

## Hydroxyquinone:NADH Oxidoreductase from *Aspergillus fumigatus*

### II. Kinetic Studies on the Enzyme\*

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The kinetic behaviour of hydroxyquinone:NADH oxidoreductase has been determined using NADH as the electron donor and a number of representative quinonoid substrates as electron acceptors. The enzymatic reactions were found to be governed by a Michaelis-Menten type of rate equation with respect to the NADH concentration, whereas the quinonoid substrates appeared to act as inhibitors competing with NADH. Some kinetic data are reported and enzymatic mechanisms giving kinetic equations that are in conformity with the experimental results are described and discussed.

Kinetic observations on the chemical reactions catalyzed by a certain enzyme provide an indispensable background for the description of the enzyme. The hydroxyquinone:NADH oxidoreductase isolated from *Aspergillus fumigatus*<sup>1</sup> has, therefore, now been characterized by kinetic studies, in which the effect of the substrate concentrations on the enzymatic reaction velocity have been determined using NADH as the electron donor and a few representative quinonoid substrates (see Table 1) as electron acceptors.

The development of kinetic theory of enzyme action further plays a more specific role as a major means of elucidating the fundamental mechanisms of the enzymatic catalysis, although it has been recognized in recent years that there are difficulties in extending the classical kinetic theory to the more complex reaction system to be considered in the present work. As pointed out by Wong and Hanes,<sup>2</sup> a common basic mechanism can be assumed to underlie the reaction in one-substrate or quasi one-substrate systems, and a limited range of rate equations may be developed for appropriate variants of this basic mechanism. A demonstration of conformity of the experimental data with one of these developed rate equations can, therefore, usually be accepted as constituting an identification of the operative mechanism of the system.

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On the other hand, in the case of two-substrate systems the great variety of possible mechanisms (a multiplicity of possible enzyme-containing intermediates and reaction sequences must be considered) and the forbidding complexity of the derived rate equations for many of these combine to make it most difficult to obtain unambiguous identifications of the operating mechanisms. Some interesting conclusions may, however, be drawn from the observed kinetic behaviour of hydroxyquinone: NADH oxidoreductase, and enzymatic mechanisms giving kinetic equations which are in conformity with the experimental data will be described and discussed.

### EXPERIMENTAL

The isolation of hydroxyquinone: NADH oxidoreductase, and the assay methods for the enzyme, have been described previously.<sup>1</sup> In the present work the NADH concentration was varied within the range  $10^{-4}$ – $10^{-5}$  M. The range of quinone concentrations tested was more limited, since the initial extinction of the reaction mixtures had to be kept below 2.0 and above 0.1 in order to obtain reliable determinations of the reaction rates. The reaction mixtures generally contained one enzyme unit (0.03–0.05 mg of protein) in a total volume of 3.5 ml; appropriate amounts of substrates were added from stock solutions in 0.05 M phosphate buffer, pH 5.3. Reactions were initiated by the addition of NADH, and incubations were carried out at pH 5.3 and 20°. The initial reaction rate for each combination of substrate concentrations was determined graphically from plots of the extinction of the reaction solution against time; measurements were performed at 525, 550, 450, 500, and 450  $m\mu$  for 2-hydroxy-3,6-dimethyl-1,4-benzoquinone, 2-hydroxy-3-methoxy-6-methyl-1,4-benzoquinone (fumigatin), 2,3-dimethoxy-5,6-dimethyl-1,4-benzoquinone (aurantiogliocladin), 4,4'-dihydroxy-2,2'-dimethyl-3,6,3',6'-dibenzoquinone (which will be called DBQ), and 5-hydroxy-1,4-naphthoquinone (juglone), respectively.

### RESULTS

The rate ( $v$ ) of enzymatic oxidation of NADH by the quinones tested (see Table 1) was found to be governed by a Michaelis-Menten type of kinetic equation with respect to the concentration ( $a$ ) of NADH. This is evident since variations of the NADH concentration at constant concentrations ( $b$ ) of the quinonoid electron acceptors yielded straight lines in Lineweaver-Burk plots

Table 1. Kinetic data for the enzymatic oxidation of NADH by five representative quinonoid substrates. The constants  $p_i$  are defined by the rate eqns. (E:1) and (E:2).

Electron acceptor	$p_1$ (M)	$p_2$	$p_3$ (M)	$p_1/p_2$ (M)
2-Hydroxy-3,6-dimethyl-1,4-benzoquinone	$10 \times 10^{-5}$	0.54	$5 \times 10^{-5}$	$1.9 \times 10^{-4}$
Fumigatin	$9 \times 10^{-5}$	0.38	—	$2.4 \times 10^{-4}$
Aurantioogliocladin	$9 \times 10^{-5}$	0.09	—	$1.0 \times 10^{-3}$
Juglone	$10 \times 10^{-5}$	0.70	—	$1.4 \times 10^{-4}$
4,4'-Dihydroxy-2,2'-dimethyl-3,6,3',6'-dibenzoquinone (DBQ)	$9 \times 10^{-5}$	2.80	—	$3.2 \times 10^{-5}$

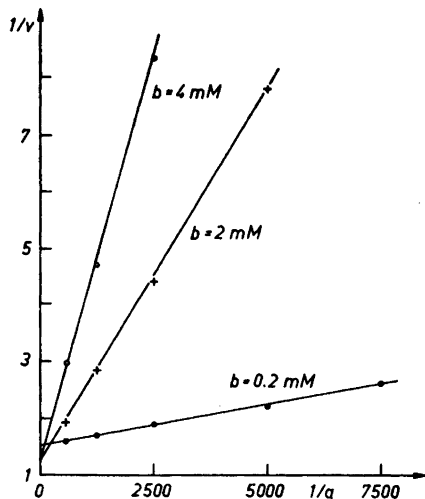


Fig. 1. Enzymatic oxidation of NADH by 2-hydroxy-3,6-dimethyl-1,4-benzoquinone. Lineweaver-Burk plots showing the relationship between the reaction rate ( $v$   $\mu\text{moles/min}$ ) and the NADH concentration ( $a$   $\text{M}$ ) at different constant concentrations ( $b$ ) of the quinonoid electron acceptor.

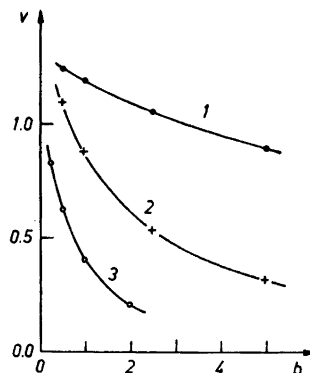


Fig. 2. Enzymatic oxidation of NADH (2  $\text{mM}$ ) by aurantiogliocladin, juglone, and DBQ (curve 1, 2, and 3, respectively). Plot of the reaction rate ( $v$   $\mu\text{moles/min}$ ) against the quinone concentration ( $b$   $\text{mM}$ ).

of  $1/v$  against  $1/a$ , as shown by the example in Fig. 1 (*cf.* Fig. 3). The reaction rate at constant NADH levels was, however, found to decrease considerably with increasing concentrations of the electron acceptor (Fig. 2), indicating that the quinonoid substrates act as inhibitors of the enzymatic reaction. The inhibition appeared generally to be competitive: at all quinone concentrations the reaction rate approached the same maximum value ( $V_m$ ) when the NADH concentration was increased to a sufficient high level. Taking fumigatin as a representative example, it was found that plots of  $V_m/v$  against  $1/a$  at different, constant, fumigatin concentrations yielded a set of straight lines which intersect the  $V_m/v$  axis at 1.0 (Fig. 3), whereas the slopes ( $\phi_2(b)$ ) of the lines were found to increase linearly with the fumigatin concentration (Fig. 4). Similar set of curves were obtained for all of the quinonoid substrates listed in Table 1, when tested at concentrations above  $5 \times 10^{-4}$   $\text{M}$ .

The above observations indicate that the enzymatic reaction is governed by the rate equation (E:1), where the constants  $p_1$  and  $p_2$  can be

$$\frac{V_m}{v} = 1 + \frac{1}{a} (p_1 + p_2 b) \quad (\text{E:1})$$

determined in the conventional way from plots analogous to the one shown in Fig. 4; the straight line obtained in this plot intersects the vertical axis at  $p_1$  and has a slope equal to  $p_2$ . Confirmatively, a plot of  $V_m/v$  against the

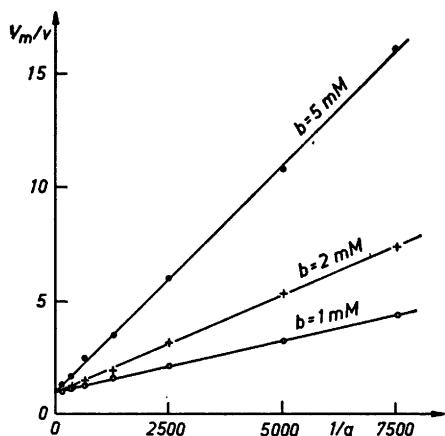


Fig. 3. Enzymatic oxidation of NADH ( $a$  M) by fumigatin. Plot of  $V_m/v$  against  $1/a$  for three different constant concentrations ( $b$ ) of fumigatin.

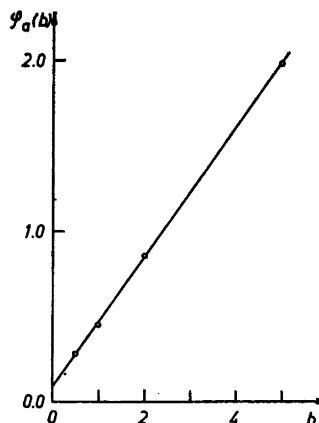


Fig. 4. Relationship between the fumigatin concentration ( $b$  mM) and the slopes ( $\phi_a(b)$  mM) of the straight lines obtained in Fig. 3.

quinone concentration ( $b$ ) gave straight lines at all NADH levels, as shown for fumigatin in Fig. 5; the lines obtained for different (constant) NADH concentrations were found to intersect at the same point, the coordinates for which can be shown to be  $(1; -p_1/p_2)$ . It is seen from eqn. (E:1) that each line in the latter plot intersects the vertical axis at  $I = 1 + p_1/a$  and has the slope  $S = p_2/a$ . The values of  $p_1$  and  $p_2$  calculated from Fig. 4 can thus be checked by plotting  $I$  and  $S$  against  $1/a$ ; the straight lines obtained have the slopes  $p_1$  and  $p_2$ , respectively (Figs. 6 and 7). Using the above methods  $p_1$  and  $p_2$  have been determined for the enzymatic oxidation of NADH by the different quinonoid substrates tested in the present work; the results

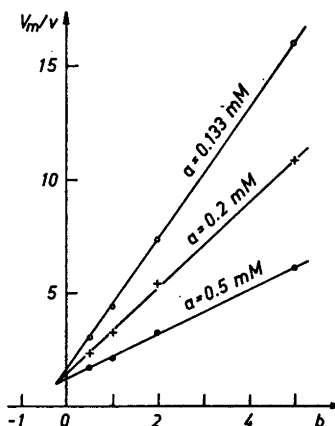


Fig. 5. Enzymatic oxidation of NADH by fumigatin ( $b$  mM). Plot of  $V_m/v$  against  $b$  for three different constant concentrations ( $a$ ) of NADH.

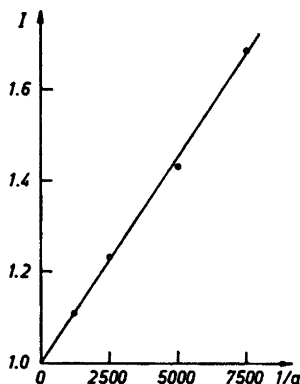


Fig. 6. Relationship between the NADH concentration ( $a$  M) and the intercepts on the vertical axis ( $I$ ) of the straight lines obtained in Fig. 5.

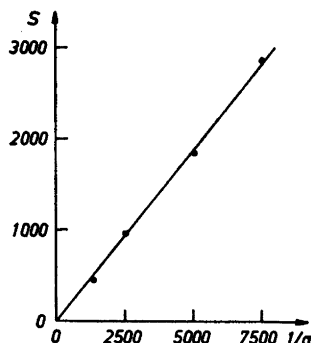


Fig. 7. Relationship between the NADH concentration ( $a$  M) and the slopes ( $S$  M) of the straight lines obtained in Fig. 5.

obtained are listed in Table 1. It is seen from the table that the  $p_1$  values are essentially independent of the structure of the electron acceptor, whereas the  $p_2$  values vary considerably. The reason for this will be discussed below.

One of the quinones tested (2-hydroxy-3,6-dimethyl-1,4-benzoquinone) was found not to follow eqn. (E:1) when assayed at concentrations below  $5 \times 10^{-4}$  M. This is evident from the Lineweaver-Burk plots given in Fig. 1; at 2-hydroxy-3,6-dimethyl-1,4-benzoquinone concentrations above  $5 \times 10^{-4}$  M the straight lines obtained intersected the vertical axis approximately at the same point ( $0; 1/V_m$ ), whereas a slightly larger intercept was obtained at a quinone concentration of  $2 \times 10^{-4}$  M. The intercept ( $\varphi_0(b)$ ) obtained in plots of  $V_m/v$  against  $1/a$  for constant quinone concentrations ( $b$ ) within the range  $10^{-4}$ – $5 \times 10^{-4}$  M seemed, in fact, to be linearly related to the inverse value of  $b$ , as shown in Fig. 8. This observation might indicate that the actual kinetic equation for hydroxyquinone:NADH oxidoreductase includes a term  $p_3/b$  (where  $p_3$  is the slope of the straight line in Fig. 8) which generally can be neglected within the range of quinone concentrations tested. The complete empirically determined rate equation would then be given by (E:2).

$$\frac{V_m}{v} = 1 + \frac{p_3}{b} + \frac{1}{a} (p_1 + p_2 b) \quad (\text{E:2})$$

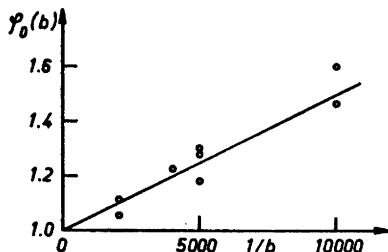
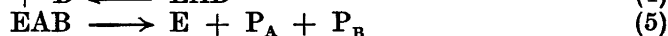
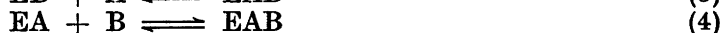


Fig. 8. Enzymatic oxidation of NADH ( $a$  M) by 2-hydroxy-3,6-dimethyl-1,4-benzoquinone. Relationship between the quinone concentration ( $b$  M) and the intercepts  $\varphi_0(b)$  of the straight lines obtained in plots of  $V_m/v$  against  $1/a$  (cf. Figs. 1 and 3).

DISCUSSION

For interpretation of the above kinetic data we consider a bimolecular reaction between the substrates A (NADH) and B (*e.g.* fumigatin) catalyzed by the enzyme E; *a*, *b*, and *e* are the total concentrations of A, B, and E, respectively. The reaction products ( $P_A$  and  $P_B$ ) are assumed to be formed in an irreversible step, and to be rapidly desorbed from the enzyme; the products formed during the action of hydroxyquinone:NADH oxidoreductase were found not to affect the enzymatic reaction velocity.<sup>1</sup> We shall further make the conventional assumption that the concentrations of the substrates are maintained constant, *i.e.* the amount of substrate which is bound to enzyme is assumed to be very small compared with the total amount of substrate present. These restrictions can be removed at the price of increasing uninteresting algebraic complexity, but with no great gain in development of principle. Let us first consider a general case where the two substrates combine reversibly with the enzyme in random order to give a ternary complex, which irreversibly breaks up to yield free enzyme and reaction products:



Assuming that  $k_{+i}$  ( $k_{-i}$ ) is the forward (backward) velocity constant of reaction (i), a steady-state treatment of this system according to the method of Briggs and Haldane<sup>3</sup> gives the general eqn. (6) for the reaction rate (*v*),

$$\frac{v}{e} = \frac{c_4 + c_5a + c_6b + c_7ab + c_8a^2 + c_9b^2 + c_{10}a^2b + c_{11}ab^2}{c_1ab + c_2a^2b + c_3ab^2} \tag{6}$$

where  $c_n$  are constants which can be expressed in terms of the velocity constants as shown in Table 2. It is seen from eqn. (6) that non-linear relationships are obtained in plots of  $1/v$  against  $1/a$  or  $1/b$ ; the above general mechanism can, obviously, not explain the kinetic behaviour of hydroxyquinone:NADH oxidoreductase.

Table 2. Relationships between the coefficients  $c_n$  defined by eqns. (6) and (15) and the forward (backward) velocity constants  $k_{+i}$  ( $k_{-i}$ ) of reaction (i).

$$\begin{aligned}
 c_1 &= k_{+1}k_{-2}k_{+3}k_{+5} + k_{-1}k_{+2}k_{+4}k_{+5} \\
 c_2 &= k_{+1}k_{+3}k_{+4}k_{+5} \\
 c_3 &= k_{+2}k_{+3}k_{+4}k_{+5} \\
 c_4 &= k_{-1}k_{-2}k_{-3} + k_{-1}k_{-2}k_{-4} + k_{-1}k_{-2}k_{+5} \\
 c_5 &= k_{+1}k_{-2}k_{-3} + k_{+1}k_{-2}k_{-4} + k_{+1}k_{-2}k_{+5} + k_{-1}k_{-2}k_{+4} + k_{-1}k_{+4}k_{+5} \\
 c_6 &= k_{-1}k_{+3}k_{-3} + k_{-1}k_{+3}k_{-4} + k_{-1}k_{+3}k_{+5} + k_{-2}k_{+3}k_{-4} + k_{-2}k_{+3}k_{+5} \\
 c_7 &= k_{+1}k_{-2}k_{+3} + k_{-1}k_{+2}k_{+4} + k_{+1}k_{+3}k_{-4} + k_{+2}k_{-2}k_{+4} + k_{+3}k_{+4}k_{+5} \\
 c_8 &= k_{+1}k_{-2}k_{+4} + k_{+1}k_{+4}k_{+5} \\
 c_9 &= k_{+2}k_{+3}k_{-4} + k_{+2}k_{+3}k_{+5} \\
 c_{10} &= k_{+1}k_{+3}k_{+4} \\
 c_{11} &= k_{+2}k_{+3}k_{+4} \\
 c_{12} &= k_{+1}k_{-2}k_{+12}k_{+13}k_{+14} \\
 c_{13} &= k_{+1}k_{+12}k_{-13} + k_{+1}k_{+12}k_{+14} \\
 c_{14} &= k_{-1}k_{-2}k_{+12}k_{+14} + k_{-2}k_{+12}k_{+13}k_{+14} \\
 c_{15} &= k_{+1}k_{-2}k_{+12}k_{+13} + k_{+1}k_{-2}k_{+12}k_{+14} \\
 c_{16} &= k_{-1}k_{+2}k_{+12}k_{+13}k_{+14} + k_{+2}k_{+12}k_{+13}k_{+14}
 \end{aligned}$$

Linear relationships will, however, be obtained if there is an obligatory (non-random) order of combination of the substrates with the enzyme to form the ternary complex. EAB can thus be assumed to be formed exclusively *via* EA; the attachment of A to the proper site of EB might, for instance, be sterically blocked by the presence of B. The rate eqn. (7) for this mechanism is readily obtained from eqn. (6) by putting  $k_{+4} = 0$ , when  $c_2, c_3, c_8, c_{10}$ , and  $c_{11}$  become zero:

$$\frac{c_1 e}{v} = c_7 + \frac{c_8}{b} + \frac{1}{a} \left( c_6 + \frac{c_4}{b} + c_9 b \right) \quad (7)$$

Inspection of eqn. (7) shows that plots of  $1/v$  against  $1/a$  give straight lines intersecting the  $1/v$  axis at  $(c_7 + c_8/b)/c_1 e$ , which represents the inverse value of the maximal reaction rate  $V_a(b)$  obtained on increasing the concentration of A for a definite concentration of B. At sufficient high concentrations of B  $V_a(b)$  will attain a limiting value  $V_m = c_1 e/c_7$ , and with introduction of this constant into eqn. (7) we get eqn. (8), where

$$\frac{V_m}{v} = 1 + \frac{c_5'}{b} + \frac{1}{a} \left( c_6' + \frac{c_4'}{b} + c_9 b' \right) \equiv \varphi_0(b) + \frac{1}{a} \varphi_a(b) \quad (8)$$

$\varphi_0(b)$  and  $\varphi_a(b)$  can be determined in the familiar way from plots of  $V_m/v$  against  $1/a$ ;  $c_n'$  stands for  $c_n/c_7$ . At sufficient low concentrations of B the term  $c_9 b'$  can be neglected and the rate equation becomes of the Michaelis-Menten type with respect to both of the substrates. Plots of  $\varphi_0(b)$  and  $\varphi_a(b)$  against  $1/b$  thus give straight lines with the intercepts (on the  $\varphi$  axis) 1 and  $c_6'$ , and the slopes  $c_5'$  and  $c_4'$ , respectively. On the other hand, at sufficient high concentrations of B we get

$$\varphi_0(b) = 1 \text{ and } \varphi_a(b) = c_6' + c_9 b' \quad (9)$$

When these conditions are fulfilled B acts as an inhibitor competing with A, and a plot of  $\varphi_a(b)$  against  $b$  gives a straight line with the slope  $c_9'$ .

All of the constants  $c_i'$  involved in eqn. (8) can thus theoretically be determined from kinetic data. The above method appears to be applicable to several two-substrate enzymatic reactions where one substrate acts as a competitive inhibitor, as for instance the reduction of methylene blue by hypoxanthine with xanthine oxidase, or the reduction of methylene blue by leucin with *L*-aminoacid oxidase of snake venom.<sup>4</sup> It is also clear that the hydroxyquinone:NADH oxidoreductase from *A. fumigatus* may be acting according to the mechanism yielding eqn. (7), provided that the conditions yielding eqn. (9) are fulfilled for the tested range of quinone concentrations. The constants  $p_1$  and  $p_2$  listed in Table 1 would then be identical with  $c_6'$  and  $c_9'$ , respectively.

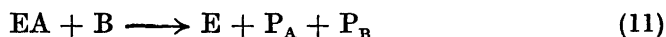
In order to investigate whether the above mechanism is unique in yielding the form of rate equation which describes the observed rate behaviour of hydroxyquinone:NADH oxidoreductase, we will now consider some other conditions leading to linear relationships in two-substrate systems. Firstly, the general system defined by (1)–(5) can be made non-random by assuming that one of the substrates combines with the free enzyme so much more rapidly than the other, that effectively there is only one path to the ternary

complex. If thus the formation of EA from E and A is eliminated by putting  $k_{+1} = 0$  the coefficients  $c_2$ ,  $c_8$ , and  $c_{10}$  become zero, and we get the kinetic eqn. (10):

$$\frac{e}{v} = \frac{c_5 + c_7b + c_{11}b^2}{c_1b + c_3b^2} + \frac{1}{a} \cdot \frac{c_4 + c_6b + c_9b^2}{c_1b + c_3b^2} \quad (10)$$

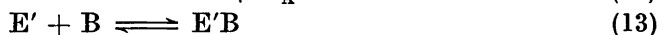
Even though it yields linear plots of  $1/v$  against  $1/a$  eqn. (10) cannot be brought into conformity with the experimental data unless both  $c_3$  and  $c_{11}$  are practically equal to zero compared with  $c_1$ ,  $c_7$ , and  $c_9$ , and inspection of Table 2 shows that the latter conditions are not fulfilled for any values of the different velocity constants.

Secondly, linear relationships may be obtained if the two-substrate reaction does not involve the formation of a ternary complex, and for such systems two basic mechanisms have to be considered. One of these is well-known from studies on peroxidase,<sup>5</sup> and is based on the principle that the binary complex EA formed according to equilibrium (1) reacts directly with B to yield free enzyme and products:



A steady-state treatment of such systems shows that linear relationships between  $1/v$  and  $1/a$  are obtained, but  $V_a(b)$  (defined as above) will always be proportional to the concentration of B (*cf.* Ref. 6). It can, therefore, be concluded that reaction (11) is not involved in the operating mechanism for hydroxyquinone:NADH oxidoreductase.

The second two-substrate mechanism not involving the formation of a ternary complex is based on the principle that EA (formed according to reaction (1)) breaks up irreversibly to  $P_A$  and  $E'$ , where  $E'$  is a modified (reduced) form of the enzyme. B must be assumed to combine reversibly with both E and  $E'$ , and the binary complex  $E'B$  (in contrast to EB) yields  $P_B$  and the free enzyme in an irreversible step. The complete system thus involves the reactions (1), (2), and (12)–(14). A steady-state treatment of this system gives the



kinetic eqn. (15), which can be transformed into eqn. (16) by introduction of

$$\frac{e}{v} = \frac{c_{13}a + c_{14}b + c_{15}ab + c_{16}b^2}{c_{12}ab} \quad (15)$$

$$\frac{V_m}{v} = 1 + \frac{c_{13}'}{b} + \frac{1}{a} (c_{14}' + c_{16}'b) \quad (16)$$

$c_n' = c_n/c_{15}$  and  $V_m = ec_{12}'$ ; the coefficients  $c_n$  can be expressed in terms of the velocity constants for the different reactions involved (see Table 2). Eqn. (16) is identical with the empirically determined rate eqn. (E:2) obtained for the enzymatic oxidation of NADH by 2-hydroxy-3,6-dimethyl-1,4-benzoquinone ( $c_{13}'$ ,  $c_{14}'$ , and  $c_{16}'$  correspond to  $p_3$ ,  $p_1$ , and  $p_2$ , respectively), and at



sufficient high concentrations of B the term  $c_{13}'/b$  can be neglected in eqn. (16), which then becomes identical with eqn. (E:1). It may thus be concluded that the kinetic behaviour of hydroxyquinone:NADH oxidoreductase also can be explained by the mechanism defined by the reactions (1), (2), and (12)–(14), *i.e.* by assuming that NADH reacts with the free enzyme to yield a reduced form E' (*e.g.* by reduction of a –S–S– bond), which is capable of carrying out the reduction of a large number of quinonoid substrates (quinones are known to be rapidly reduced by –SH compounds such as cysteine or glutathione).

Since the rate behaviour of hydroxyquinone:NADH oxidoreductase cannot be studied at sufficient low concentrations of the quinonoid substrates, it has not been possible to distinguish between the above two mechanisms which yield kinetic equations ((8) and (16), respectively) that are in conformity with the experimental data. It can, however, be concluded that both NADH and the quinonoid substrates combine reversibly with the free enzyme, and that the binary enzyme-quinone complex is inactive. The forward reaction in which the enzyme-NADH complex (EA) is involved, further, appears to be the rate-determining step in the enzymatic process, *i.e.* the reaction velocity is approximately proportional to the concentration of EA. A steady-state treatment of the simplified system defined by the reactions (1), (2), and (17) gives, in fact, the



kinetic eqn. (18), where  $V_m = k_{+17}e$ ,  $K_m = (k_{-1} + k_{+17})/k_{+1}$  and

$$\frac{V_m}{v} = 1 + \frac{K_m}{a} \left( 1 + \frac{b}{K_b} \right) \quad (18)$$

$K_b = k_{-2}/k_{+2}$ . From this point of view the reaction can be described by means of a conventional Michaelis-Menten constant  $K_m$  (with reference to the binary complex EA) and an inhibitor constant  $K_b$  (the dissociation constant of EB). Comparison with eqn. (E:1) shows that  $K_m$  is equal to  $p_1$ , which explains why  $p_1$  was found to be essentially independent of the structure of the quinonoid electron acceptor.  $K_b$ , on the other hand, is obtained as  $p_1/p_2$  and varies for the different quinones tested (see Table 1); it may be noted that  $(1; -K_b)$  are the coordinates for the points of intersection of the straight lines obtained in plots of  $V_m/v$  against  $b$  (Fig. 5).

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