

## Effect of NADH on the Ceruloplasmin Catalyzed Oxidation of Dopamine and Noradrenaline

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The kinetics of the ceruloplasmin catalyzed oxidation of dopamine and noradrenaline in the absence and presence of NADH has been investigated. Straight lines were obtained when the reciprocal activity was plotted against the reciprocal substrate concentration. NADH had an activating effect on the rate of substrate oxidation, and the activity increased with increasing NADH concentration.  $V_{\max}$  did not change when the concentration of NADH was altered. The apparent  $K_m$  values were lower in the presence of NADH, and decreased with increasing NADH concentration.

A reduction of a primary oxidation product of dopamine (noradrenaline) by NADH may account for the kinetics observed.

Ceruloplasmin (E. C. 1. 12. 3.) has oxidase activity against three principal classes of substrates; Fe(II), aromatic diamines and diphenols. The kinetics of the enzyme catalyzed oxidation of Fe(II)<sup>1-3</sup> and *N,N*-dimethyl-*p*-phenylenediamine<sup>4-7</sup> (DPD), a commonly used substrate of the diamine class, has been extensively studied by several investigators. The present paper deals with the ceruloplasmin catalyzed oxidation of dopamine and noradrenaline, two substrates of the diphenol class.

Walaas *et al.*<sup>8</sup> demonstrated that dihydronicotinamide adenine dinucleotide (NADH) is oxidized in the presence of ceruloplasmin and dopamine (noradrenaline), and that the activity can be measured as the rate of NADH oxidation. NADH, at relatively low concentrations, did not seem to have any effect on the apparent Michaelis constants ( $K_m$ ). The present study, however, demonstrates that NADH at higher concentrations affects the kinetic parameters of the enzyme catalyzed oxidation of dopamine and noradrenaline, and a mechanism is proposed accounting for the kinetics observed.

### EXPERIMENTAL

*Materials.* Human ceruloplasmin was obtained from AB Kabi, Stockholm. The enzyme was crystallized three times in our laboratory, and was 95 % pure as determined from the ratio of the extinctions at 610 m $\mu$  and 280 m $\mu$ . Enzyme concentrations were calculated from the extinction at 610 m $\mu$  using  $\epsilon_{610} = 1.09 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ .<sup>9</sup>

Dopamine (3-hydroxytyramine), HCl and NADH were purchased from Sigma Chemical Co. 1-Noradrenaline *d*-bitartrate monohydrate and deferoxamine-B-methane sulfonate (Desferal) were obtained from Phillips-Roxane and Ciba, respectively. All aqueous solutions were prepared in deionized glass-distilled water. Buffer solutions were passed through Chelex-100 (Bio-Rad) chelating resin prior to use in the experiments.

*Assays.* The assay mixture used for determining the molar absorption of dopaminochrome (480  $m\mu$ ) and noradrenochrome (490  $m\mu$ ) contained 1  $\mu$ M ceruloplasmin and substrate concentrations ranging from 25  $\mu$ M to 125  $\mu$ M in 0.05 M sodium acetate buffer, pH 5.9, at 30° (Fig. 1).

The ceruloplasmin activity, measured as the rate of chrome formation, was followed spectrophotometrically at 480  $m\mu$  and 490  $m\mu$  in the case of dopamine and noradrenaline, respectively. The system contained 0.27  $\mu$ M ceruloplasmin and 0.5 mM Desferal in 0.05 M sodium acetate buffer, pH 5.9, at 30°. The substrate concentration ranged from 0.25 mM to 5.0 mM. Desferal was added in order to prevent the activating effect of trace amounts of iron ions.<sup>10</sup> The concentration used did not affect the enzyme activity.

Stock solutions of substrate (25 mM) were made immediately before use and had a total volume of 2.0 ml. In order to stabilize the solution 5  $\mu$ l EDTA (1.0 mM) was added. When NADH was present in the assay, the activity was measured as the rate of NADH oxidation at 340  $m\mu$  ( $\epsilon_{340} = 6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ ).

In some experiments the time course of the reaction was studied, when access of oxygen from the air was prevented by covering the reaction mixture in the cell with a layer of liquid paraffin. The system contained 10  $\mu$ M ceruloplasmin, 10 mM dopamine, 0.26 mM oxygen and 0.5 mM Desferal in 0.2 M sodium acetate buffer, pH 5.9, at 20°. The chrome formation was followed spectrophotometrically at 480  $m\mu$ . Another system contained 0.78 mM NADH in addition to the other reagents. The time course of this reaction was followed at 340  $m\mu$ .

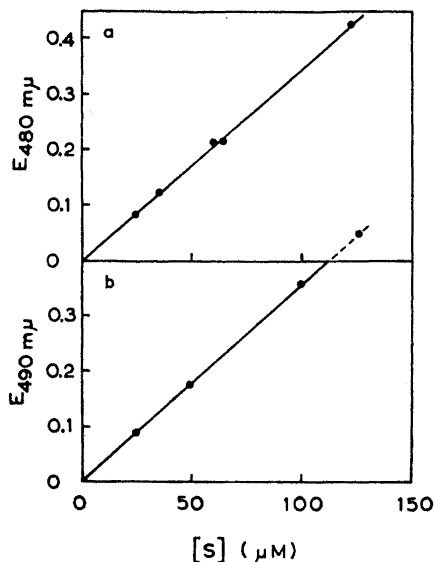
Spectrophotometric measurements were performed with a Beckman DK-1 recording spectrophotometer, equipped with a thermocell (1.0 cm light path).

## RESULTS

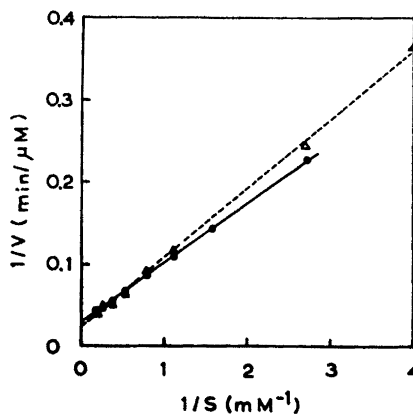
*Molar absorption of dopaminochrome and noradrenochrome.* Dopamine and noradrenaline are oxidized to reddish-brown coloured chrome products in the presence of ceruloplasmin. Dopaminochrome has an absorption maximum at 480  $m\mu$  and noradrenochrome at 490  $m\mu$ . The chrome products are rather stable under the conditions used, but on standing overnight a dark-coloured precipitate is formed. In order to calculate the rate of chrome formation, the molar absorption of dopaminochrome and noradrenochrome was determined. This was performed by measuring the final absorbance readings at 480  $m\mu$  (dopamine) and 490  $m\mu$  (noradrenaline), after all the substrate had been converted to chrome, and plotting the final absorbance readings against substrate concentration, according to Osaki.<sup>11</sup> Judging from the data in Fig. 1 the value  $3.50 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$  was adopted as the molar absorption of dopaminochrome at 480  $m\mu$  and  $3.58 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$  for noradrenochrome at 490  $m\mu$ .

*Kinetics of chrome formation.* Straight lines were obtained when the reciprocal activity, measured as the rate of chrome formation, was plotted against the reciprocal substrate concentration ( $1/V$  vs.  $1/S$ ) as demonstrated in Fig. 2. The apparent Michaelis constant,  $K_m$ , was 2.5 mM for dopamine and 3.5 mM for noradrenaline. The maximum activity,  $V_{\text{max}}$ , was slightly higher for noradrenaline.

*Effect of NADH on the ceruloplasmin activity.* NADH is oxidized when added to reaction mixtures containing ceruloplasmin and dopamine (noradrenaline), preventing the formation of chrome, as reported by Walaas *et al.*<sup>8</sup>

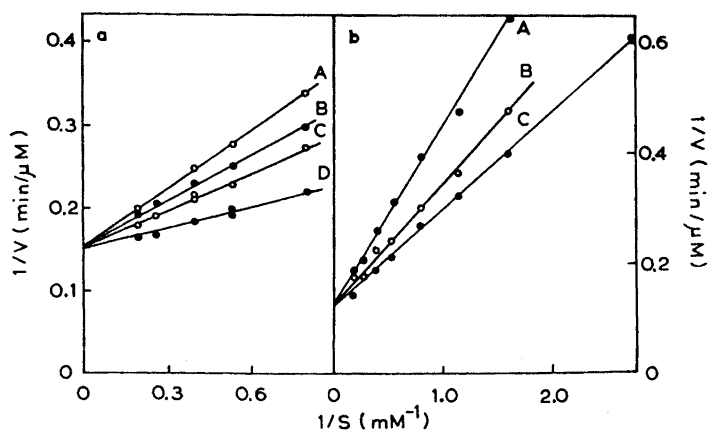


*Fig. 1.* Final absorbance readings at 480  $m\mu$  (dopamine) and 490  $m\mu$  (noradrenaline) as a function of substrate concentration.



*Fig. 2.* Double reciprocal plot of the activity, measured as the rate of chrome formation. Filled circles represent dopamine and open triangles noradrenaline.

The enzymic activity, measured as the rate of NADH oxidation at 340  $m\mu$ , was estimated for several substrate- and NADH concentrations, and in Fig. 3 the reciprocal activity is plotted against the reciprocal substrate concentration.



*Fig. 3.* The reciprocal activity, measured as the rate of NADH oxidation, plotted against the reciprocal dopamine concentration (a) and noradrenaline concentration (b). In the former case the NADH concentration was 0.10 mM (A), 0.15 mM (B), 0.23 mM (C) and 0.32 mM (D), and in the latter 0.10 mM (A), 0.23 mM (B) and 0.29 mM (C).

Straight lines were obtained also in this case, indicating that the oxidation of dopamine and noradrenaline follows ordinary Michaelis-Menten kinetics.

The rate of NADH oxidation was considerably higher than the rate of chrome formation at constant enzyme concentration, and the activity increased with increasing NADH concentration, as seen from Fig. 3. The maximum activity,  $V_{\max}$ , however, remained constant.

The  $K_m$  value for both substrates was lowered when the NADH concentration increased (Table 1). By plotting  $K_m$  against the reciprocal NADH con-

Table 1. Kinetic parameters for the ceruloplasmin catalyzed oxidation of dopamine and noradrenaline.

Dopamine			Noradrenaline		
(NADH) mM	Apparent $K_m$ mM	$V_{\max}/(CP_0)^a$ min <sup>-1</sup>	(NADH) mM	Apparent $K_m$ mM	$V_{\max}/(CP_0)$ min <sup>-1</sup>
0	2.5	13	0	3.5	16
0.10	1.60	25	0.10	2.6	30
0.15	1.25	25			
0.23	0.95	25	0.23	1.7	30
0.32	0.70	25	0.29	1.4	30
$\infty$	0.3		$\infty$	0.9	

<sup>a</sup> Total ceruloplasmin concentration.

centration (Fig. 4) approximate values for  $K_m$  at infinite NADH concentration ( $1/(\text{NADH})=0$ ) could be estimated. The kinetic parameters are listed in Table 1.  $K_m$  values obtained in the presence of NADH were lower than those determined in the absence of NADH for both substrates.

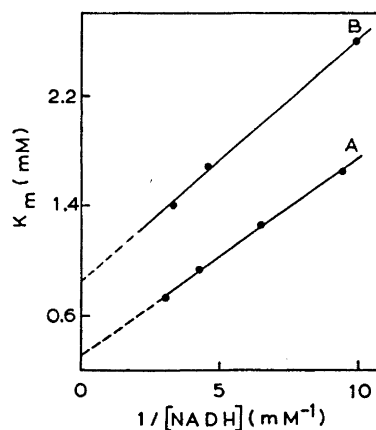


Fig. 4. Apparent Michaelis constants for dopamine (A) and noradrenaline (B) plotted against the reciprocal NADH concentration.

*Time course of dopamine oxidation in the absence and presence of NADH, when access of oxygen is prevented.* Ceruloplasmin has a very high affinity for molecular oxygen,<sup>1</sup> which is used for reoxidizing protein bound cuprous ions after reduction by substrate. The amount of chrome formed in a reaction mixture containing dopamine and a limited amount of oxygen (0.26 mM) is determined in Fig. 5a. When all oxygen is consumed the curve levels off, and

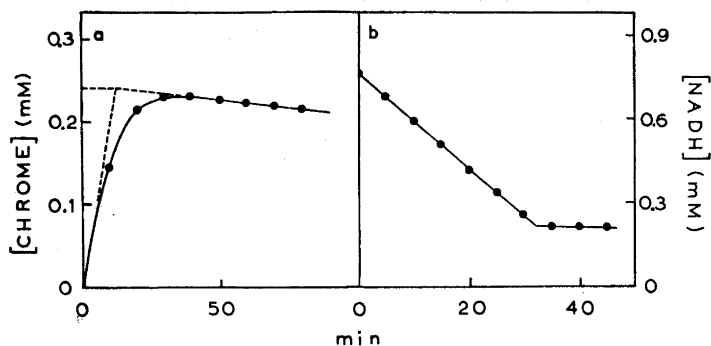


Fig. 5. Time course of the chrome formation (a) and NADH oxidation (b) when access of oxygen is prevented. Conditions as described in Experimental.

0.24 mM chrome has been generated. Consequently one molecule of oxygen is used up during formation of one molecule of chrome ( $(\text{chrome})/(\text{oxygen}) = 0.93$ ). In the presence of excess NADH, the amount of NADH oxidized is 0.53 mM when the oxygen is used up as estimated from the time course of the reaction (Fig. 5b). Thus two molecules of NADH are oxidized when one molecule of oxygen is reduced to water ( $(\text{NADH})/(\text{oxygen}) = 2.0$ ).

#### DISCUSSION

The ceruloplasmin catalyzed oxidation of Fe (II) and DPD exhibits non-linear kinetics in typical  $V$  vs.  $V/S$  and  $1/V$  vs.  $1/S$  plots. Curzon<sup>4</sup> and Walaas *et al.*,<sup>5</sup> studying the interaction with DPD, attributed the nonlinearity to the presence of two different active sites, acting on the same substrate. However, investigations by Pettersson and Pettersson<sup>6,7</sup> suggest that the curvature observed can be ascribed to a major influence of the first oxidation product,  $\text{DPD}^+$ , on the reaction velocities at low DPD concentrations.

In the case of Fe (II), Huber and Frieden<sup>12</sup> proposed a mechanism based on substrate activation in order to account for the curves obtained in a  $V$  vs.  $V/S$  plot.

The kinetics observed when dopamine and noradrenaline were used as substrate was quite different, since ordinary straight lines were obtained in double reciprocal plots (Figs. 2 and 3). This type of kinetics is usually observed when only one kind of active site reacts with substrate.

The proposed formation of chrome from dopamine is shown in Fig. 6.<sup>13</sup> Two protons and two electrons are removed from dopamine and a quinone is

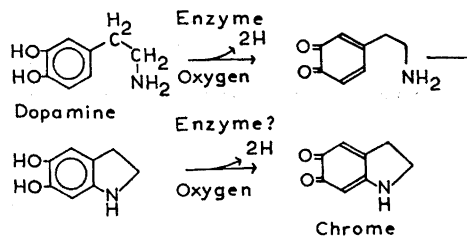
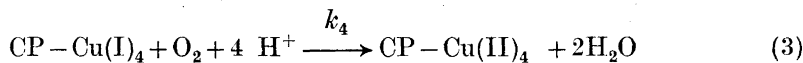
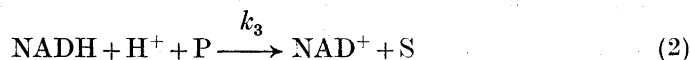
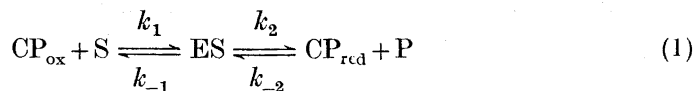


Fig. 6. The proposed mechanism of formation of chrome from dopamine.

formed. After a rearrangement of the molecule another two protons and electrons are removed and chrome is formed. In both oxidative steps oxygen is consumed. A total of four electrons are removed during formation of one molecule of chrome. This is the number of electrons necessary for reducing one molecule of oxygen to water and fits with the (chrome)/(oxygen) ratio of 0.93 calculated from Fig. 5a. The initial oxidation is catalyzed by ceruloplasmin. Whether ceruloplasmin is capable of catalyzing the second oxidative step is not known. NADH, a two electron donor, probably acts by reducing one of the oxidative steps in the formation of chrome. According to Fig. 5b the ratio between the amount of NADH oxidized and oxygen used is 2.0, suggesting that NADH generates dopamine from a primary product, as proposed by Walaas and Walaas,<sup>14</sup> who reported that addition of NADH to adrenochrome and noradrenochrome had no effect on the visible spectrum of the chrome products.

These observations are in accordance with the following scheme:



CP, ES, P and C represent ceruloplasmin, enzyme-substrate complex, primary product and chrome, respectively. The rate constants for the different reactions are shown in the scheme. In the absence of NADH the primary product is converted to chrome (eqn. 4).

At initial steady state the rate of NADH oxidation is

$$V = - \frac{d(\text{NADH})}{dt} = k_3(\text{NADH})(\text{H}^+)(\text{P}) \quad (5)$$

$$= k_2(\text{ES}) - k_{-2}(\text{CP}_{\text{red}})(\text{P}) \quad (6)$$

From eqns. 5 and 6 one gets

$$(P) = \frac{k_2(ES)}{k_3(NADH)(H^+) + k_{-2}(CP_{red})} \quad (7)$$

By inserting this expression into eqn. 5 the rate equation is transformed to

$$V = \frac{k_2(ES)}{1 + \frac{k_{-2}(CP_{red})}{k_3(NADH)(H^+)}} \quad (8)$$

According to eqn. 8 an increase in the NADH concentration results in an increased activity, as observed experimentally (Fig. 3). At infinite NADH concentration the reaction follows simple kinetics, since  $V$  is equal to  $k_2(ES)$  in this case. The proper Michaelis constant is thus the one estimated from the ordinate intersection point in Fig. 4, where  $K_m$  (apparent Michaelis constant) is plotted against  $1/(NADH)$  (Table 1). Since this value is obtained by extrapolation it must be regarded as rather approximate. It is also evident from eqn. 8 that  $V_{max}$  will remain constant regardless of NADH concentration, since the amount of free, reduced enzyme will decrease with increasing substrate concentration as the enzyme becomes saturated. The decrease in apparent  $K_m$  with increasing concentration of NADH is to be expected, since the activity increases while  $V_{max}$  is constant.

The apparent  $K_m$  values for dopamine and noradrenaline determined previously in the presence of NADH<sup>8</sup> are somewhat higher than those reported in this paper. This may be due to a lower NADH concentration used in the earlier experiments.

The comparative study of the ceruloplasmin catalyzed oxidation of dopamine and noradrenaline in the presence and absence of NADH demonstrates that NADH has an activating effect on the rate of substrate oxidation (Table 1).

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