proteins present in this segment of the electron-transfer chain.

As a result of the changed position of the equilibrium, the two values of V, which are equal to k_4 and $k_2 \cdot k_4/k_{-2}$ in Type I and Type II, respectively, differ by three orders of magnitude. The values of K_m(NADH), equal to k_4/k_1 and K_s in Type I and Type II, respectively, are also different (see Table III). These differences, together with the double substrate inhibition in Type I and the 60-fold increase of k_3 in Type II may be largely overlooked if the activities of the two preparations are compared at a single concentration of donor and acceptor, e.g. 100 μM NADH and 1 mM ferricyanide, since they have effects in different directions. Thus despite a difference by a factor of about 10^4 in the value of k_2, the measured activities with these concentrations of substrate and acceptor differ by a factor of only about ten.

NADH-cytochrome c reductase activity of Type-II dehydrogenase

Since the results obtained with cytochrome *c* as acceptor are qualitatively the same as found with ferricyanide, they may be explained in terms of the minimum hypothesis stated above, where the intercepts on the abscissa in Fig. 8 and on the

ordinate in Fig. 7 for zero cytochrome *c* concentration equal k_4/k_3 and k_{-2}/k_2k_4, respectively. If we assume for k_4 the value $\geqslant 10^4\ s^{-1}$, calculated for the unitarian mechanism in Table III, k_3 for cytochrome *c* becomes $\geqslant 3.3 \cdot 10^9\ M^{-1} \cdot s^{-1}$, approaching that of a diffusion-controlled reaction [17]. This thirty times higher value of k_3 for cytochrome *c* compared with ferricyanide stresses the electron-accepting capacities of the former [18].

On the basis of the same assumptions it may be calculated from the data in Table I that k_{-2}/k_2 is ten times higher with cytochrome *c* than with ferricyanide at pH 8.0 and twice at pH 8.5. Since the concentration of the electron-accepting protein cytochrome *c* is about 1000 times that of the enzyme, these effects on k_{-2}/k_2 may be due to aspecific binding of cytochrome *c* to the flavoprotein subunit resulting in a lowering (raising) of the redox potential of the latter. Since cytochrome *c* has an isoelectric point of 9.8, it would be expected to bind less effectively at higher pH, which is consistent with the smaller effect on k_{-2}/k_2 at pH 8.5.

Thus, the increase in cytochrome *c* reductase activity during transformation of Type I to Type II appears to be caused by an enormous increase in the accessibility of cytochrome *c* to the reduced flavoprotein, which overshadows the consequences of the decrease of the value of k_2.

NADH-2,6-dichloroindophenol activity of Type-II NADH dehydrogenase

Although Fig. 9 seems to be quite different from Figs. 5 and 7, the proposed minimum hypothesis may still be applied. From the intersection point on the ordinate in Fig. 9, V may be calculated as $3.3 \cdot 10^2\ s^{-1}$. This value, representing a turnover number at infinite concentrations of donor and acceptor, is about two orders of magnitude higher than the value of V calculated with ferricyanide or cytochrome *c* by extrapolation to zero acceptor concentration. In fact it approximates to the value of k_2 ($1.1 \cdot 10^3\ s^{-1}$) in Table III. It appears, then, that with 2,6-dichloroindophenol in the concentration range measured, the ordered mechanism predominates, so that in Eqn. 1 k_4 may be neglected with respect to k_3a and, at infinite concentrations of donor and acceptor, $TN^{-1} = 1/k_2 + 1/k_4 = 1/k_2$ (see Table III) is obtained. That such differences are found with different acceptors is not unexpected, since the value of k_3 is determined by the accessibility of the acceptor to the reduced enzyme, and the convenient concentration range of a is determined by the A ($mM^{-1} \cdot cm^{-1}$) of the particular acceptor at its optimum wavelength.

From the intercepts on the ordinate in Fig. 9, a K_m(acceptor) of 100 μM may be estimated. This is much lower than the value of 1.4 mM reported for Type-I dehydrogenase in the preceding paper [7], indicating that, like ferricyanide and cytochrome *c*, the indophenol accepts electrons from the reduced flavoprotein subunit at a more accessible site, consistent with the absence of inhibition by NADH.

Unlike ferricyanide and cytochrome *c*, the indophenol acts as an inhibitor, competitive with respect to NADH. In the preceding paper it was concluded that this acceptor binds at or near the NADH-binding site of Type-I dehydrogenase and, as a consequence, the rate at infinite concentration of donor and acceptor yields the rate constant of the oxidation of reduced enzyme by bound acceptor. Although Type-II dehydrogenase exposes sites to the indophenol that are more accessible and therefore more rapidly oxidized, the binding near the NADH-binding sites still exists and largely obscures the ordered mechanism.

TABLE IV

COMPARISON OF PROPERTIES AND RATE CONSTANTS OF TYPE-I AND TYPE-II NADH DEHYDROGENASE AS MEASURED IN THE PRESENCE OF DIFFERENT ELECTRON ACCEPTORS

Enzyme	Acceptor	V	K_m(NADH)	Mechanism	Effect of NADH	Competitive substrate inhibition by	
						NADH	Acceptor
Type I							
Complex I	Ferricyanide	k_4	k_4/k_1	Ping pong	Hyperbolic	Hyperbolic	+
Complex I	Indophenol	k_5*	k_4/k_1	Ping pong	Hyperbolic	Hyperbolic	+
Q-deficient Complex I	Ferricyanide	k_4	k_4/k_1	Ping pong	Sigmoidal	Sigmoidal	+
Type II							
	Indophenol	k_2	k_{-1}/k_1	Ordered	Hyperbolic	—	Sigmoidal
	Ferricyanide	k_2k_4/k_{-2}**	k_{-1}/k_1	Mixture of ordered and ping pong	Sigmoidal	—	—
	Cytochrome *c*	k_2k_4/k_{-2}**	k_{-1}/k_1	Mixture of ordered and ping pong	Sigmoidal	—	—

* k_5 represents the rate constant of oxidation of reduced enzyme by bound acceptor.
** Extrapolated to zero acceptor concentrations.

The straight lines in Fig. 9 show that, under the conditions used here, binding of NADH does not induce a conformational change in the Type-II dehydrogenase. However, judging by the sharp increase in the slope of these lines at high indophenol concentrations, binding of the indophenol at or near the NADH-binding sites becomes stronger at higher concentrations, suggesting that, like ubiquinone, the two-electron acceptor indophenol acts as an allosteric effector for the enzyme. That the K_m (NADH) of the NADH dehydrogenase is different in the presence of a one-electron or a two-electron acceptor was pointed out previously by Hatefi and Stempel [8].

For comparison, some of the properties and rate constants of Type-I and Type-II NADH dehydrogenase, as measured in the presence of the electron acceptors studied in this paper, are listed in Table IV.

EXPERIMENTAL

Heart-muscle particles were prepared essentially as described by Keilin and Hartree [19]. Complex I was isolated from these particles according to the procedure of Hatefi et al. [20]. Type-II NADH dehydrogenase was resolved from Complex I essentially according to the method of Hatefi and Stempel [8], using 0.5 M $NaClO_4$ [21] instead of 2.5 M urea. The resolution was carried out in a medium containing 0.66 M sucrose, 50 mM Tris · HCl buffer (pH 8.0), 1 mM EDTA and 1 mM dithiothreitol. All solutions were de-aerated before use and the enzyme solution was kept under N_2 throughout the isolation procedure. Small quantities of the enzyme were stored at the temperature of liquid N_2 and thawed just before use.

Protein was determined by the biuret method after trichloroacetic acid precipitation as described by Cleland and Slater [22]. The enzyme concentration is expressed on the basis of FMN concentration, determined fluorimetrically as acid-extractable flavin, as described in the previous paper [7].

The NADH-ferricyanide activity was measured at 25 °C as described in the previous paper [7]. The assay was started by adding appropriate amounts of the undiluted enzyme solution with a syringe. Control assays were carried out every 30 min to correct for loss in enzymic activity during storage at 0 °C. After 8 h at 0 °C the activity had decreased by 30 %.

The NADH-dichloroindophenol activity was measured in the same way, the reduction being followed at 600 nm ($A_{ox-red} = 21\ mM^{-1} \cdot cm^{-1}$).

The NADH-cytochrome *c* reductase activity was measured by following the absorbance at 550 nm, using 21.1 $mM^{-1} \cdot cm^{-1}$ as the absorption coefficient for cytochrome *c* (reduced minus oxidized) [23]. Assays were carried out in a medium containing 1 mM EDTA, 20 mM phosphate (pH 8.0), or 20 mM glycylglycine buffer at higher pH.

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