The Oxidation of *NN*-Dimethyl-*p*-phenylenediamine by Oxidizing Agents and by Caeruloplasmin

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1. The oxidation of *NN*-dimethyl-*p*-phenylenediamine (DPD) by inorganic oxidants and by caeruloplasmin was studied. Some experiments were also made with *NNN'*-tetramethyl-*p*-phenylenediamine (TPD). 2. E_{550}^{mK} of the first free radical oxidation product of DPD (DPD⁺) was 9.8 and E_{550}^{mK} of the corresponding product of TPD (TPD⁺) was 12.5. 3. The non-enzymic decomposition of DPD⁺ was studied with respect to temperature, pH, concentration and DPD/DPD⁺ ratio, thus defining conditions for enzyme experiments under which DPD⁺ extinction at 550m μ was proportional to enzyme activity. 4. Rates of oxidation of DPD to DPD⁺ by caeruloplasmin were constant over a range of DPD concentrations. At low DPD concentrations a lag period occurred, which was eliminated by addition of DPD⁺. 5. A lag period was not observed with TPD, but at low TPD concentrations the rate of TPD⁺ formation was greater when TPD⁺ was added. This suggests that TPD⁺ may compete weakly as a substrate with TPD and may be oxidized further by the enzyme before a non-enzymic reaction with TPD to form more TPD⁺. 6. With DPD sulphate or acetate or TPD sulphate as substrate, Lineweaver-Burk plots were curved. With DPD hydrochloride the chloride ion caused inhibition at higher concentrations, opposing the curvature. 7. Curved Lineweaver-Burk plots were interpreted in terms of two types of substrate binding site with different K_m values but similar V_{max} values. 8. The apparent thermodynamic changes associated with enzyme-substrate-complex formation at the sites with higher K_m suggest that considerable conformational change may occur on binding at these sites. 9. With substrate concentrations at which only the low- K_m sites are involved 2mol. of DPD⁺/mol. of caeruloplasmin are formed before a steady state is established. At higher substrate concentrations up to 3.2mol. of DPD⁺/mol. of caeruloplasmin are formed at this initial stage. 10. Results are discussed in relation to caeruloplasmin structures in which (a) two valence-changing and two permanently cuprous copper atoms are more accessible than the remaining four copper atoms or (b) binding of substrate at one site hinders access of substrate to another site.

Caeruloplasmin, the blue copper oxidase of plasma, has weak enzymic activity against various aromatic dihydroxy and diamino compounds. The oxidase activity and blue colour are related to the presence of four cupric copper atoms bound in an unusual way, as indicated by the hyperfine electron-spin-resonance spectrum (Broman, Malmström, Aasa & Vånngård, 1962). During enzyme activity these atoms oscillate between the cupric and cuprous forms (Broman, Malmström, Aasa & Vånngård, 1963). The molecule also contains four permanently cuprous atoms whose function is less clear, though it has been suggested that they are involved in substrate binding (Broman *et al.* 1963). Investigations of the kinetics of inhibition by azide have shown that the four valence-changing copper atoms are

probably close together (Curzon, 1966). Caeruloplasmin kinetics have usually been studied by using phenylenediamines as substrates and determining the coloured free-radical oxidation products spectrophotometrically. These methods derive from a study of the oxidation of phenylenediamines by Michaelis, Schubert & Granick (1939), who considered that the diamines and their first and second oxidation products are in equilibrium and that the first oxidation products, free radicals formed by abstraction of one electron, were more stable than most free radicals because of resonance. In general, stability was highest at pH 3.5-6. To define conditions under which spectrophotometric determination of the free radical could be used as a quantitative measure of caeruloplasmin activity,

more detailed information on the stability of the free radical in relation to pH, temperature, concentration and unoxidized diamine is needed. Non-linear oxidation of substrates by caeruloplasmin has been reported under specific conditions (Holmberg & Laurell, 1951; Aprison, Hanson & Austin, 1959), and it is not clear whether this is the result of changes in the state of the enzyme or of instability of the free radical. Further investigation of the oxidation of phenylenediamines by caeruloplasmin is also required because many previous kinetic studies were made with narrow ranges of substrate concentration or were carried out before the effect of traces of iron on the enzyme activity (Curzon, 1961) was known. This paper describes a study of the oxidation of DPD* (a) by oxidizing agents and (b) by oxygen in the presence of caeruloplasmin. A more limited series of experiments with TPD are also reported. The parent substance *p*-phenylenediamine was not investigated as its oxidation by caeruloplasmin is more complex (Peisach & Levine, 1963; Nicolau, Hörer, Thomas, Stroescu & Pascaru, 1964).

MATERIALS AND METHODS

Caeruloplasmin. Purified human caeruloplasmin (American Red Cross, Batch 1995) was dialysed against 10 mM-NaCl, ultrafiltered and non-caeruloplasmin copper removed with chelating resin as described by Curzon (1966). $E_{305}^{1\text{cm}}$ was 1.2–1.4 and the E_{605}/E_{280} ratio was 0.039, which indicates a purity of about 88% (Curzon & Vallet, 1960; Deutsch, Kasper & Walsh, 1962). $E_{305}^{1\text{cm}}$ was 1.40/mM-caeruloplasmin copper. In a single experiment caeruloplasmin from A. B. Kabi (Stockholm, Sweden) was used. This was treated as above, E_{605}/E_{280} 0.031 (70% pure). Caeruloplasmin copper content was determined by the method of Rice (1960). Caeruloplasmin copper concentrations given in the text are eight times the actual caeruloplasmin concentration, there being 8 atoms of copper/molecule.

Chemical oxidizing agents. These were prepared as approx. 0.1 N solutions and standardized with thiosulphate before dilution to required concentrations.

Substrates. Sulphate and acetate salts of substrates were prepared via the free bases (Curzon, 1966) from *NN*-dimethyl-*p*-phenylenediamine dihydrochloride (Eastman Kodak, Rochester, N.Y., U.S.A.) and *NNN'*-tetramethyl-*p*-phenylenediamine dihydrochloride (British Drug Houses Ltd., Poole, Dorset). Substrates were stored as 0.1 M solutions at -25° and brought to required pH values immediately before use with 0.1 N-NaOH or H_2SO_4 . Concentrations of DPD solutions were checked occasionally by means of the absorption peak at 240 m μ ($E_{240}^{1\text{cm}}$ 10.4).

Spectrophotometry. A Unicam SP.800 recording spectrophotometer with a thermostatically controlled cell housing

was used. Lower extinctions were obtained on a Honeywell slave recorder by using scale expansion. Determinations of extinction changes between 5 and 30 sec. of starting the enzyme reaction were made as follows. The slave recorder was allowed to run at 1 in./3.6 sec. and the final component of the incubation mixture rapidly added to the cuvette contents through a small hole drilled in the top of the cell housing. A syringe pipette (Aimer Products, London, N.W. 1) fitted with a thermostat water jacket and a glass and plastic needle dipping beneath the cuvette contents, but not in the light-path, was used for this purpose. In four trials extinctions were within 0.01 and 0.005 of the final value 5 and 8 sec. respectively after addition of a phenol red solution to water in the cuvette.

Oxidase activity of caeruloplasmin. This was determined by incubating mixtures containing EDTA as previously described (Curzon, 1966) and measuring extinctions at 550 m μ and 563 m μ , with DPD and TPD respectively as substrates. EDTA was necessary to prevent enhancement of activity by traces of ferrous iron (Curzon, 1961; Levine & Peisach, 1963). Substrates were usually used in the form of sulphates instead of the more commonly used hydrochlorides, to avoid the considerable inhibition at higher substrate concentrations (Fig. 6). Enzyme was added last to the incubation mixtures except when determinations were made during the first 30 sec. of reaction (see above); DPD was then added last. Enzyme activities were obtained as $\Delta E/\text{min.}$ measured during the period of linear increase of extinction and were converted, when necessary, into moles of product/atom of caeruloplasmin copper/sec. by using the determined molar extinctions of products. Under the conditions used, errors due to non-enzymic decomposition of product were negligible. All points shown in Figures represent duplicate determinations.

Reactions with oxidizing agents. The conditions were similar to those used for the determinations of oxidase activity. All points shown in Figures represent duplicate determinations.

RESULTS

Effect of oxidizing agents on DPD. At pH 5.5 and 25° , bromine, iodine, ferricyanide, dichromate, chloramine T or caeruloplasmin all oxidized DPD to the red DPD^+ , which faded on addition of excess of oxidant. The spectrum of DPD^+ was the same whether bromine, iodine or caeruloplasmin was used. It had peaks at 550 and 515 m μ and a shoulder at 480 m μ as described by Michaelis *et al.* (1939), who used bromine. A threefold molar excess of DPD over bromine was necessary for maximal colour formation. With DPD/bromine ratios of 2 and 1 moles/g. atom, extinctions were about 10% and 30% lower respectively, presumably because of formation of a colourless second oxidation product. The results of Michaelis *et al.* (1939) show that the two univalent oxidation steps may overlap slightly. The addition (up to 0.2 m-equiv./l.) of bromine, iodine or ferricyanide to 1 mM-DPD resulted in development of colour within 1 min. Chloramine T or dichromate, however, required 12–15 min. for colour development. When bromine or iodine was used E_{550} was

* Abbreviations: DPD, *NN*-dimethyl-*p*-phenylenediamine; TPD, *NNN'*-tetramethyl-*p*-phenylenediamine; DPD^+ , first free-radical oxidation product of DPD; TPD^+ , first free-radical oxidation product of TPD.

Fig. 1. Effect of oxidizing agents on DPD at 25°. To 1.0 ml. of 25 mM-acetate, pH 5.5, containing 10 μ M-EDTA, was added 1.0 ml. of 2.5 mM-DPD dihydrochloride and 0.5 ml. of oxidizing agent solution to give the final concentrations shown. $E_{550}^{1\text{ cm}}$ was recorded at 1 min. intervals and extrapolated to zero time. Oxidizing agents: ○, iodine; △, bromine; □, ferri cyanide.

directly proportional to the amount of oxidant (Fig. 1), though the extinctions when bromine was used were about 10% lower than with iodine, probably because of losses from volatilization. At 37°, extinctions obtained with bromine were still lower whereas those with iodine were unaffected by temperatures between 5° and 37°. Ferri cyanide gave considerably less colour than the halogens. As the addition of ferrocyanide caused fading, an equilibrium system was probably present. A value of 9.8 for $E_{550}^{1\text{ cm}}$ of DPD⁺ may be obtained from the top curve in Fig. 1, if one assumes that side reactions do not occur. The constancy of extinctions resulting from iodine oxidation over a range of 32° justifies this assumption. Michaelis *et al.* (1939) reported a value of 6.2 in methanol-acetic acid, and Ceriotti & Spandrio (1961) found that $E_{515}^{1\text{ cm}}$ was 9.5 at pH 2.7 (the 515 and 550 m μ peaks are of similar heights). In the present work the use of iodine as oxidant under the conditions of Michaelis *et al.* (1939) gave $E_{550}^{1\text{ cm}}$ 10.2, and under the conditions of Ceriotti & Spandrio (1961) $E_{515}^{1\text{ cm}}$ was 9.6 and $E_{550}^{1\text{ cm}}$ was 9.8. The latter value has been used to convert product extinction into concentration.

Treatment of TPD with iodine or bromine gave TPD⁺ spectra with peaks at 612 m μ and 563 m μ and

Fig. 2. Rate of non-enzymic decolourization of DPD⁺. DPD dihydrochloride was oxidized by bromine, $E_{550}^{1\text{ cm}}$ was recorded at 1 min. intervals. Bromine concentration in the incubation mixture was 0.02–0.23 M. Temperatures: ○, 37°; △, 25°; □, 5°. General conditions were as in Fig. 1.

$E_{563}^{1\text{ cm}}$ 12.5. Michaelis *et al.* (1939) found peaks at 606 and 560 m μ with $E_{560}^{1\text{ cm}}$ 12.2 in methanol-acetic acid.

Stability of DPD⁺ and TPD⁺. Bromine was used as oxidizing agent in most of these experiments. The red DPD⁺ faded slowly at pH 5.5, the decline over the first 10 min. after adding oxidant being linear, and only a small fraction of the initial extinction. Fig. 2 shows that the rate of fall of E_{550} was proportional to the initial E_{550} , i.e. the fading reaction is unimolecular or pseudo-unimolecular. At 25° the first-order rate constant did not vary significantly as the ratio equiv. of bromine/mol. of DPD was varied between 0.1 and 1.0. Therefore the stability of DPD⁺ is independent of the concentration of residual unchanged DPD. Variation of pH between 2.70 and 7.62 did not affect E_{550} when extrapolated to zero time. The rate of fading rose sharply with pH (Table 1). This finding (in agreement with Michaelis *et al.* 1939), together with the unimolecular kinetics, suggested that hydrolysis by OH⁻ may be responsible for the fading. Less clear results were obtained with TPD⁺, which was studied only at pH 5.5 and 25°. The net effect, however, was insufficient to cause significant TPD⁺ decomposition in the enzyme experiments.

Effect of DPD⁺ decomposition on the determination of caeruloplasmin activity. The determination of caeruloplasmin activity by measurement of the red colour of DPD⁺ formed by the action of caeruloplasmin upon DPD is valid only if the decomposition of DPD⁺ may be neglected. It may be shown that $(\text{DPD}^+ \text{ formed})/(\text{DPD}^+ \text{ determined}) = 2/(1 + e^{-kt})$

when DPD^+ is formed at a constant rate. From this the values shown in Table 2 may be obtained. At 37° or below and at pH 5.5, errors are negligible if reaction times do not exceed 5 min.

Time-course of oxidation of DPD and TPD by caeruloplasmin at various substrate concentrations. At 25° , with acetate-EDTA buffer, pH 5.5, 0.5–0.6 μM -enzyme, and with DPD sulphate at a concentration between 10 and 1 mM, the rates of formation of DPD^+ were constant. Above and below this range of substrate concentration deviations occurred. The rate appeared to decrease after the first few minutes with 20 mM substrate though interpretation was difficult because of high extinctions. Confirmation was obtained with 0.1 μM - and 0.2 μM -enzyme, when a definite decrease of rate was apparent after 6 min. With substrate concentrations 0.2 mM and below a lag period occurred, the rate increasing during the first 2–3 min. then being constant. The lag was unaffected by increasing the sulphate concentration to that present when higher substrate concentrations were used. Neither was it affected by preincubation of enzyme with buffer for 5 min. before addition of substrate. The lag was eliminated when the sub-

strate was partly oxidized to DPD^+ by iodine before addition of enzyme (Table 3). Extrapolation to the time of addition of caeruloplasmin gave extinctions of DPD^+ formed by iodine oxidation, as would be expected from Fig. 1. Thus there was no evidence of reduction of DPD^+ by the incubation system. These results suggest that DPD^+ is necessary for full enzymic activity. At higher substrate concentrations a lag would not be easily detected because of the faster rate of DPD^+ formation and because of the presence of more DPD^+ produced by non-enzymic oxidation of DPD.

After DPD was oxidized to DPD^+ by caeruloplasmin decolourization of DPD^+ occurred. This was much more rapid than the slow fading discussed above. If azide, which inhibits caeruloplasmin, was added during decolourization then further decolourization was inhibited; but if more enzyme was added the decolourization was accelerated. Addition of DPD to the decolourized solution caused immediate appearance of DPD^+ . This suggests an enzyme-independent equilibrium of the type $\text{DPD} + \text{DPD}^{2+} \rightleftharpoons 2\text{DPD}^+$. The linear relationship

Table 1. *Effect of pH on the decomposition of DPD^+ at 25°*

The reaction mixture contained 1 mM-DPD dihydrochloride, 10 mM buffer (except at pH 2.7, when 2 mM-HCl was used), 4 μM -EDTA, 0.15 M-equiv./l. of bromine. $E_{550}^{1\text{cm.}}$ was measured at 1 min. intervals and k_d , the first-order rate constant for decomposition of DPD^+ , was calculated.

Buffer	$10^3 k_d$ (min. ⁻¹)
pH 2.7, HCl	3.0
pH 3.82, acetate	2.8
pH 4.55, acetate	2.8
pH 5.5, acetate	5.5
pH 6.0, acetate	8.5
pH 7.62, phosphate	200*
pH 7.62, tris- H_2SO_4	50*
pH 8.42, tris- H_2SO_4	800*

* Accurate values of k_d were unobtainable because of interference by non-specific absorption.

Table 3. *Effect of DPD^+ on the rate of oxidation of 0.2 mM-NN-dimethyl-p-phenylenediamine by caeruloplasmin at 25°*

To 1.0 ml. of 2.5 mM-acetate buffer, pH 5.5, containing 10 μM -EDTA, was added 0.5 ml. of DPD sulphate and 0.5 ml. of water or iodine (0.1–0.5 mM) so that molarities of DPD and DPD^+ in the final mixture were as shown. A portion (0.5 ml.) of 3.4 μM -caeruloplasmin was rapidly added and E_{550} recorded continuously against a blank containing neither iodine nor enzyme. Recording was started 15 sec. later. v_a , v_b are the rates of DPD^+ formation during the periods 15 sec.–2 min. and 3–7 min. respectively after addition of enzyme.

Molarities at zero time (mM)		v_a/v_b
DPD ⁺	DPD	
—	0.2	0.73
0.02	0.2	0.96
0.04	0.2	1.00
0.10	0.2	1.00

Table 2. *Effect of DPD^+ decomposition on the determination of caeruloplasmin activity*

Conditions		$10^3 k_d$ (min. ⁻¹)	(DPD ⁺ formed)/(DPD ⁺ determined)		
Temp.	Buffer		5 min.	15 min.	30 min.
5°	pH 5.50, acetate	1.6	1.00	1.01	1.02
25	pH 5.50 acetate	5.5	1.01	1.04	1.09
37	pH 5.50, acetate	12	1.03	1.07	1.18
25	pH 7.62, tris- H_2SO_4	50	1.13	1.37	1.67

between iodine concentrations and E_{550} in Fig. 1 indicates that this equilibrium is well to the right. Enzymic depletion of DPD would eventually shift the equilibrium to the left, causing decolourization. Therefore the above experiments do not show whether DPD^+ is, itself, a substrate.

When TPD sulphate from 40 mM to 0.02 mM was used as substrate, with the same general conditions as in the DPD experiments, rates of formation of TPD^+ were constant for at least 5 min. after addition of enzyme. Time-courses of development of TPD^+ at lower concentrations are shown in Fig. 3. With any initial substrate concentration the rate of formation of product decreased only slightly with TPD depletion. For example, when the initial concentration of TPD was 0.2 mM the initial rate of TPD^+ formation was $9.7 \mu\text{moles/min.}$, which was unchanged when TPD was depleted to 0.1 mM. However, with an initial concentration of 0.1 mM-TPD the initial rate of TPD^+ formation was $7.5 \mu\text{moles/min.}$ This effect was not noted with DPD. Michaelis *et al.* (1939) described a side reaction

during the later stages of inorganic oxidation of TPD in which a trimethyl-*p*-phenylenediamine radical, with peaks at 563 and $578 m\mu$, is formed. If this reaction occurred during caeruloplasmin action then kinetic data could be misinterpreted. Therefore 0.1 mM-TPD was oxidized by caeruloplasmin while the spectrum between 650 and $500 m\mu$ was scanned at 2 min. intervals. At no stage was any change in the shape of the spectrum observed. During the subsequent decolourization the $614 m\mu$ peak declined faster than the $563 m\mu$ peak, which gradually broadened and shifted to about $555 m\mu$. This change is consistent with formation of the trimethyl radical, though as it was only apparent after much greater incubation times than were usually used, it may be ignored. The rate of formation of TPD^+ from 0.1 mM-TPD was unaffected by increasing the EDTA concentration to 0.4 mM. When TPD was partly oxidized by iodine before addition of enzyme then the rate of TPD^+ formation was the same as that found after a TPD plus TPD^+ mixture of the same composition was allowed to develop by the action of enzyme (Fig. 3). This rate was greater than that found when the same TPD concentration was used without TPD^+ . Thus the rate of oxidation of TPD depends upon the concentrations of both TPD and TPD^+ . Fig. 4 shows that the effect of TPD^+ on caeruloplasmin activity decreases as the ratio TPD/TPD^+ increases, which suggests that TPD and TPD^+ may be competing substrates at low TPD concentration.

Fig. 3. Effect of TPD^+ on the oxidation of low concentrations of TPD sulphate by caeruloplasmin at 25° . Caeruloplasmin copper, $4.3 \mu\text{M}$. $\circ$, 0.2 mM-TPD; $\triangle$, 0.1 mM-TPD; $\square$, 0.05 mM-TPD; $\bullet$, 0.1 mM-TPD + 0.1 mM- TPD^+ ; $\blacktriangle$, 0.05 mM-TPD + 0.05 mM- TPD^+ . Separate abscissa scales are drawn on the Figure for time after addition of enzyme for $\bullet$ (upper scale) and for $\blacktriangle$ (lower scale). When enzyme was added to $\text{TPD} + \text{TPD}^+$ the total amount of TPD^+ is shown. TPD^+ was measured by $E_{668}^{1\text{cm}}$. General conditions were as in Fig. 1, caeruloplasmin solution being used instead of oxidizing agent.

Fig. 4. Effect of TPD concentration on the increase of initial rate of TPD^+ formation by TPD^+ at 25° . Caeruloplasmin copper, $4.8 \mu\text{M}$. $\circ$, No TPD^+ ; $\triangle$, 0.05 mM- TPD^+ . General conditions were as in Fig. 3.

Fig. 5. Effect of caeruloplasmin concentration on the rate of oxidation of DPD sulphate at 25°. Substrate concentrations: O, 20 mM; Δ , 0.2 mM. General conditions were as in Fig. 3.

Fig. 6. Lineweaver-Burk plots at 25°. Caeruloplasmin copper: $4.6 \mu\text{M}$. Substrates: O, DPD dihydrochloride; Δ , DPD sulphate. General conditions were as in Fig. 3.

Effect of caeruloplasmin concentration on activity. The rate of DPD oxidation was proportional to enzyme concentration at both high and low substrate concentrations (Fig. 5).

Effect of pH on activity. The pH optimum was between pH 5 and 5.8 with 10 mM-DPD at 37°, 25° and 7.5°. Similar results were obtained at 25° with substrate concentrations between 10 mM and 0.1 mM.

Effect of substrate concentration on activity. Fig. 6 shows the relationship between DPD concentration

Fig. 7. Lineweaver-Burk plots of the low- K_m site of caeruloplasmin at various temperatures. Substrate, DPD sulphate. Temperatures: O, 37°; Δ , 30°; $\square$, 25°; $\bullet$, 18°; $\blacktriangle$, 7.5°. Caeruloplasmin copper concentrations: $1.9 \mu\text{M}$ (37°); $2.8 \mu\text{M}$ (30°); $4.6 \mu\text{M}$ (25°); $9 \mu\text{M}$ (18°); $41 \mu\text{M}$ (7.5°). Sodium chloride was kept constant at 0.5 mM.

and activity at 25° in the Lineweaver-Burk form. With DPD sulphate or acetate (not shown) downward curvature occurred. This was not observed with DPD hydrochloride, when at the higher substrate concentrations inhibition by chloride opposes the curvature. Sulphate and acetate have much smaller effects on activity than chloride (Curzon, 1960, 1966). High activity at higher DPD concentrations was not related to contaminant iron as increasing the EDTA concentration to 0.16 mM had no significant effect. Curved Lineweaver-Burk plots were also obtained when TPD was used as substrate or when Kabi caeruloplasmin was used. With DPD, curved Lineweaver-Burk plots were also obtained at 7.5°, 18°, 30° and 37°. Variation of activity with substrate concentration was unaffected by keeping SO_4^{2-} concentration constant, except at 7.5° when about 15% inhibition occurred at the lower substrate concentrations, when SO_4^{2-} concentration was made up to that present at the highest substrate concentration used.

Lineweaver-Burk plots of this form may be interpreted in terms of two or more substrate-binding sites. If it is assumed that two kinds of site are present and that binding of substrate at one site does not affect binding at the other (Dixon & Webb, 1964):

$$V_i = \frac{V_1}{1 + K_1/S} + \frac{V_2}{1 + K_2/S}$$

where V_i is the observed velocity and V_1 , V_2 and K_1 , K_2 the maximum velocities and Michaelis con-

stants for the first and second kinds of binding site respectively. If $K_2 < K_1$ then V_2 and K_2 may be obtained by extrapolation from the linear part of the Lineweaver-Burk plots at lower substrate concentration (Fig. 7) as under these conditions only the sites with higher affinity for substrate contribute significantly to the activity.

An alternative form of the above equation is eqn. (1).

$$\frac{1}{V_1 - \frac{V_2}{1 + K_2/S}} = \frac{1}{V_1} \left(\frac{K_1}{S} + 1 \right) \quad (1)$$

By plotting the left-hand side against $1/S$ at higher substrate concentrations, a Lineweaver-Burk plot is obtained for activity at the first site only (Fig. 8). Thus V_1 and K_1 may be derived graphically.

Fig. 9 shows the Arrhenius plots for the variation of the 'association constant' of the enzyme-substrate reaction with temperature. As it is not known if the caeruloplasmin-DPD reaction obeys Michaelis kinetics, the thermodynamic constants derived from these lines are not necessarily related to the enzyme-substrate equilibrium. However, it is apparent that the interactions at the different sites are thermodynamically distinct. Thus at the low- K_m site(s) ΔH for formation of the enzyme-substrate complex is -4 kcal./mole and ΔS is 7 cal./mole/degree. At the higher K_m site(s) ΔH is 7 kcal./mole and ΔS 33 cal./mole/degree. Fig. 10 shows the Arrhenius plots for breakdown of the enzyme-substrate complex to products. These are identical at both types of site with $\Delta H^* 16$ kcal./

mole and $\Delta S^* -9$ cal./mole/degree. (To determine ΔS^* , k_2 with respect to the absolute concentration of enzyme is needed. Although most enzymes have one active site/molecule, caeruloplasmin has four active sites in the sense that 4 mol. of product are produced by a complete turnover of an enzyme molecule. Therefore k_2 is given by $1/4$ DPD⁺ formed/mol. of caeruloplasmin/sec. = $2 \times$ DPD⁺ formed/atom of copper/sec.)

DPD⁺ concentration and caeruloplasmin colour at

Fig. 9. Arrhenius plot of the association constants ($1/K_m$) of caeruloplasmin with DPD. $\circ$, High- K_m site; Δ , low- K_m site.

Fig. 8. Lineweaver-Burk plot of the high- K_m site of caeruloplasmin at various temperatures. Reaction velocities were calculated from equation 1. Symbols are as described in Fig. 7.

Fig. 10. Arrhenius plots for the velocity constant for the breakdown of the caeruloplasmin-DPD complex to DPD⁺. $\circ$, High- K_m site; Δ , low- K_m site.

Fig. 11. Rate of oxidation of DPD sulphate by caeruloplasmin at 7.5°. DPD concentrations: (a) 22 mM; (b) 3.1 mM; (c) 0.12 mM. The bottom curve shows the change of E_{605} of the caeruloplasmin in the presence of 0.12 mM-DPD. At the higher DPD concentrations shown E_{605} was too low to measure. Caeruloplasmin copper was 41 μM ($E_{605}^{1\text{cm}} 0.055$). General conditions were as in Fig. 3 except that the buffer was 1.0 ml. of 25 mM-acetate, pH 5.5, containing 35 μM -EDTA.

7.5°. The formation of DPD^+ and the decrease in E_{605} of the enzyme at 7.5° were measured during the first 30 sec. after addition of DPD. It was not possible to measure ΔE_{605} at higher temperatures as E_{605} was too low at enzyme concentrations convenient for DPD^+ determination. At 7.5° the reaction occurred in two stages (Fig. 11): first a rapid formation of DPD^+ associated with fading at 605 m μ , then a linear rate of formation of product during which time the enzyme remained decolourized. At higher DPD concentrations the first stage is over within the 8 sec. which elapses before extinctions can be measured by the method used. At lower DPD concentration the time-course of part of the first stage was demonstrable. By extrapolating the linear part of the curve to zero time, product formation in this stage may be determined. At the highest DPD concentration (as in Fig. 8) the extrapolated E_{550} corresponds to 3.2 mol of DPD^+ /mol. of caeruloplasmin. At the highest DPD concentration of the lower range (Fig. 7), at which the low- K_m site(s) are assumed to be saturated, E_{550} corresponds to 2 mol. of DPD^+ /mol. of enzyme.

Inhibition of caeruloplasmin by azide at high substrate concentration. One azide molecule completely inhibits a caeruloplasmin molecule, which suggests that re-oxidation of all four valence-changing copper atoms is prevented and that they are probably close together when in the reduced state (Curzon, 1966). Since at the highest substrate concentrations previously used (4 mM) the binding

sites of higher K_m would not have been saturated, the number of azide molecules for complete inhibition may have been underestimated. However, by using 38 mM-DPD sulphate and plotting the inhibition by various concentrations of azide as before, i.e. by the graphic method of Easson & Stedman (1936), it was found that one azide molecule inhibited 1.1 mol. of caeruloplasmin. Thus one azide molecule completely inhibits caeruloplasmin when both kinds of substrate-binding site are almost saturated.

DISCUSSION

In early studies of the oxidase activity of caeruloplasmin (Holmberg & Laurell, 1951), *p*-phenylenediamine was used as substrate. As Michaelis *et al.* (1939) found the DPD oxidation product to be stabler than that derived from *p*-phenylenediamine, Akerfeldt (1957) introduced DPD as a substrate. More recently, Peisach & Levine (1963) showed that when *p*-phenylenediamine is oxidized by caeruloplasmin considerable conversion of the free radical formed into secondary products occurs within a few minutes. Also, Nicolau *et al.* (1964) reported that the *p*-phenylenediamine oxidation products formed by caeruloplasmin and by inorganic oxidizing agents show spectral differences. These complications do not occur when DPD is used as substrate. Though the red colour of DPD^+ slowly fades, resulting errors are only slight under a wide range of conditions and may, if necessary, easily be corrected for.

An initial lag in rate of product formation has previously been reported (Holmberg & Laurell, 1951; Aprison *et al.* 1959). Elimination of the lag when DPD^+ was present in the incubation mixture before addition of enzyme is difficult to explain except in terms of a conformational change induced by the product. With TPD as substrate the lag was not observed but TPD^+ caused increased activity at very low TPD concentration. Under these conditions TPD^+ may itself be a substrate being converted into a higher oxidation state and then reacting non-enzymically with TPD to form more TPD^+ . Mechanisms of this kind have been discussed with respect to the oxidation of *p*-phenylenediamine by caeruloplasmin (Peisach & Levine, 1963). At higher TPD concentrations, TPD^+ has no effect. This may be explained if TPD competes successfully for the enzyme with TPD^+ . Though caeruloplasmin caused DPD^+ to fade there was insufficient evidence to show that this was due to direct action of the enzyme on DPD^+ . E. Walaas, R. Løvstad & O. Walaas (unpublished work) have shown that DPD^+ is a substrate but only in the absence of DPD.

With DPD or TPD as substrate, Lineweaver-Burk plots have slopes which decrease as the substrate concentration decreases. In general this may

arise because different active sites catalyse the same reaction, but with different K_m values. The sites may be on the same or on different molecules. Heterogeneity of caeruloplasmin, genetic or constitutional (Morell & Scheinberg, 1960; Broman, 1964; Imlah, 1964) or through artifact formation (Curzon & Vallet, 1960; Broman, 1964), has been reported. However, variant forms have the same K_m values (Richterich, Temperli & Aebi, 1962; Broman, 1964). Therefore it is more likely that the Lineweaver-Burk plots are non-linear because there are different active sites on the same enzyme molecule. When a caeruloplasmin molecule reacts with the substrate, four electrons are transferred to the enzyme, one from each substrate molecule. Therefore caeruloplasmin may have four substrate-binding sites and the curved Lineweaver-Burk plots may arise because different sites have different K_m values. Curvature has not been reported in previous kinetic studies with caeruloplasmin (Holmberg & Laurell, 1951; Aprison *et al.* 1959; Curzon, 1960, 1966; Hanson, Austin & Aprison, 1959; Osaki, 1961; Hörer, Thomas, Mira & Nicolau, 1964; Osaki, McDermott & Frieden, 1964; Peisach & Levine, 1965), probably because narrower ranges of substrate concentration were generally used. When the substrate is used as the hydrochloride, chloride ion inhibits at higher concentrations and this opposes the curvature (Fig. 6). Furthermore, the apparent K_m values for the two kinds of site converge with rising temperature and so curvature is more apparent at lower temperatures. Concurrently with the work described here, Walaas *et al.* (1967) obtained curved Lineweaver-Burk plots with DPD at 10° which they also explain in terms of two sites.

Thermodynamic constants relating to oxidase activity of caeruloplasmin must be interpreted with caution in the absence of sufficient information concerning the mechanism. Marked differences are apparent between the thermodynamic constants derived from K_m values thought to be associated with the two kinds of binding site. As the constants associated with breakdown of the enzyme-substrate complexes to products are the same for both kinds of site it is probable that though K_m may include a kinetic term, any differences between K_m values derive from differences in the equilibria between enzyme and substrate. The thermodynamic constants associated with substrate binding at the low- K_m site(s) are not unusual. The differences between these values and the corresponding ones at the high- K_m site(s) indicate an endothermic change and a quite large entropy increase when substrate is bound at this site. The large entropy change may mean that a considerable conformation change occurs in binding at this site. The similarity of the V_{max} values and of the thermodynamic changes associated with breakdown of the enzyme-

substrate complexes to products at both sites suggests that, though they may differ in accessibility to substrate, they are of similar structure.

It has been suggested that the permanently cuprous atoms of caeruloplasmin are involved in substrate binding (Broman *et al.* 1963), though probably in an indirect manner through ligand groups (Broman, 1966). The substrate-binding sites of low and high K_m may therefore be associated respectively with two more accessible cuprous atoms and two less accessible cuprous atoms. The reduction of caeruloplasmin by catecholamines has also been shown to occur in two stages corresponding to two types of site, one of which is more accessible than the other (Walaas, Walaas & Løvstad, 1966). Similarly, proton relaxation studies on caeruloplasmin during reduction by ascorbic acid suggest that two of the cupric copper atoms (i.e. the valence-changing atoms) are more accessible to water molecules than are the other two (Blumberg, Eisinger, Aisen, Morell & Scheinberg, 1963). The two accessible cuprous atoms and the two accessible cupric atoms are possibly identical with the four copper atoms made easily diffusible by chymotryptic digestion (Curzon, 1958; Marriott & Perkins, 1966). Kinetic study of the inhibition of caeruloplasmin by azide indicates that the four valence-changing copper atoms are sufficiently close together when in the cuprous state, to be simultaneously re-oxidized by an oxygen molecule (Curzon, 1966). This implies that the less accessible valence-changing copper atoms may be near to the more accessible ones, but deeper within a cleft.

Another possible interpretation of the thermodynamic data and the two K_m values derives from the likelihood that all eight caeruloplasmin copper atoms are in the same region of the molecule (Curzon, 1966). So, although all the binding sites might be identical and equally accessible, the binding of substrate at one site might hinder access of a second substrate molecule to an adjacent site. This would be reflected in a larger K_m at the second site. Opening out or disordering of the molecule in the binding area would be necessary for binding of the second substrate molecule, and the observed large entropy increase would be expected.

In experiments at 7.5°, caeruloplasmin was almost completely decolorized by substrate concentrations far below the K_m of the less accessible site. As the intensity of the blue colour of caeruloplasmin is proportional to the concentration of cupric copper (Ehrenberg, Malmström, Broman & Mosbach, 1962; Blumberg *et al.* 1963; Kasper, Deutsch & Beinert, 1963), this indicates that complete reduction of the cupric copper atoms is possible when not all the substrate-binding sites are involved. The slower reduction at low substrate concentration than at

high substrate concentration would be expected if, in the former case, two consecutive cycles at the low- K_m sites are necessary for complete reduction whereas only one cycle is necessary in the latter. At high substrate concentration the DPD^+ formed, extrapolated to zero-time, was up to 3.2 mol./mol. of caeruloplasmin whereas at low substrate concentration, when only the low- K_m sites are involved, 2 mol. of $\text{DPD}^+/\text{mol.}$ of caeruloplasmin were formed. This agrees with above interpretation. Complete reduction of cupric copper atoms at low substrate concentration implies that electrons may be donated to more than one cupric copper atom via the same binding site.

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