*Biochimica et Biophysica Acta*, 440 (1976) 1–15


BBA 47108

# STEADY-STATE KINETICS OF HIGH MOLECULAR WEIGHT (TYPE-I) NADH DEHYDROGENASE

G. DOOIJEWAARD and E. C. SLATER

*Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam, Plantage Muidergracht 12, Amsterdam (The Netherlands)*

(Received November 4th, 1975)

## SUMMARY

(1) Studies of the steady-state kinetics of the NADH dehydrogenase activity of Complex I (NADH: Q oxidoreductase) revealed that the reaction mechanism with the one-electron acceptor ferricyanide or the two-electron acceptor 2,6-dichloroindophenol is ping pong bi bi, with double substrate inhibition. NADH inhibits the reaction of the reduced form of the flavoprotein with the acceptors, and the acceptors prevent NADH from reacting with the oxidized form. This implies that both NADH and acceptors react with the same site on NADH dehydrogenase.

(2) The velocity at infinite NADH and acceptor concentrations (corrected for the double substrate inhibition) is much larger with ferricyanide than with the indophenol. It is concluded that the latter binds to the reduced enzyme. Thus, with ferricyanide the rate constant measured refers to the dissociation of bound $NAD^+$ from the reduced enzyme ($k_2$) and with the indophenol to the rate constant of oxidation of reduced enzyme by bound acceptor ($k_4$). The latter value is not an estimate for the situation in vivo, where ubiquinone is the acceptor.

(3) The rate constant of the dissociation of bound $NAD^+$ from the reduced enzyme ($k_2$) increases with pH. It is suggested that an ionizing group on the enzyme is involved in the dissociation.

(4) After extraction of ubiquinone from Complex I with pentane the curve relating activity at infinite ferricyanide concentration to NADH concentration changes from hyperbolic to sigmoidal. The hyperbolic curve is restored by reincorporating ubiquinone. It is concluded that ubiquinone is an effector for NADH dehydrogenase.

## INTRODUCTION

Two types of preparation of NADH dehydrogenase have been isolated from mitochondria or submitochondrial particles [1–4]. NADH dehydrogenase isolated by phospholipase digestion of heart particles [5, 6] at 30 °C or by dispersal with cholate

Abbreviation: DC1P, 2,6-dichloroindophenol.

and fractionation with ammonium sulphate [7] contains about 1.2 nmol flavin/mg protein (Type I). After phospholipase digestion at 37 °C or by treatment with acid ethanol, urea, thiourea or proteolytic enzymes, a preparation of NADH dehydrogenase containing more than 10 nmol flavin/mg protein [2] (Type II) may be isolated. In addition to flavin, Type-I dehydrogenase contains many Fe-S centres. Orme-Johnson et al. [8] and Ohnishi et al. [9–11] identified at least five types with different EPR characteristics and redox potential. In Type-II dehydrogenase most of these centres are lacking as judged by the low Fe-S content [12, 13]. Furthermore, Type I is characterized by a high NADH-ferricyanide activity and a low antimycin-insensitive NADH-cytochrome *c* reductase activity, whereas in Type II the turnover number with ferricyanide is at least an order of magnitude lower and the cytochrome *c* reductase activity is much greater.

Kaniuga [14] pointed out in 1963 that the difference between the two types of preparation could be explained in two ways. The two alternatives may now be expressed. (1) The Type-II dehydrogenase is a subunit of the Type-I dehydrogenase, which is a large protein made up of a large number of subunits. This subunit is isolated without gross changes in tertiary structure, except insofar as the tertiary structure is influenced by the quaternary structure, but its acceptor specificity is changed by release from the restraints imposed on it when associated with other subunits in the Type-I dehydrogenase. (2) The Type-II dehydrogenase is irreversibly modified, perhaps by breakage or forming of a covalent bond, when it is separated from the other subunits, in such a way that its acceptor specificity is changed.

Singer [6] strongly advocates the second explanation. Hatefi and coworkers [15–18], on the other hand, favour the first.

In parellel work, which has been reported briefly in a symposium report [19] and which will be described in detail in a paper to be submitted later, it is shown that two of the six or more subunits present in Type-I preparations are retained in the Type-II preparation. One of these is the FMN-containing subunit.

Whichever is the correct explanation of the striking differences in the activity and acceptor specificity of the two types of NADH dehydrogenase, it is of interest to determine which of the partial reactions that must be involved in the enzyme mechanism (such as reaction with NADH, reduction of flavin by bound NADH, oxidation of flavin by acceptor and dissociation of bound $NAD^+$) are affected. Indeed, it might be expected that this would give an indication of which of the two possible explanations given above is correct. In this and the accompanying paper [20], an attempt is made to do this by comparing the steady-state kinetics of the two types of dehydrogenase. It is shown [20] that Type-II dehydrogenase is isolated from Type I without gross changes in tertiary structure, as judged by the unaltered rate constants for dissociation of NADH and $NAD^+$ and association of NADH. The difference in kinetics can be explained by a greater accessibility (by two orders of magnitude) of the smaller protein to acceptors and by a lower rate of intramolecular electron transfer (by four orders of magnitude).

This paper deals with Type-I dehydrogenase. Some of this work has been presented elsewhere [19, 21, 22].

RESULTS

*NADH-ferricyanide activity of Type-I dehydrogenase*

Fig. 1 shows that the NADH-ferricyanide reductase activity of Complex I (Type-I dehydrogenase) remains constant for a sufficient period to enable the accurate measurement of the initial rate, when the reaction is started by addition of enzyme. Under the conditions of Fig. 1A, the reaction is virtually zero order until nearly all the NADH is consumed. In the experiment shown in Fig. 1B, where the ratio of ferricyanide and NADH concentrations was 2.5 times that in Fig. 1A, the rate increased slightly during the course of the reaction. All rates in this paper refer to the initial rate.

The reaction is inhibited by both NADH (Fig. 2A), in agreement with Minakami et al. [23], and ferricyanide (Fig. 2B). The Dixon plot given in Fig. 3

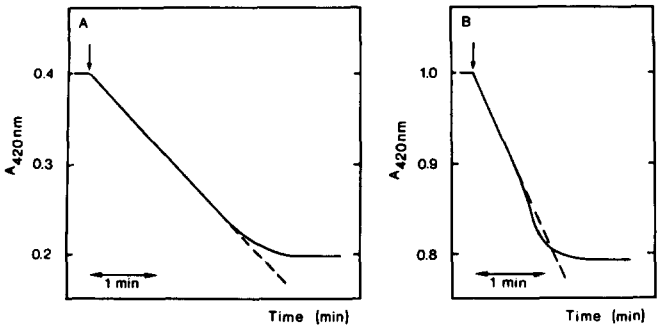


Fig. 1. Recorder traces of NADH-ferricyanide activity assay of Complex I, plotting the absorbance of ferricyanide at 420 nm as function of time. NADH, 100 $\mu$M; ferricyanide, 400 $\mu$M (A) or 1 mM (B). The reaction mixture contained in addition 20 mM sodium phosphate and 1 mM EDTA. The pH was 7.55. 5.7 $\mu$g Complex I added at the arrows.

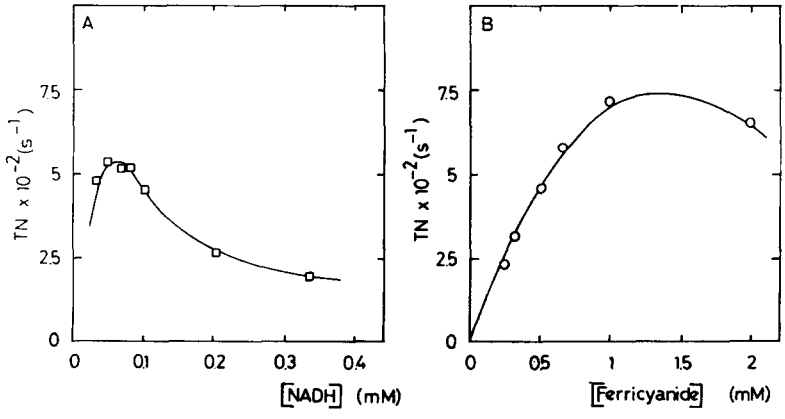


Fig. 2. The initial rate of the NADH-ferricyanide activity as function of NADH (A) and ferricyanide (B) concentration at fixed concentration of the other reactant (0.5 mM ferricyanide in A and 0.1 mM NADH in B). The initial rate is expressed as turnover number (TN) in mol NADH oxidized/mol Complex I (FMN basis) per s. Enzyme concentration, 0.6 nM; pH 7.55.

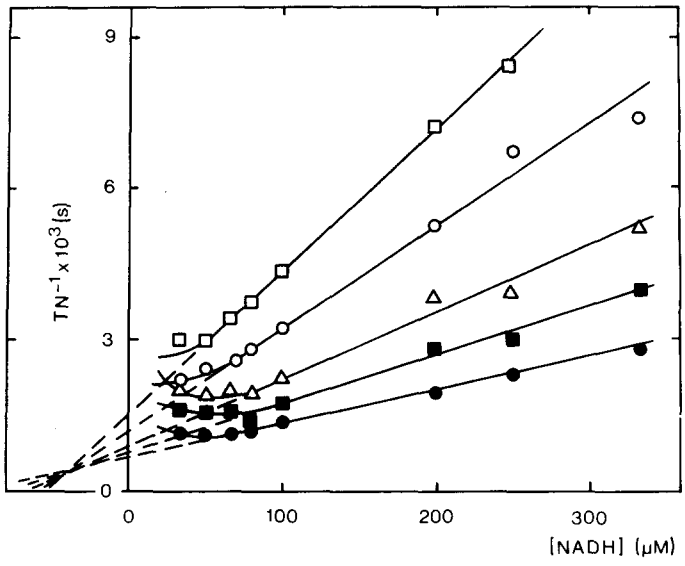


Fig. 3. Dixon plot of initial rate of NADH-ferricyanide activity as function of NADH concentration at fixed concentrations of ferricyanide of (□) 0.25 mM, (○) 0.33 mM, (△) 0.50 mM, (■) 0.67 mM and (●) 1.0 mM. Conditions as described in Fig. 2.

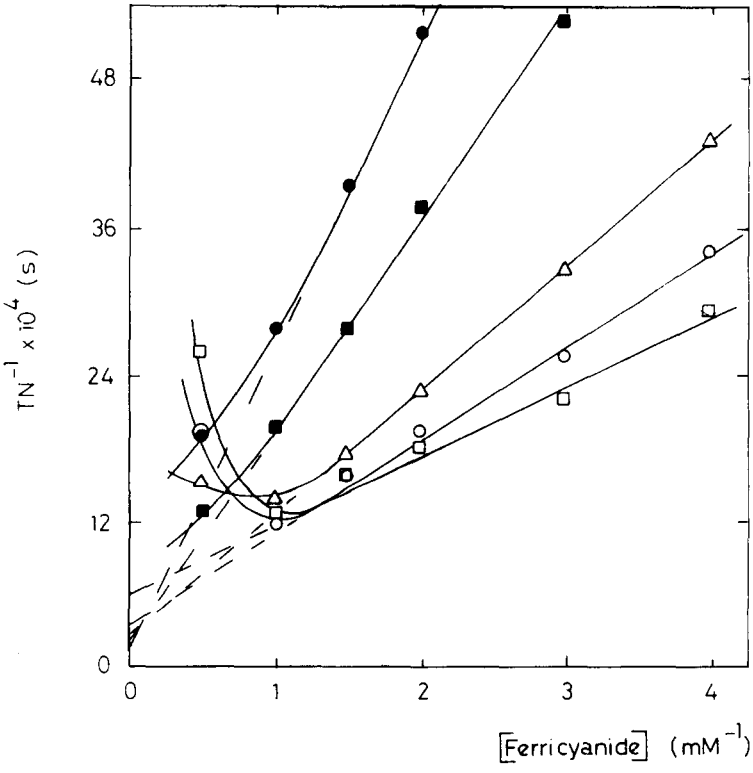


Fig. 4. Lineweaver-Burk plot of initial rate of NADH-ferricyanide activity as function of ferricyanide concentration at fixed concentrations of NADH of (□) 33 μM, (○) 67 μM, (△) 100 μM, (■) 200 μM and (●) 333 μM. Conditions as described in Fig. 2.

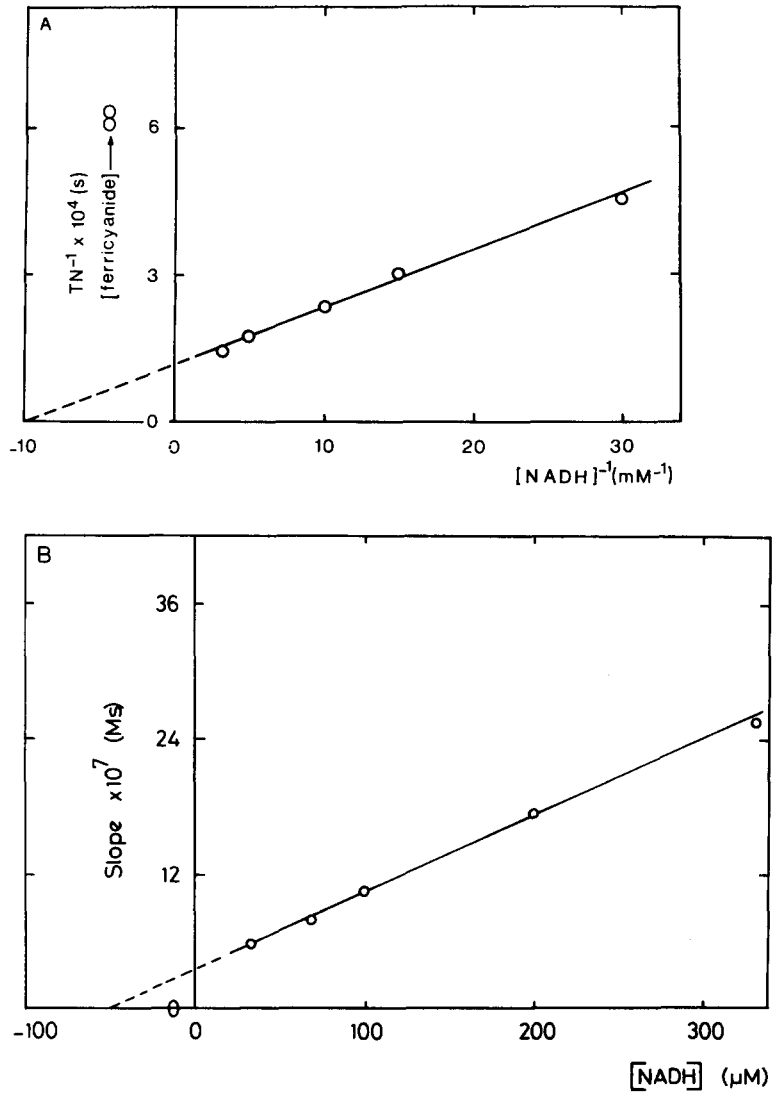


Fig. 5. Derivative plot of Fig. 4. The intercepts on the ordinate (A) and the slopes (B) of the straight portions of the lines in Fig. 4 are plotted against the reciprocal of the NADH concentration (A) and the NADH concentration (B).

yields a $K_i$ (NADH) equal to 40 $\mu$M. In Fig. 4 the same data are expressed in a double-reciprocal plot. In Figs. 5A and 5B the intercepts on the ordinate and the slopes, respectively, of the straight portions of the lines in Fig. 4 are plotted against the reciprocal of the NADH concentration and the NADH concentration, respectively.

The data are consistent with a ping-pong mechanism with double-substrate inhibition, as formulated in Scheme A and Eqn. 1:

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

$$Fe^{3+} + E_{red} \overset{k_3}{\rightarrow} [E_{semi}] + Fe^{2+}$$

$$Fe^{3+} + [E_{semi}] \overset{fast}{\rightarrow} E_{ox} + Fe^{2+}$$

$$Fe^{3+} + E_{ox} \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} [Fe^{3+} \cdot E_{ox}]$$

$$NADH + E_{red} \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} [NADH \cdot E_{red}]$$

$$K_m\,(NADH) = k_2/k_1 \qquad K_i\,(NADH) = k_{-6}/k_6 \qquad K_i\,(ferri) = k_{-5}/k_5$$

$$V = k_2$$

Scheme A

$$TN^{-1} = \frac{1}{k_2}\left\{1 + \frac{K_m(NADH)}{[NADH]}\left(1 + \frac{[Fe^{3+}]}{K_i(ferri)}\right)\right\} + \frac{1}{k_3[Fe^{3+}]}\left(1 + \frac{[NADH]}{K_i(NADH)}\right) \quad (1)$$

(TN = turnover number)

in which two stable forms of the enzyme, oxidized and reduced, are involved. NADH is able to bind to both forms, but only the complex with oxidized enzyme leads to further reaction, yielding the reduced form and $NAD^+$. Ferricyanide is also able to interact with both forms, but only the reaction with the reduced enzyme leads to further reaction, resulting in the oxidized form. Thus both NADH and ferricyanide inhibit the reaction competitively. NADH inhibits the reaction of the reduced form of the flavoprotein with ferricyanide, and the acceptor prevents NADH from reacting with the oxidized enzyme. This implies that NADH and ferricyanide affect the same site on Type-I dehydrogenase.

According to this mechanism, the turnover number, calculated for infinite concentration of both substrates and disregarding the double substrate inhibition, is equal to $k_2$, the rate constant for dissociation of bound $NAD^+$ from reduced enzyme. This is a parameter of the enzyme activity that is not influenced by the acceptor. Its value is given by the inverse of the intercept on the ordinate in Fig. 5A, namely $8.3 \cdot 10^3\ s^{-1}$. From the intercept of this line on the abscissa ($-1/K_m$) the $K_m$ for NADH may be calculated as 100 $\mu$M. The intercepts of the line in Fig. 5B yield on the ordinate, $1/k_3$, and on the abscissa $-K_i$(NADH).

Table I summarizes the rate constants, obtained from the appropriate derivative

TABLE I

RATE CONSTANTS OF TYPE-I NADH DEHYDROGENASE CALCULATED FROM NADH-FERRICYANIDE ACTIVITY AT DIFFERENT pH VALUES

| pH | $k_2(s^{-1})$ | $K_m$(NADH) ($\mu$M) | $K_i$(NADH) ($\mu$M) | $k_3(M^{-1} \cdot s^{-1})$ |
|---|---|---|---|---|
| 7.0 | $4.2 \cdot 10^3$ | 90 | 25 | $5.5 \cdot 10^6$ |
| 7.55 | $8.3 \cdot 10^3$ | 100 | 50 | $2.9 \cdot 10^6$ |
| 8.2 | $\geqq 10^4$ | — | 80 | $1.8 \cdot 10^6$ |

plots, at different pH values. $k_2$ increases with pH, reaching values of more than $10^4$ $s^{-1}$ above pH 8, whereas $K_m$ for NADH is rather pH-independent, at least between pH 7.0 and 7.55. The affinity of NADH for the reduced enzyme ($K_i$) diminishes by a factor of about 3 between pH 7 and 8.2. The second-order rate constant ($k_3$) for the reaction of ferricyanide with reduced enzyme also declines 3-fold.

*NADH-2,6-dichloroindophenol activity of Type-I dehydrogenase*

In order to test if the same mechanism for Type-I dehydrogenase holds for other acceptors, measurements were also made with 2,6-dichloroindophenol. Fig. 6 shows that a double-reciprocal plot of turnover number against acceptor concentration is qualitatively similar to that with ferricyanide. When, however, the rate constants appropriate for the mechanism given in Scheme A are calculated in the same way as for ferricyanide, the turnover at infinite concentration of both substrates is 500 $s^{-1}$, less than 5 % of that found in the ferricyanide assay at the same pH ($\geqslant 10^4$ $s^{-1}$). In order to explain this discrepancy an enzyme-acceptor complex is postulated in Scheme B and Eqn. 2.

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

$$\mathrm{DCIP}+\mathrm{E_{red}} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} [\mathrm{DCIP}\cdot\mathrm{E_{red}}] \overset{k_4}{\rightarrow} \mathrm{DCIP}+\mathrm{E_{ox}}$$

$$\mathrm{DCIP}+\mathrm{E_{ox}} \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} [\mathrm{DCIP}\cdot\mathrm{E_{ox}}]$$

$$\mathrm{NADH}+\mathrm{E_{red}} \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} [\mathrm{NADH}\cdot\mathrm{E_{red}}]$$

$$K_m(\mathrm{NADH}) = k_2/k_1 \qquad K_m(\mathrm{DCIP}) = \frac{k_{-3}+k_4}{k_3} \qquad K_i(\mathrm{NADH}) = k_{-6}/k_6$$

$$K_i(\mathrm{DCIP}) = k_{-5}/k_5$$

$$V = \frac{k_2 k_4}{k_2+k_4}$$

Scheme B

$$\mathrm{TN}^{-1} = \frac{1}{k_2}\left\{1+\frac{K_m(\mathrm{NADH})}{[\mathrm{NADH}]}\left(1+\frac{[\mathrm{DCIP}]}{K_i(\mathrm{DCIP})}\right)\right\} + \frac{1}{k_4}\left\{1+\frac{K_m(\mathrm{DCIP})}{[\mathrm{DCIP}]}\left(1+\frac{[\mathrm{NADH}]}{K_i(\mathrm{NADH})}\right)\right\} \qquad (2)$$

As a consequence the rate equation reduces to $(1/k_2+1/k_4)$ at infinite concentration of both substrates (ignoring the double substrate inhibition). The derivative plots yield in this case $K_m(\mathrm{NADH})/k_2$, $K_m(\mathrm{DCIP})/k_4$ and $(1/k_2+1/k_4)$, from which the individual rate constants cannot be calculated without a further assumption. For the calculation of the constants in Table II the $k_2$ ($\geqslant 10^4$ $s^{-1}$) from the NADH-ferricyanide assay (Table I) was used. Obviously the turnover number at infinite substrate concentrations represents in this case $k_4$ rather than $k_2$, and the acceptor-independent

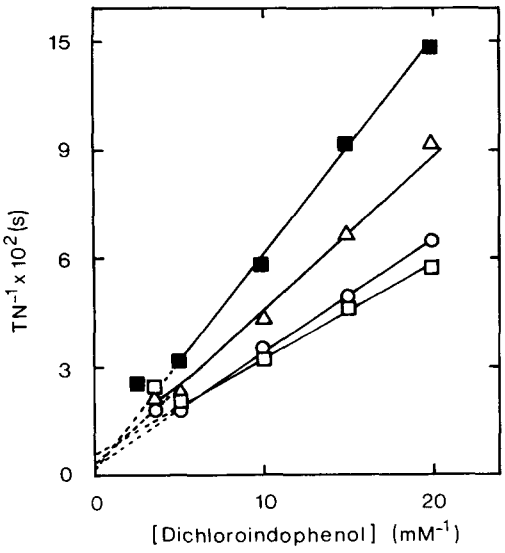


Fig. 6. Lineweaver-Burk plot of initial rate of NADH-2,6-dichloroindophenol activity as function of the indophenol concentration at fixed concentrations of NADH of (□) 4 $\mu$M, (○) 12.5 $\mu$M, (△) 50 $\mu$M and (■) 100 $\mu$M, pH 8.0. Enzyme concentration, 4.8 nM. Further conditions as described in Fig. 2.

TABLE II

RATE CONSTANTS OF TYPE-I NADH DEHYDROGENASE AT pH 8.0, CALCULATED FROM THE NADH-2,6-DICHLOROINDOPHENOL (DCIP) ACTIVITY ASSAY

| $k_2(s^{-1})$ | $k_4(s^{-1})$ | $V(s^{-1})$ | $K_m$(NADH) ($\mu$M) | $K_i$(NADH) ($\mu$M) | $K_m$(DCIP) (mM) |
|---|---|---|---|---|---|
| $\geq 10^4$* | $5.4 \cdot 10^2$ | $5 \cdot 10^2$ | $\geq 140$ | 75 | 1.4 |

* Obtained from Table I.

rate constant of dissociation of bound $NAD^+$ from the reduced enzyme is not measured with this acceptor.

*NADH-ferricyanide activity of ubiquinone-depleted Type-I dehydrogenase*

Since ferricyanide reacts before the rotenone block and rotenone inhibits the reduction of ubiquinone by NADH, one would not expect that extraction of ubiquinone from Complex I would change the steady-state kinetics. Complex I was dialysed against 100 mM KCl for 3 h and then lyophilized. The lyophilized preparation was extracted with pentane 4 times according to the method of Szarkowska [24] and then resuspended in Tris-sucrose buffer. In Fig. 7A the rate, extrapolated to infinite ferricyanide concentration, is plotted against NADH concentration for the untreated Complex I, lyophilized complex, extracted lyophilized complex and extracted complex in which ubiquinone was reincorporated. Fig. 7B is the reciprocal plot. The rate constants, obtained from derivative plots appropriate for Scheme A, are compiled in Table III.

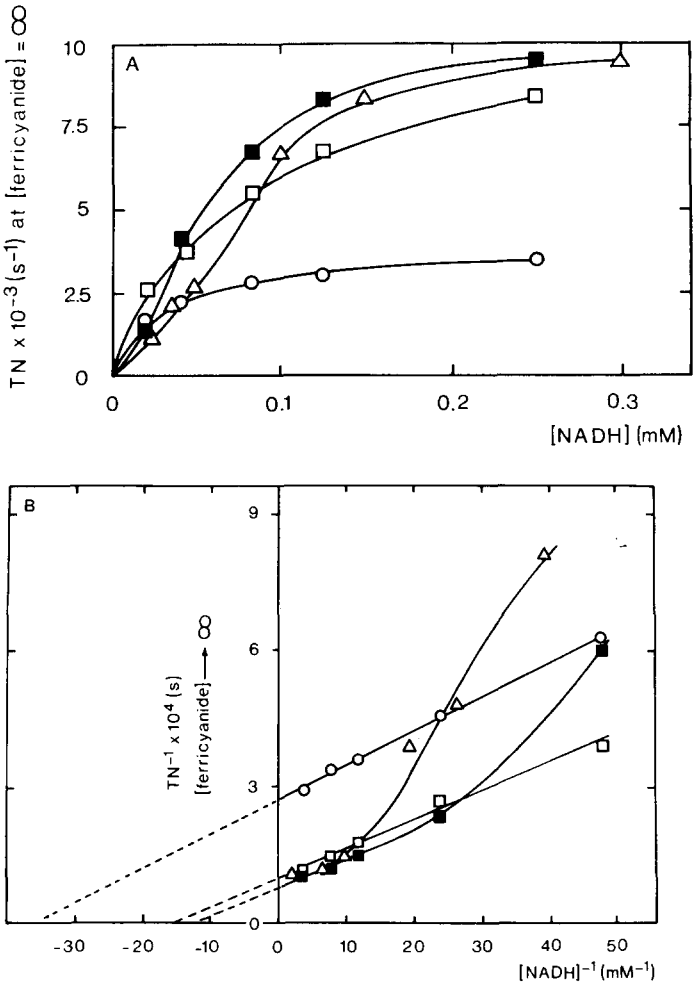


Fig. 7. Plot of initial rate of NADH-ferricyanide reductase activity at infinite ferricyanide concentration as function of NADH concentration (A) and double-reciprocal plot (B) for untreated Complex I (□), lyophilized complex (○), ubiquinone-extracted lyophilized complex (△) and extracted complex in which ubiquinone was reincorporated (■), pH 7.5. Enzyme concentrations, 0.6, 0.7, 2.0 and 1.0 nM, respectively. Further conditions as described in Fig. 2.

The data for untreated Complex I are similar to those given in Table I. The lyophilization procedure alone seems to have a marked effect, the maximum turnover number ($k_2$) decreasing by more than 50 % in agreement with Singer [6]. The $K_m$(NADH) declines to about the same extent so that the lines in Fig. 7B are parallel. The increase in $K_i$(NADH) and decrease in $k_3$ show that the reactivity of the reduced enzyme with NADH and acceptor are also affected. However, the effects on the $K_i$(NADH) and $k_3$ are already found after the dialysis against KCl before lyophilization and do not disappear after subsequent dialysis against Tris-sucrose buffer; those on $k_2$ and $K_m$(NADH) are reversed by dialysis of the KCl-containing lyophilized enzyme against Tris-sucrose buffer (not shown here).

TABLE III

EFFECT OF UBIQUINONE DEPLETION ON RATE CONSTANTS OF TYPE-I NADH DEHYDROGENASE AT pH 7.5

| Treatment | $k_2(s^{-1})$ | $K_m$(NADH) ($\mu$M) | $K_i$(NADH) ($\mu$M) | $k_3(M^{-1} \cdot s^{-1})$ |
|---|---|---|---|---|
| None | $9.8 \cdot 10^3$ | 62.5 | 35 | $6.7 \cdot 10^6$ |
| Lyophilized | $3.7 \cdot 10^3$ | 28 | 60 | $3.3 \cdot 10^6$ |
| Pentane extraction | $12.8 \cdot 10^3$* | — | 80* | $3.3 \cdot 10^6$* |
| Ubiquinone reincorporated | $12.8 \cdot 10^3$ | 77 | 50 | $3.3 \cdot 10^6$ |

* Obtained at [NADH] > 100 $\mu$M.

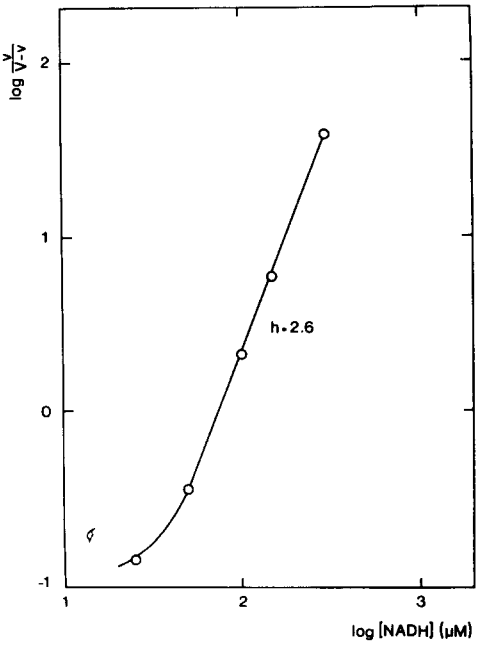


Fig. 8. Hill plot for the NADH-ferricyanide reductase activity of pentane-extracted Complex I as a function of NADH concentration. Conditions as described in Fig. 7. $V$ was determined by extrapolation of the initial rates in Fig. 7B to infinite NADH concentration.

After extraction of ubiquinone from lyophilized Complex I, the curve relating activity at infinite ferricyanide concentration to NADH concentration becomes sigmoidal (Fig. 7A). At NADH concentrations higher than 100 $\mu$M the original activity in Complex I is restored (see Fig. 7B). The Hill plot in Fig. 8 illustrates the sigmoidicity, the slope $h$ being equal to 2.6.

Reincorporation of an excess of Q largely restores the original hyperbola (Fig. 7A). $k_2$ and $K_m$(NADH) in the ubiquinone-reincorporated complex resemble those in Complex I but the increased $K_i$(NADH) and decreased $k_3$ resulting from the dialysis against KCl are not affected.

## DISCUSSION

The most important conclusion to be drawn from the experiments described in this paper is that the mechanism of action of Type-I NADH dehydrogenase with artificial electron acceptors is ping pong bi bi with double substrate inhibition [25]. Although this mechanism is adequate as a minimum hypothesis to compare rate constants under different conditions with respect to pH, acceptor and type of preparation, it cannot be excluded that a more complicated mechanism with more intermediates is operating.

A ping-pong mechanism with double substrate inhibition yields hyperbolae in double-reciprocal plots but, by extrapolation of the straight-line portions at the hyperbolae corresponding to low substrate concentrations, the rate constants of the uninhibited reaction can be extracted, if $K_i$(substrate) is appreciably higher than the substrate concentration. Thus, from Fig. 4, the double-reciprocal plot of rate against ferricyanide concentration, and from Figs. 5A and 5B, $k_2$, $K_m$(NADH), $k_3$ and $K_i$(NADH) can be determined. $K_i$(ferricyanide) could not, however, be determined in this way, since $K_i$(NADH) is too low (see Table I). The increase of the hyperbolae in Fig. 4 below 1 $mM^{-1}$ ferricyanide suggests that it is about 1 mM and, indeed, curves calculated on the basis of Eqn. 1, $K_i$(ferricyanide) = 1 mM, and the rate constants given in Table I agreed closely with the experimental curves in Fig. 4 (correlation coefficient, 0.98).

Further support for the proposed ping-pong mechanism is obtained by an examination of the time course of the reaction. By integration of the rate equation appropriate to Scheme A, Eqn. 3 relating the time of the reaction to the amount of NADH oxidized is obtained.

$$t = \frac{1}{[\mathrm{FMN}]}\left\{\underbrace{\frac{1}{k_2}\left(1+\frac{2K_m(\mathrm{NADH})}{K_i(\mathrm{ferri})}\right)(a-x)}_{\text{term 1}} - \underbrace{\frac{1}{k_2}\left(1+\frac{(b-2a)}{K_i(\mathrm{ferri})}\right)K_m(\mathrm{NADH})\ln\left(\frac{x}{a}\right)}_{\text{term 2}}\right.$$
$$\left. + \underbrace{\frac{(a-x)}{2k_3\,K_i(\mathrm{NADH})}}_{\text{term 3}} - \underbrace{\frac{1}{2k_3}\left(1-\frac{(b-2a)}{2K_i(\mathrm{NADH})}\right)\ln\left(1-\frac{2(a-x)}{b}\right)}_{\text{term 4}}\right\} \qquad (3)$$

where

$[\mathrm{NADH}]_{t=0} = a$
$[\mathrm{ferri}]_{t=0} = b$
$[\mathrm{NADH}]_{t=t} = x$

The time $t$ is a function of all the kinetic constants, the initial NADH and acceptor concentrations, the NADH concentration at time $t$ and the fraction of the initial concentrations of NADH and ferricyanide present at time $t$. Fig. 9 shows how a theoretical time course, neglecting product inhibition, is built up of the four separate terms in Eqn. 3. The first and second terms include reactions of the oxidized enzyme, and the third and fourth those of the reduced enzyme. The first and third terms show a linear relationship and the second and fourth a logarithmic relationship between time and NADH concentration. When NADH is exhausted the second term becomes

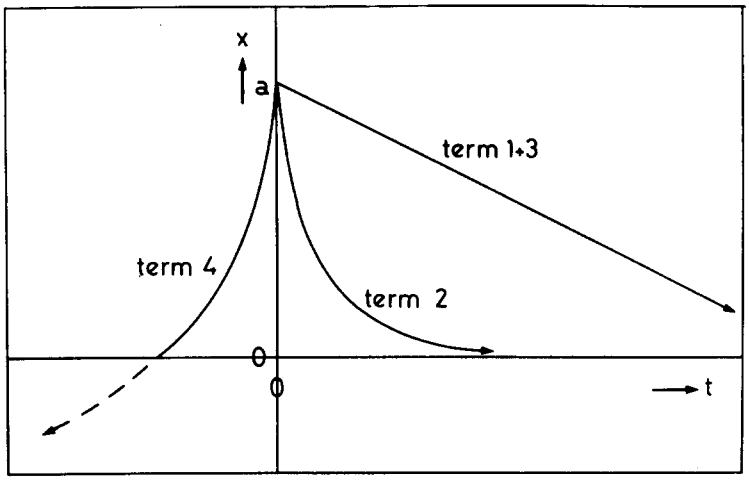


Fig. 9. Theoretical plots of the NADH concentration at time $t$ (denoted by $x$), as function of the time of the reaction ($t$), showing how the recorder trace in Figs. 1A and 1B is built up of the four separate terms in Eqn. 3, the integrated rate equation.

infinite, whereas the fourth term approaches a constant value. If ferricyanide is sufficiently in excess, so that $(b-2a) > 2K_i$(NADH), then term 4 is negative, i.e. it contributes to an increase in the reaction rate. With a moderate excess of ferricyanide over NADH the decrease in rate (term 2) and the increase (term 4) may cancel one another resulting in an apparent zero-order reaction (see Fig. 1A). During the course of the reaction the ferricyanide concentration increases relative to that of NADH, as a result of which the reduction of oxidized enzyme by NADH will be more inhibited, whereas the oxidation of the reduced enzyme by ferricyanide will be less inhibited. With a greater excess of ferricyanide (concentration ratio about 10), the increase in the fourth term of Eqn. 3 can completely overcome the decrease in the second term, resulting in a slight increase in the rate during the course of the reaction (see Fig. 1B).

The conclusion that NADH dehydrogenase operates by a ping-pong mechanism is supported by data in the literature. Kean et al. [26] provided evidence for such a mechanism for the NADH:acetylpyridine-adenine dinucleotide oxidoreductase activity of Type-I NADH dehydrogenase. Minakami et al. [27] presented data for the reduced acetylpyridine-adenine dinucleotide: ferricyanide oxidoreductase activity that may also be interpreted in terms of a ping-pong mechanism. Double substrate inhibition has been found by Tottmar and Ragan [28] with NADH dehydrogenase isolated from *Candida utilis*.

It is worth mentioning that Kean et al. [26] also reported that the rate at infinite ferricyanide concentration increases with NADH concentration ($K_m \approx 80$ $\mu$M), in agreement with our results, but in disagreement with the earlier interpretation of Minakami et al. [23], who drew straight lines through the measured points, intersecting at the ordinate in the double-reciprocal plot of rate against ferricyanide concentration at different NADH concentrations. We agree, however, with the conclusion of Minakami et al. [23, 27] that ferricyanide is a satisfactory electron acceptor to measure the NADH dehydrogenase activity. Our results suggest that at infinite concentrations of both substrates, $k_2$, the rate constant for the dissociation of bound

$NAD^+$ from the reduced enzyme, is obtained (see also ref. 20). This is a parameter of the enzyme activity that is not influenced by the acceptor. With dichloroindophenol as acceptor, however, much lower rates are obtained at infinite concentrations of both substrates, presumably because of a binding of this acceptor to the reduced enzyme.

In agreement with Minakami et al. [23] we observed an effect of pH on the NADH-ferricyanide activity. From Table I it is possible to distinguish between changes that may occur in vivo and those due to the use of the artificial acceptor. The rate of dissociation of bound $NAD^+$ from the reduced enzyme ($k_2$) increases with increasing pH between 7 and 8, but less than the increase of the $OH^-$ concentration, suggesting that some nucleophilic group on the enzyme or its prosthetic group with a p$K$ in the region of 7–8 facilitates the dissociation. Histidine is a possible candidate. Since $K_m$(NADH) which, according to Scheme A, represents $k_2/k_1$, is not affected by variation of pH between 7 and 7.55, it may be concluded that the rate of binding of NADH to the oxidized enzyme ($k_1$) is affected in the same way.

Dialysis of the enzyme against KCl results in an irreversible inhibition of the reactions of the reduced enzyme ($K_i$(NADH) is increased and $k_3$ decreased) without any effect on those of the oxidized enzyme. Lyophilization of the enzyme in KCl results in a halving of both $K_m$(NADH) ($k_2/k_1$ according to Scheme A) and $k_2$ (cf. Singer and Gutman [6]), but this is reversed by dialysis of the lyophilized enzyme against Tris-sucrose buffer. Thus this effect is due to reversible inhibition by KCl of $k_2$ of the lyophilized preparations rather than to damage caused by the lyophilization itself.

The experiments discussed above were carried out with the acceptor in vivo, ubiquinone, bound to the enzyme. Extraction of the ubiquinone has a marked effect on the affinity of oxidized enzyme for NADH, the saturation curve for NADH becoming sigmoidal (see Fig. 7A). The $h$ value of 2.6 obtained from the Hill plot (Fig. 8) suggests that more than one interacting binding site for NADH is present. Reincorporation of an excess of Q restores the original hyperbola. Since Q influences $K_m$(NADH) but not $k_2$ (see Table III), it may be concluded that it is an effector for the binding of NADH to NADH dehydrogenase. Fig. 7 shows that the sigmoidal effect of Q extraction disappears at NADH concentrations above 100 $\mu$M. This may explain why it was not observed by Minakami et al. [23] in their preparation of soluble NADH dehydrogenase, which is free from ubiquinone.

The double substrate inhibition shows that NADH and ferricyanide compete for the active site in both oxidized and reduced enzyme. It is unlikely that two compounds differing in chemical structure as much as NADH and ferricyanide would bind to the same site in the enzyme. However, competitive-inhibition kinetics would also be found when the two compounds compete for entry, via the same cleft in the protein, to the active site. We suggest, then, that under the conditions studied the active centre(s) can be reached by the substrates only via one cleft. This is understandable for a multi-enzyme complex made up of a flavoprotein and many Fe-S proteins, protected by a hydrophobic sheath of lipids and structural proteins [12]. The observation [29] that reactivity with ferricyanide involves some of the non-heme iron components is not inconsistent with this picture, since a rapid electron flow between the different centres would be possible.

## EXPERIMENTAL

Heart-muscle particles were prepared according to the method of Keilin and Hartree [30]. Complex I was isolated from these particles according to the procedure of Hatefi et al. [7]. It was depleted of ubiquinone by dialysis for 3 h against 1000 vols. of 100 mM KCl with three changes of the dialysate, lyophilization and extraction 4 times with *n*-pentane according to the method of Szarkowska [24]. Q-10 was reincorporated into the pentane-extracted preparation by stirring the dried material for 5 min at room temperature with Q-10 in *n*-pentane. The pentane was then removed in a rotary evaporator under reduced pressure. The dry powders of Q-depleted and Q-reincorporated Complex I were homogenized in 0.2 M sucrose/50 mM Tris · HCl buffer (pH 8.0).

Protein was determined by the biuret method after precipitation with trichloroacetic acid [31]. The enzyme concentration is expressed on the basis of FMN concentration, determined fluorimetrically as acid-extractable flavin, using a calibration curve made with known FMN concentrations. Fluorescence was measured with an Eppendorf fluorimeter (primary filter 405+436 nm, secondary filter 500–3000 nm).

The NADH-ferricyanide activity was measured at 25 °C according to the method of Minakami et al. [23], the reduction of $K_3Fe(CN)_6$ being followed at 420 nm ($A = 1.03\ mM^{-1} \cdot cm^{-1}$). Absorbance measurements were carried out with a Zeiss PMQ II spectrophotometer, equipped with a scale expander and a Varicord recorder. The assay mixture contained 1 mM EDTA and 20 mM sodium phosphate, at the pH indicated in the legends. The total volume was 3 ml. The assay was started by adding 50 µl of an appropriately diluted enzyme solution. If necessary, corrections were made for the rate of the nonenzymic reduction of ferricyanide by NADH.

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

All materials were obtained from commercial sources and were used without further purification.

## ACKNOWLEDGEMENTS

The authors wish to thank Dr B. F. van Gelder for his continuous interest and valuable discussion, Mr J. H. Nijhoff for help with some of the experiments and Mrs G. J. M. de Bruin and Mr P. J. van Dijk for skilful technical assistance. This work was supported in part by grants from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) under auspices of the Netherlands Foundation for Chemical Research. (S.O.N.).

## REFERENCES

1 Mahler, H. R., Sarkar, N. K., Vernon, L. P. and Alberty, R. A. (1952) J. Biol. Chem. 199, 585–597
2 King, T. E., Howard, R. L., Kettman, Jr., J., Hegdekar, B. M., Kuboyama, M., Nickel, K. S. and Possehl, E. A. (1966) in Flavins and Flavoproteins (Slater, E. C., ed.), BBA Library, Vol. 8, pp. 441–481, Elsevier, Amsterdam
3 Slater, E. C. (1966) in Flavins and Flavoproteins (Slater, E. C., ed.), BBA Library, Vol. 8, pp. 482–495, Elsevier, Amsterdam

4 Singer, T. P. and Gutman, M. (1970) in Pyridine Nucleotide-Dependent Dehydrogenases (Sund, H., ed.), pp. 375–391, Springer Verlag, Berlin
5 Ringler, R. L., Minakami, S. and Singer, T. P. (1963) J. Biol. Chem. 238, 801–810
6 Singer, T. P. and Gutman, M. (1971) Adv. Enzymol. 34, 79–153
7 Hatefi, Y., Haavik, A. G. and Griffith, D. E. (1962) J. Biol. Chem. 237, 1676–1680
8 Orme-Johnson, N. R., Orme-Johnson, W. H., Hansen, R. E., Beinert, H. and Hatefi, Y. (1971) Biochem. Biophys. Res. Commun. 44, 446–452
9 Ohnishi, T., Wilson, D. F., Asakura, T. and Chance, B. (1972) Biochem. Biophys. Res. Commun. 46, 1631–1638
10 Ohnishi, T., Asakura, T., Wilson, D. F. and Chance, B. (1972) FEBS Lett. 21, 59–62
11 Ohnishi, T. and Pring, M. (1974) in Dynamics of Energy-Transducing Membranes (Ernster, L., Estabrook, R. W. and Slater, E. C., eds.), BBA Library, Vol. 13, pp. 169–180, Elsevier, Amsterdam
12 Hatefi, Y. and Stempel, K. E. (1969) J. Biol. Chem. 244, 2350–2357
13 Kumar, S. A., Appaji Rao, N., Felton, S. P. and Huennekens, F. M. (1968) Arch. Biochem. Biophys. 125, 436–448
14 Kaniuga, Z. (1963) Biochim. Biophys. Acta 73, 550–564
15 Hatefi, Y. and Stempel, K. E. (1967) Biochem. Biophys. Res. Commun. 26, 301–308
16 Davis, K. A. and Hatefi, Y. (1969) Biochemistry 8, 3355–3361
17 Hatefi, Y. and Hanstein, W. G. (1969) Proc. Natl. Acad. Sci. U.S. 62, 1129–1136
18 Hatefi, Y. (1973) Abstr. 9th Intern. Congr. Biochem., Stockholm, Aktiebolaget Egnellska Boktryckeriet, Stockholm, p. 13
19 Slater, E. C. (1975) in Electron-Transfer Chains and Oxidative Phosphorylation (Quagliariello, E., Papa, S., Palmieri, F., Slater, E. C. and Siliprandi, N., eds.), pp. 3–14, North-Holland, Amsterdam
20 Dooijewaard, G. and Slater, E. C. (1976) Biochim. Biophys. Acta 440, 16–35
21 Dooijewaard, G., Slater, E. C. and Nijhoff, J. H. (1972) Abstr. 8th Meeting Fed. Eur. Biochem. Soc., Amsterdam, North-Holland, Amsterdam, no. 446
22 Dooijewaard, G. (1973) Abstr. 9th Intern. Congr. Biochem., Stockholm 1973, Aktiebolaget Egnellska Boktryckeriet, Stockholm, p. 227
23 Minakami, S., Ringler, R. L. and Singer, T. P. (1962) J. Biol. Chem. 237, 569–576
24 Szarkowska, L. (1966) Arch. Biochem. Biophys. 113, 519–525
25 Cleland, W. W. (1970) in The Enzymes (Boyer, P. D., ed.) Vol. 2, 3rd edn., pp. 1–65, Academic Press, New York
26 Kean, E. A., Gutman, M. and Singer, T. P. (1971) J. Biol. Chem. 246, 2346–2353
27 Minakami, S., Cremona, T., Ringler, R. L. and Singer, T. P. (1963) J. Biol. Chem. 238, 1529–1537
28 Tottmar, S. O. C. and Ragan, C. I. (1971) Biochem. J. 124, 853–865
29 Beinert, H., Palmer, G., Cremona, T. and Singer, T. P. (1965) J. Biol. Chem. 240, 475–480
30 Keilin, D. and Hartree, E. F. (1947) Biochem. J. 41, 500–502
31 Cleland, K. W. and Slater, E. C. (1953) Biochem. J. 53, 547–556