A Method for Obtaining Linear Reciprocal Plots with Caeruloplasmin and its Application in a Study of the Kinetic Parameters of Caeruloplasmin Substrates

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(Received 11 January 1972)

1. The curved plots of $1/v$ against $1/[S]$ obtained when caeruloplasmin oxidizes *NN*-dimethyl-*p*-phenylenediamine were investigated. The first free-radical oxidation product of caeruloplasmin oxidation of *NN*-dimethyl-*p*-phenylenediamine is required for curvature, as straight-line plots were obtained when activities were measured either before appreciable free-radical product had appeared or in the presence of ascorbate, which reduced it back to *NN*-dimethyl-*p*-phenylenediamine. 2. In the presence of ascorbate linear reciprocal-plots were obtained with all of the 37 substrates tested. $V_{\max}$ values varied over only an eightfold range and those for the 20 *p*-amino compounds over only a twofold range. K_m values, however, varied over a 10^4 -fold range. The small range of $V_{\max}$ values indicates that the rate-limiting step in caeruloplasmin action is relatively independent of the nature of the substrate. K_m values suggest that substrates bind primarily by ring electrons, although certain side-chain groups increased the K_m in a manner unrelated to likely changes of ring-electron densities. A mechanism involving repulsion between negative charges on the substrate and the enzyme was supported by the variation of the K_m of 5-hydroxyindol-3-ylacetic acid with pH.

Caeruloplasmin (ferroxidase), the blue copper-containing glycoprotein of plasma, has oxidase activity towards various aromatic polyphenols and polyamines (Holmberg & Laurell, 1951) and also towards Fe^{2+} (Curzon & O'Reilly, 1960). It has been extensively investigated by both visible and electron-spin-resonance spectroscopy (see Malkin & Malmström, 1970) and by inhibition studies (Curzon, 1960; Curzon & Speyer, 1968; Speyer & Curzon, 1968). There have also been two studies of the kinetic parameters of a range of caeruloplasmin substrates (Levine & Peisach, 1962; Peisach & Levine, 1965). However, as traces of contaminating iron can increase caeruloplasmin activity towards a wide range of substrates via a caeruloplasmin-iron coupled system (Curzon, 1961), and thus true activities can only be determined in the presence of a chelator such as EDTA (McDermott *et al.*, 1968), previous substrate studies cannot be interpreted. Other problems derive from the curves obtained when reciprocals of substrate concentration and activity are plotted against each other (Osaki, 1966; Curzon, 1967; Walaas *et al.*, 1967). These have been taken to indicate that caeruloplasmin has two kinds of active site (Curzon, 1967; Walaas *et al.*, 1967) or that caeruloplasmin preparations contain two kinetically different species (Løvstad, 1969) present in different proportions in subjects of different ages.

However, in the present study, various purified caeruloplasmins all gave similarly curved kinetic plots. Also, with *NN*-dimethyl-*p*-phenylenediamine as substrate the curvature was shown to be associated with the formation of the first free-radical oxidation product of caeruloplasmin action on it. When the free-radical product is not allowed to accumulate then a straight-line plot of $1/v$ against $1/[S]$ is obtained. Pettersson (1969, 1970*a*) and Pettersson & Pettersson (1969) have similarly found by mathematical analysis of the spectral changes during oxidation of *NN*-dimethyl-*p*-phenylenediamine by caeruloplasmin that curved plots are due to the free-radical product.

By determining caeruloplasmin activity in the presence of both EDTA and ascorbate the effects of both iron and products of enzyme action on activity were eliminated and the kinetic properties of a range of substrates were compared and discussed. A preliminary report of part of this work has appeared (Curzon & Young, 1971).

Materials and Methods

Caeruloplasmin

Human plasma from which the anti-haemophilic factor had been removed by freezing was supplied by the Blood Products Laboratory, Lister Institute (Aldenharn, Herts., U.K.), and 400-ml batches were purified on DEAE-Sephadex A-50 (Pharmacia,

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Table 1. Kinetic parameters of caeruloplasmin substrates

Experimental details are given in the Materials and Methods section. The sources of chemicals were: A, BDH Chemicals (Poole, Dorset, U.K.); B, Calbiochem (London W.1, U.K.); C, R. N. Emanuel (Alperton, Middx., U.K.); D, gift from Mr. C. W. Amery (Research Department, Gillette, Reading, Berks., U.K.); E, Koch-Light Laboratories (Colnbrook, Bucks., U.K.); F, Kodak (Kirkby, Liverpool, U.K.); G, gift from Dr. L. Williams (Research Department, Kodak, Wealdstone, Middx., U.K.); H, Regis Chemical Co. (Chicago, Ill., U.S.A.); I, Roche Products (Welwyn Garden City, Herts., U.K.); J, Sigma Chemical Co. (London S.W.6, U.K.). Many compounds had to be purified, because they were brown or black owing to contamination by oxidation products. All solids were purified until they were white or nearly white (except 2-nitro- <i>p</i> -phenylenediamine which is brown when pure). Liquids were purified until colourless. As Cl ⁻ ions inhibit caeruloplasmin (Curzon, 1960) those compounds obtained as hydrochlorides were converted into free bases and then to sulphate salts. Crystallization of free bases was from the following solvents: 1, water; 2, ethanol; 3, ethanol then acetone; 4, chloroform; 5, chloroform then benzene; 6, diethyl ether; 7, toluene. Other procedures were: 8, hydrochlorides converted into the purified free bases by the method of Curzon & Speyer (1967); 9, hydrochlorides dissolved in the minimum quantity of water and the free base precipitated by addition of stoichiometric quantities of 5M-NaOH and filtered off. When compounds were recrystallized from water, charcoal (Norit A; Kodak, Kirkby, Liverpool, U.K.) was added to the hot solution, which was then stirred for 2 min and filtered while still hot. The weight of charcoal added was half that of the compound. 10, Free bases distilled; 11, free base dissolved in the minimum quantity of 1M-H ₂ SO ₄ in 80% (v/v) ethanol at 4°C, and the sulphate salt precipitated by slow addition of diethyl ether with stirring; 12, hydrochloride converted into free base by using DEAE-cellulose. A 50 ml volume of 20 mm-hydrochloride through which N ₂ had been bubbled was poured into a column (5 cm × 2 cm diam.) of DEAE-cellulose (Kodak, Kirkby, Liverpool, U.K.) in the OH ⁻ form. The brown leading band of eluate was discarded and the next 10 ml of Cl ⁻ -free eluate were used. All compounds were dried in a vacuum desiccator before use. All the amino compounds, except the three obtained as sulphate salts (see below), were dissolved in H ₂ SO ₄ soln. to give a solution equimolar with the H ₂ SO ₄ . Other compounds were dissolved in water. Solutions were adjusted to pH 5.5 with 1M-NaOH that had been treated with Chelex 100 metal-binding resin (100–200 mesh; BioRad) stored under N ₂ at 4°C and diluted appropriately for use. <i>NN'</i> -Di- <i>s</i> -butyl- <i>p</i> -phenylenediamine, <i>N</i> -ethyl- <i>N</i> -(2-hydroxyethyl)- <i>p</i> -phenylenediamine and <i>N</i> -ethyl- <i>N</i> -2-(<i>S</i> -methylsulphonamido) ethyl- <i>p</i> -phenylenediamine, which were obtained as the purified sulphates, were dissolved in water and adjusted to pH 5.5 as above. Because the solids may not have contained equimolar concentrations of sulphate and phenylenediamine the concentration of phenylenediamine in these solutions was checked by spectrophotometric titration with I ₂ . On addition of I ₂ solution all three compounds behaved in the same way as <i>NN</i> -dimethyl- <i>p</i> -phenylenediamine (Pettersson, 1968), i.e. they gave a coloured first oxidation product, which on addition of more I ₂ gave a colourless second oxidation product. It was assumed that the normality of I ₂ giving the maximum colour was equal to the molarity of phenylenediamine.						
<i>p</i> -Amino compounds	Source	Purification	M.p. (°C)*	$V_{\max.}$ (e/Cu atom per min)	K_m (μ M)	pK
<i>N</i> -Acetyl- <i>p</i> -phenylenediamine	A	1	162–162.5°C (163–164°C)	3.42	12300	4.5
<i>p</i> -Aminophenol	A	3	183–184°C (186°C)	3.53	1540	5.3
<i>p</i> -Anisidine	A	1	55–56°C (57.2°C)	4.05	6140	5.4
2-Chloro- <i>p</i> -phenylenediamine	C	8	169°C (170°C)	5.35	241	5.3
<i>NN'</i> -Di- <i>s</i> -butyl- <i>p</i> -phenylenediamine	F	11		6.05	620	7.2
2,5-Dichloro- <i>p</i> -phenylenediamine	D	6	169°C (170°C)	3.94	740	3.7
<i>NN</i> -Diethyl- <i>p</i> -phenylenediamine	A	9	160–162°C (160–162°C)	3.18	556	7.8
		then 10				
<i>NN'</i> -Dimethyl- <i>p</i> -phenylenediamine	F	8		4.33	164	6.6
<i>NN</i> -Dimethyl- <i>p</i> -phenylenediamine	F	8		5.13	203	6.4
Durenediamine	D	9	148°C (149°C)	6.00	171	5.9
		then 4				

<i>N</i> -Ethyl- <i>N</i> -(2-hydroxyethyl)- <i>p</i> -phenylenediamine	G			5.49	110	7.4
<i>N</i> -Ethyl- <i>N</i> -2-(<i>S</i> -methylsulphonamido)-ethyl- <i>p</i> -phenylenediamine	G			6.06	87.2	6.5
2-Methoxy- <i>p</i> -phenylenediamine	D	9 then 6		6.20	161	6.0
<i>N</i> -(<i>p</i> -Methoxyphenyl)- <i>p</i> -phenylenediamine	A	8		6.50	20.6	5.3
2-Methyl- <i>p</i> -phenylenediamine	F	8		5.49	213	6.3
2-Nitro- <i>p</i> -phenylenediamine	D	1	143°C (143°C)	6.93	1260	4.5
<i>p</i> -Phenylenediamine	F	5	138.5–140°C (140°C)	4.44	292	6.3
<i>p</i> -Phenylenediamine-2-sulphonic acid	G	1		3.83	2620	5.6
<i>N</i> -Phenyl- <i>p</i> -phenylenediamine	F	9		4.78	47.7	5.3
<i>NNN'</i> - <i>N'</i> -Tetramethyl- <i>p</i> -phenylenediamine	F	8		5.06	197	6.4
Catechols						
L-Adrenaline	A			2.29	2550	
Catechol	A	2	102–103°C (105°C)	8.98	282000	
3,4-Dihydroxyphenylacetic acid	B			†	±250000	
3,4-Dihydroxyphenylalanine (dopa)	I			†	~20000	
3,4-Dihydroxyphenethylamine	J	12		7.54	2850	
4-Methylcatechol	A	1		6.81	60300	
L-Noradrenaline	J			2.71	2810	
5-Hydroxyindoles						
5-Hydroxyindol-3-ylacetic acid	J			1.50	8340	
5-Hydroxytryptamine	J			5.68	908	
5-Hydroxytryptophan	E			1.78	16300	
5-Hydroxytryptophol	H			2.87	5100	
Other compounds						
<i>m</i> -Aminophenol	A	7		4.03	199000	4.4
<i>o</i> -Aminophenol	A	1	122–123°C (123°C)	3.60	2880	4.7
Ascorbate	A		173–174°C (174°C)	4.07	5200	
<i>NN</i> -Dimethyl- <i>m</i> -phenylenediamine	F	9 then 10		4.02	3050	5.3
<i>NN</i> -Dimethylaniline	A	10		†	±25000	
<i>p</i> -Methoxyphenol	A	2	53°C (53°C)	†	±700000	
<i>m</i> -Phenylenediamine	F	9		5.60	36000	5.1
<i>o</i> -Phenylenediamine	F	8		1.30	2950	4.8
Pyrogallol	A			10.80	57900	
Quinol	A	1	170–171°C (170°C)	5.64	65700	
Resorcinol (100mm)	A	4	109°C (111°C)			
Gentisic acid (100mm)	A	1	204–205°C (205°C)			
Aniline (2mm)	A	10				
Mercaptoethanol (100mm)	B					

Activity undetectable

* Values in parentheses are from the *Handbook of Chemistry and Physics* (1964).† $V_{\max}$ not determinable because of limited solubility and/or K_m .

London W.5, U.K.) as described by Stokes (1967), followed by further purification on hydroxyapatite (BioRad Laboratories, St. Albans, Herts., U.K.) as described by Broman (1958). Two caeruloplasmin components were eluted from hydroxyapatite columns, the major one containing 85% of the total caeruloplasmin eluted. These were dialysed against 20mM-sodium acetate buffer, pH 5.5, for 24h, and traces of non-caeruloplasmin copper and other metals were removed on a column (1cm × 1cm) of Chelex 100 resin (100–200 mesh; BioRad Laboratories) equilibrated with the same buffer. Procedures were performed and caeruloplasmin was stored at 4°C. Typical preparations had an E_{605}/E_{280} ratio of 0.039, indicating at least 85% purity. Caeruloplasmin concentrations were determined from E_{605} measurements, assuming E_{605} of 21.4 for 1g of Cu/l (Deutsch *et al.*, 1962) and 6Cu atoms/molecule of caeruloplasmin (Magdoff-Fairchild *et al.*, 1969). Unless otherwise specified the major component separated on hydroxyapatite was used.

Neonatal serum was obtained as cord serum from Queen Charlotte's Hospital (London W.14, U.K.) and caeruloplasmin was purified from 120ml as above.

Adult serum was bulked material from patients of The National Hospital (Queen Square, London W.C.1, U.K.).

Substrates

Sources of substrates and methods used for their purification are given in Table 1.

Determination of caeruloplasmin activity

Spectrophotometric measurements. Spectrophotometric measurements were made at 25°C by using a Unicam SP. 800 spectrophotometer with a constant-temperature cell housing and scale-expansion accessory. Unless otherwise specified 1cm-path-length cuvettes were used. Caeruloplasmin activity against *NN*-dimethyl-*p*-phenylenediamine was determined by using the absorption peak of its first free-radical product at 550nm as described by Curzon & Speyer (1968).

In experiments on serum, solutions in 0.1M-sodium acetate buffer, pH 5.9, containing 0.5mM-1,10-phenanthroline to bind iron were used. The reaction was started by adding 0.2ml of serum to give a total volume of 2.5ml. These conditions were identical with those used by Løvstad (1969). Other conditions differed from those of Løvstad (1969), as the temperature was maintained at 25°C instead of 15°C and *NN*-dimethyl-*p*-phenylenediamine sulphate instead of the hydrochloride was used. Chloride contributed by the serum was present at about 8mM. Activity was determined by measuring the increase

of E_{550} . Initially lags in colour development were obtained with neonatal serum, especially at low *NN*-dimethyl-*p*-phenylenediamine concentrations. This did not occur after dialysis of the serum against a buffer solution (143mM-NaCl–5.7mM-KCl–10mM-Na₂HPO₄–10mM-glucose adjusted to pH 7.4 with HCl). Lags were probably due to diffusible material reducing the free-radical product formed back to *NN*-dimethyl-*p*-phenylenediamine, the product not accumulating until the reducing material was oxidized. As all subsequent experiments on neonatal serum were done with dialysed material, the adult serum was also dialysed before use.

Oxygen electrode. Activity was determined by measuring the rate of uptake of O₂ required for the reoxidation of the enzyme reduced by reaction with the substrate. This method may be used as the K_m for O₂ is 3.9μM (Frieden *et al.*, 1965), which is much smaller than the O₂ concentration at which determinations were made (60μM). A Beckman model 777 oxygen analyser with a Beckmann 39065 sensor coupled to a Honeywell 25.4cm (10in) potentiometric recorder was used. The reaction medium (9ml) was contained in a cylindrical chamber surrounded by a water jacket at 25°C and stirred magnetically at the bottom. The electrode was introduced at the top of the chamber, a gas-tight fit being obtained by means of a tapered Fluon gasket. The reaction vessel was made to specification by Aimer Products (London N.W.1, U.K.). The electrode was calibrated by the method of Dixon & Kleppe (1965). The apparatus was usually adjusted so that a full-scale deflexion on the recorder corresponded to a change of 10μM-O₂.

Experiments on the oxidation of *NN*-dimethyl-*p*-phenylenediamine by purified caeruloplasmin were performed in a medium containing 10mM-sodium acetate buffer, pH 5.5, and various substrate concentrations. Sulphate was maintained at 10mM with sodium sulphate solution. EDTA (100μM) was present to prevent enhancement of activity by traces of contaminating iron (Curzon, 1961). Caeruloplasmin was added last to the reaction mixture after the rate of non-enzymic oxidation had been measured.

In experiments on the series of substrates the activity of each compound was first measured at one concentration with both 50μM- and 100μM-EDTA present. As activities were in all cases essentially identical at both EDTA concentrations, all substrates exhibited true and not iron-mediated activity, which would have been decreased at the higher EDTA concentration. Except in preliminary experiments, 50μM-ascorbate was present in the reaction mixture to prevent the accumulation of products that might also be substrates. Although in general sulphate was maintained at 10mM this concentration was exceeded in the cases of three substances with high K_m values, i.e. *m*-aminophenol, *m*-phenylenediamine and *N*-

acetyl-*p*-phenylenediamine. However, sulphate has little effect on caeruloplasmin activity (Curzon, 1960).

Rates of O_2 uptake were measured with at least five concentrations of each substrate. The concentration of caeruloplasmin, which was constant for any one substrate, was between 0.2 and $1.0\mu M$. Non-enzymic oxidation of substrates was allowed for and was less than 3% of the enzymic rate for all substrates and concentrations used, except for *NN*-dimethyl-*p*-phenylenediamine, when it was 7% at the highest concentrations used.

Plotting of results

V_{max} and K_m values were determined by the method of Lineweaver & Burk (1934).

In the comparison of substrates the values of the intercepts taken were those of the regression line of $1/v$ on $1/[S]$. Study of three substrates, namely 3,4-dihydroxyphenylacetic acid, *NN*-dimethylaniline and *p*-methoxyphenol, was restricted by their limited solubilities. Their activities were therefore measured at concentrations near saturation and approximate lower limits for the K_m values were calculated by using the Michaelis-Menten equation, the concentration of caeruloplasmin used and a low value of the V_{max} of $1.3e/Cu$ atom per min (electron transfers/Cu atom per min). V_{max} values of caeruloplasmin towards all 37 substrates tested varied between 1.3 and $10.8e/Cu$ atom per min (see below).

Measurements with dopa (3,4-dihydroxyphenylalanine) were also restricted by its low solubility, and an approximate K_m value was determined by measuring the activity at two concentrations by using a super-saturated solution. This was prepared by dissolving dopa in 50mM-NaOH to give a 50mM solution and adjusting the pH to 5.5 with 0.5M- H_2SO_4 . The solution was used within 5min of preparation. Crystallization was not apparent until 10min after the pH had been adjusted to 5.5.

Determination of pK values

Values of pK were determined at $25^\circ C$ by titrating 10ml of 10mM solutions of substrates with 20mM-NaOH or 10mM- H_2SO_4 by using an EIL pH-meter with a glass electrode.

Results

Oxidation of *NN*-dimethyl-*p*-phenylenediamine by caeruloplasmin

Preliminary experiments. With *NN*-dimethyl-*p*-phenylenediamine as substrate curved plots of $1/v$ against $1/[S]$ were found with adult and neonatal sera and also with caeruloplasmin purified from either adult or neonatal sera. Curvature was also found with

both the major and minor caeruloplasmin components eluted from hydroxyapatite. Curvature was identical for all the purified preparations.

Substrate properties of the first free-radical oxidation product of *NN*-dimethyl-*p*-phenylenediamine. The above results suggest that the curved plots of $1/v$ against $1/[S]$ are not due to kinetically-distinct caeruloplasmin species. The following results indicate that curvature with *NN*-dimethyl-*p*-phenylenediamine is caused by the substrate activity of the first product of its oxidation, i.e. from the presence of two substrates rather than from distinct enzyme species or active sites.

Walaas *et al.* (1967) followed the appearance and subsequent disappearance of the first oxidation product during caeruloplasmin action on *NN*-dimethyl-*p*-phenylenediamine and showed that the product reduced caeruloplasmin and was oxidized further to the colourless second product. When I_2 is used to oxidize *NN*-dimethyl-*p*-phenylenediamine (Fig. 1) the plot of E_{550} against I_2 added has a similar form to that of Walaas *et al.* (1967) for E_{550} against time of oxidation by caeruloplasmin, there being no sharp discontinuity corresponding to complete oxidation to the first product. This is consistent with a non-enzymic equilibrium between substrates and products (Pettersson, 1968):

Fig. 1. Oxidation of *NN*-dimethyl-*p*-phenylenediamine by I_2

I_2 solution to the normalities shown was added to *NN*-dimethyl-*p*-phenylenediamine solution to give a final concentration of *NN*-dimethyl-*p*-phenylenediamine plus its oxidation products of $100\mu M$. Other conditions were as described for caeruloplasmin oxidase activity determinations in the Materials and Methods section.

Equilibrium is attained quickly, E_{550} reaching its final value within 5 s of the addition of I_2 . In the presence of a relatively large proportion of unoxidized *NN*-dimethyl-*p*-phenylenediamine the second product is largely reduced back to the first one. Thus the rate of appearance of the first product measures activity towards a mixture of *NN*-dimethyl-*p*-phenylenediamine and first product, the increasing competition of the latter for the active site as lower *NN*-dimethyl-*p*-phenylenediamine concentrations are used causing the plot of $1/v$ against $1/[S]$ to curve.

The degree of curvature depends on the concentration of the first product at which measurements were made, and should be negligible at sufficiently low concentrations of it. This was investigated experimentally. Initial activities could not be used owing to rapid initial reduction of caeruloplasmin by substrate (Curzon, 1967). Therefore the rate of first product formation was measured between 15 and 30 s after the addition of caeruloplasmin to the reaction medium, first product concentrations being less than

Fig. 2. Initial rates of oxidation of *NN*-dimethyl-*p*-phenylenediamine by caeruloplasmin

A plot of $1/v$ against $1/[S]$ is shown. The caeruloplasmin concentration was $0.415 \mu\text{M}$. Other conditions were as described in the Materials and Methods section. Activities were measured by the rate of increase of the first product (E_{550}) between 15 and 30 s after the addition of caeruloplasmin.

$5 \mu\text{M}$ as determined by using $\epsilon_{550} \ 9.8 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Curzon, 1967). The plot approximates to a straight line (Fig. 2). A V_{max} of 3.42 e/Cu atom per min and a K_m of $142 \mu\text{M}$ -*NN*-dimethyl-*p*-phenylenediamine were calculated from it.

Oxidation of NN-dimethyl-p-phenylenediamine by caeruloplasmin in the presence of ascorbate. When caeruloplasmin oxidizes *NN*-dimethyl-*p*-phenylenediamine in the presence of low ascorbate concentrations, the first product formed is reduced back to it by the ascorbate and does not accumulate until the latter has been oxidized (Humoller *et al.*, 1958). The rate of oxidation thus measures enzyme activity towards *NN*-dimethyl-*p*-phenylenediamine alone. The ascorbate does not interfere with the interaction between caeruloplasmin and *NN*-dimethyl-*p*-phenylenediamine as, in agreement with McDermott *et al.* (1968), the rate of O_2 uptake was negligible with up to

Fig. 3. Caeruloplasmin activity towards mixtures of *NN*-dimethyl-*p*-phenylenediamine and its first product

Curve A, initial *NN*-dimethyl-*p*-phenylenediamine concentration $70 \mu\text{M}$ and initial first product concentration $30 \mu\text{M}$; curve B, initial *NN*-dimethyl-*p*-phenylenediamine concentration $100 \mu\text{M}$ and no product added; curve C, initial *NN*-dimethyl-*p*-phenylenediamine concentration $100 \mu\text{M}$ and initial ascorbate concentration $50 \mu\text{M}$. In all experiments the caeruloplasmin concentration was $0.17 \mu\text{M}$. Other conditions were as described in the Materials and Methods section. Caeruloplasmin activity was measured by the rate of O_2 uptake.

300 μM -ascorbate in the presence of 1 μM -caeruloplasmin. Also, rates of oxidation by caeruloplasmin of both 60 mM- and 1 mM-*NN*-dimethyl-*p*-phenylenediamine were unaffected by varying the ascorbate concentration between 20 and 300 μM . At higher concentrations of ascorbate, however, it has significant substrate properties with a K_m of 4.7 mM (Curzon & Young, 1972).

Fig. 3 shows the activities (measured by O_2 uptake) of caeruloplasmin under three conditions: towards *NN*-dimethyl-*p*-phenylenediamine in the presence of ascorbate (i.e. *NN*-dimethyl-*p*-phenylenediamine alone as substrate), towards *NN*-dimethyl-*p*-phenylenediamine without ascorbate (i.e. with first product accumulating) and finally towards a mixture of *NN*-dimethyl-*p*-phenylenediamine and first product (i.e. with the concentration of the latter high enough to give a constant activity). During the accumulation of first product the activity increases from the low constant value obtained in its absence towards the high constant value obtained with the mixture. This demonstrates that the activity in the presence of ascorbate is effectively the activity in the absence of the first product. It also explains the previously found initial period of low activity that was abolished by addition of first product (Curzon, 1967).

When caeruloplasmin activity towards a range of *NN*-dimethyl-*p*-phenylenediamine concentrations was determined in the presence of ascorbate a straight-line plot of $1/v$ against $1/[S]$ was obtained (Fig. 4). This provides direct evidence for the hypothesis that curved plots are caused by the presence of first product and that caeruloplasmin has only one type of active site. Values obtained from the plot are $V_{\max}$, 5.26 e/Cu atom per min and K_m , 203 μM -*NN*-dimethyl-*p*-phenylenediamine.

A similar method was utilized in the work described below.

Determination of the kinetic parameters of a series of caeruloplasmin substrates

Preliminary experiments. In preliminary studies with ascorbate absent from the reaction medium the activity of caeruloplasmin against low concentrations of all the *p*-phenylenediamines and aminophenols and some of the polyphenols tested increased with reaction time, as noted for *NN*-dimethyl-*p*-phenylenediamine (Curzon, 1967). This did not occur in the presence of ascorbate, when activities remained constant with respect to time. When substrates were oxidized to products with spectra in the visible region (e.g. all the *p*-phenylenediamines) 50 μM -ascorbate prevented the formation of spectrophotometrically detectable products.

Plots of $1/v$ against $1/[S]$ in the presence of ascorbate were straight lines for all 37 substrates studied.

Fig. 4. Oxidation of *NN*-dimethyl-*p*-phenylenediamine by caeruloplasmin in the presence of ascorbate

A plot of $1/v$ against $1/[S]$ is shown. The caeruloplasmin concentration was 0.11 μM and the initial ascorbate concentration was 50 μM in all experiments. Other conditions were as described in the Materials and Methods section. Caeruloplasmin activity was measured by the rate of O_2 uptake.

This strengthens the hypothesis that curved plots obtained with *NN*-dimethyl-*p*-phenylenediamine in the absence of ascorbate are due to the first product.

Table 1 gives the K_m and $V_{\max}$ values determined from the reciprocal plots for the various substrates. The pK values for compounds investigated with aromatic amino groups are given, as not all of these substances are in the same ionic form at pH 5.5 and some occur as a mixture of ionic forms at this pH.

Variation in $V_{\max}$ values. Whereas the K_m values vary from 20 μM to 280 000 μM , i.e. 10^4 -fold, the $V_{\max}$ values vary only eightfold, from 1.3 to 10.8 e/Cu atom per min. This is illustrated by a plot of $\log V_{\max}$ against $\log K_m$ for the 37 compounds for which values were determined (Fig. 5a). Thus a striking lack of effect on $V_{\max}$ of the position and nature of substituents at the aromatic ring of substrates is indicated. Even alteration of the ring from a benzene to an indole nucleus has little effect. $V_{\max}$ values of the 20 *p*-amino compounds studied varied only about twofold

Fig. 5. Relationships between K_m and V_{max} for caeruloplasmin substrates

(a) Plot of $\log K_m$ against $\log V_{max}$ for all 37 compounds for which both values were determined. Mean $\log V_{max}$ is 0.639 (s.d. ± 0.195) e/Cu atom per min. The correlation between $\log K_m$ and $\log V_{max}$ was not significant ($r = 0.003$). (b) Similar plot for the 20 *p*-amino compounds only. Mean $\log V_{max}$ is 0.687 (s.d. ± 0.100) e/Cu atom per min. The correlation between $\log K_m$ and $\log V_{max}$ was significant ($r = -0.538$, $P < 0.05$).

(Fig. 5b). The small variation of V_{max} for this group of compounds showed no significant relationship with pK values or with the size, position or electronic character of their substituents. However, there was a significant negative correlation between $\log K_m$ and $\log V_{max}$.

Substrates without *p*-amino groups showed somewhat larger variation of V_{max} , although eight out of 17 of these values were within the range found for the substrates containing *p*-amino groups. A number of generalizations about the relationship between structure and V_{max} can be made for the substrates without *p*-amino groups. Thus the catecholic substrates catechol, 3,4-dihydroxyphenethylamine, 4-methyl-

catechol and pyrogallol have rather high V_{max} values (6.81–10.80 e/Cu atom per min), although the catechols L-adrenaline and L-noradrenaline, which also have a side-chain hydroxyl group, have relatively low values of 2.29 and 2.71 e/Cu atom per min respectively. Similarly, in the group of 5-hydroxyindoles, 5-hydroxytryptophol, which also has a side-chain hydroxyl group, has a low V_{max} of 2.87 e/Cu atom per min and the value for 5-hydroxytryptamine is well within the range found for the *p*-amino compounds. The lowest values found were those of *o*-phenylenediamine and of 5-hydroxyindol-3-ylacetic acid and 5-hydroxytryptophan, the two 5-hydroxyindoles with charged carboxyl groups.

Variation in K_m values. Unlike V_{max} , K_m values of caeruloplasmin substrates vary markedly. Values for *p*-amino compounds are generally lower than those of the other classes of substances, with little overlap, only three of the former group having K_m values greater than $2000\ \mu\text{M}$ and only one of the latter having a K_m value less than $2000\ \mu\text{M}$. Even within the class of *p*-amino compounds K_m values varied over a 600-fold range. The two substrates with the lowest K_m values were the only two *p*-amino compounds containing two benzene rings, namely *N*-(*p*-methoxyphenyl)-*p*-phenylenediamine and *N*-phenyl-*p*-phenylenediamine.

The work of Gunnarsson *et al.* (1970) on *p*-phenylenediamines indicates that protonated and unprotonated forms of the same substance may both be caeruloplasmin substrates, each with different K_m values. Therefore K_m values of some of the amino compounds obtained in the present study could be composite. Comparisons of *pK* and $\log K_m$ values (Table 1) also suggest that caeruloplasmin does not oxidize substrates exclusively in either their protonated or their unprotonated form. Thus, for example, *NN*-diethyl-*p*-phenylenediamine and 2,5-dichloro-*p*-phenylenediamine have comparable K_m values, although at pH 5.5 an amino group of the former is effectively completely protonated whereas those of the latter are completely unprotonated.

According to the calculations of Pettersson (1970*b*) and Gunnarsson *et al.* (1970), caeruloplasmin substrates have particularly high energies of their highest occupied molecular orbital. They obtained a significant correlation between energy of the highest occupied molecular orbital and $\log K_m$ by using values for four *NN*-dimethyl-*p*-phenylenediamine species and four other substrates. It appears, however, that factors other than energy of the highest occupied molecular orbital may also influence the K_m . Thus negatively charged carboxyl groups on the side chain of indoles are associated not only with low V_{max} , (see above) but also with high K_m values (Table 1). It is unlikely that this results from an effect on ring electrons, as the charged groups are all separated from

the indole ring by at least one methylene group. A similar relationship occurs among the catecholamines, the K_m for 3,4-dihydroxyphenylalanine being much lower than that for 3,4-dihydroxyphenylacetic acid.

Effect of pH on kinetic parameters of caeruloplasmin substrates. The finding of particularly low V_{max} , and high K_m values for substrates such as 5-hydroxyindol-3-ylacetic acid having ionized carboxyl groups was investigated further. Table 2 shows the kinetic parameters of 5-hydroxyindol-3-ylacetic acid and 5-hydroxytryptamine at various pH values. Whereas the K_m for the latter altered only slightly between pH 5.5 and pH 4.0, the V_{max} fell sharply as pH declined. The latter finding agrees with results obtained with *NN*-dimethyl-*p*-phenylenediamine and its first product (Gunnarsson *et al.*, 1970). In contrast with the above results, the K_m for 5-hydroxyindol-3-ylacetic acid fell sharply with decreasing pH but V_{max} remained relatively constant. As the pH decreased the K_m values of 5-hydroxytryptamine and 5-hydroxyindol-3-ylacetic acid converged. The significance of these relationships is considered in the Discussion section.

Discussion

Results strongly indicate that the curved plots of $1/v$ against $1/[S]$ previously obtained with caeruloplasmin result from the presence of products of caeruloplasmin action that are themselves also substrates and not from the presence of different active sites either on the same (Curzon, 1967; Walaas *et al.*, 1967) or different (Løvstad, 1969) enzyme molecules. Although other examples are known of enzymes that react further with their first products, in the case of caeruloplasmin this process is obscured by non-enzymic equilibria between substrates and products.

Reciprocal plots for the oxidation of *NN*-dimethyl-*p*-phenylenediamines by caeruloplasmin are straight lines if measurements are made at very low concentrations of the first product and become more curved as measurements are made at higher first-product concentrations. The different kinetic parameters for

Table 2. Variation with pH of kinetic parameters of 5-hydroxyindol-3-ylacetic acid and 5-hydroxytryptamine with caeruloplasmin

K_m and V_{max} values were determined as described in the Materials and Methods section, except that buffers of the pH values shown were used.

pH	5-Hydroxyindol-3-ylacetic acid		5-Hydroxytryptamine	
	V_{max} , (e/Cu atom per min)	K_m (mM)	V_{max} , (e/Cu atom per min)	K_m (μM)
5.5	1.50	8.34	5.68	908
5.0	1.97	6.96	4.59	744
4.5	1.34	2.72	3.31	795
4.0	1.48	1.50	2.70	683

NN-dimethyl-*p*-phenylenediamine reported by Løvstad (1969) for caeruloplasmin from neonatal, adult and pregnant sources and the different values reported by other authors (Curzon, 1967; Walaas *et al.*, 1967) may well be due to measurements being made at different first-product concentrations.

The straight-line plots obtained complement the mathematical analysis made by Pettersson (1969, 1970*a,b*) and by Pettersson & Pettersson (1969), who showed that reaction rates at various concentrations of *NN*-dimethyl-*p*-phenylenediamine and its products were consistent with *NN*-dimethyl-*p*-phenylenediamine and its first product being substrates and the second product being an inhibitor. Huber & Frieden (1970) have proposed a different explanation for the curved reciprocal plot for Fe^{2+} , which, unlike that obtained with other substances, occurs even in the presence of ascorbate. Here the curvature can hardly be due to the product Fe^{3+} also being a substrate, and it was suggested to result from Fe^{2+} binding both to an active and to an activating site.

Mathematical analysis of spectrophotometric results as used by Pettersson (1969, 1970*a,b*) is difficult to apply to many substrates, as most of their free-radical products that have been investigated are much less stable than the first product of *NN*-dimethyl-*p*-phenylenediamine oxidation (Michaelis *et al.*, 1939). The method of determining caeruloplasmin activities in the presence of ascorbate described in the present study can, however, readily be applied to many substrates. The finding, obtained by using this method, that V_{max} values varied only over a narrow range suggests that during cycling of the enzyme a common and rate-limiting change occurs relatively independently of substrate structure. This rate-limiting change could perhaps be identical with that responsible for the slow reoxidation of the enzyme after its reduction by substrate (Osaki & Walaas, 1967). This was not due to slow dissociation of the enzyme-product complex, as the product (Fe^{3+}) was removed as a transferrin complex before reoxidized enzyme was detectable. A slow conformation change before reoxidation rather than a slow reoxidation step is suggested by the much faster reoxidation of enzyme after storage in the reduced state (Broman *et al.*, 1963).

Osaki & Walaas (1967) obtained a velocity of the limiting step with Fe^{2+} as substrate that was much higher than any velocity found in the present study. Huber & Frieden (1970), however, showed that this was consistent with Fe^{2+} binding to both activating and active sites, and obtained a rate constant for the rate-limiting step for the unactivated enzyme with Fe^{2+} as substrate of 0.17 s^{-1} , which is equivalent to a V_{max} of 6.8 e/Cu atom per min, a value well within the range for the other substrates (Table 1).

The lowest V_{max} values found were for 5-hydroxy-

indol-3-ylacetic acid and 5-hydroxytryptophan, both of which have ionized side-chain carboxyl groups at pH 5.5. Although in general the V_{max} for caeruloplasmin substrates falls as pH decreases below 5.5, the V_{max} for 5-hydroxyindol-3-ylacetic acid remained relatively constant. This suggests that the low values for 5-hydroxyindol-3-ylacetic acid and 5-hydroxytryptophan are caused by their ionized carboxyl groups, the influence of which decreases as they become protonated. Thus as the pH decreases the value for 5-hydroxyindol-3-ylacetic acid converges with that of 5-hydroxytryptamine, which is structurally similar to the former compound but has no side-chain carboxyl group. Charged carboxyl groups possibly decrease the rate of the rate-limiting step.

Previous observations of very low velocities of caeruloplasmin action in comparison with most other enzymes is confirmed. If this slowness is due to a rate-limiting step relatively independent of substrate structure, then it is improbable that rates with any substrates would be much greater. The possibility, however, of physiologically significant enzyme properties of caeruloplasmin cannot be discounted, as its concentration in human plasma is high, averaging 24 mg/100 ml according to Scheinberg *et al.* (1957), and there is evidence for physiological oxidation of Fe^{2+} by caeruloplasmin (Roesser *et al.*, 1969; Osaki & Johnson, 1969).

The K_m values found do not indicate rigid steric restrictions for the binding of caeruloplasmin substrates. Thus the completely N-substituted *NNN'*-tetramethyl-*p*-phenylenediamine has a K_m comparable with that of *p*-phenylenediamine or partially N-substituted *p*-phenylenediamine derivatives. Similarly, *p*-phenylenediamine derivatives with particularly large substituent groups on their N atoms do not necessarily have high K_m values, e.g. *N*-(*p*-methoxyphenyl)-*p*-phenylenediamine had the smallest K_m found. Again, the K_m values of *o*-, *m*- and *p*-diamines, aminophenols and dihydric phenols are in the order not of steric but of electronic similarity, i.e. $K_m(\textit{para}) < K_m(\textit{ortho}) < K_m(\textit{meta})$. These observations, and the demonstration by Gunnarsson *et al.* (1971) of the substrate properties of *NN*-dimethylaniline, which has only one group attached to the benzene ring, all suggest that binding of the substrate to the enzyme is by the substrate rings (possibly the π electrons) rather than by ring substituents. Presumably substituents influence binding of substrates to the enzyme by altering ring-electron densities or energies. Thus ring-substituted *p*-phenylenediamine derivatives with the strongly electron-withdrawing 2-nitro and 2-sulphonic acid groups have much higher K_m values than those of other 2-substituted *p*-phenylenediamine derivatives.

The lack of obvious steric restraints on substrates is in striking contrast with the marked steric restrictions on inhibitors (Curzon & Speyer, 1968), and

suggests that substrates and inhibitors bind either to different enzyme intermediates or to different sites. This is consistent with the lack of inhibitors showing competitive kinetics, except for cyanide, which nevertheless appears not to have a competitive mechanism of inhibition (Speyer & Curzon, 1968).

Substrates not grossly different in size and only differing in regions separated from their aromatic rings by a methylene group, and which are likely therefore to have comparable characteristics of their ring electrons, may have strikingly different K_m values. Thus 5-hydroxyindol-3-ylacetic acid and 5-hydroxytryptophan have much higher values than has 5-hydroxytryptamine, and among the catechols dopa and 3,4-dihydroxyphenylacetic acid have much higher values than has 3,4-dihydroxyphenethylamine. That the negatively charged side-chain carboxyl groups of the above substrates with relatively high K_m values are probably responsible for these values is indicated by the relationships between the K_m values of 5-hydroxyindol-3-ylacetic acid and 5-hydroxytryptamine and pH (Table 2). Thus whereas the K_m for the latter remains relatively constant from pH 5.5 down to pH 4.0 the K_m for the former falls sixfold over the same pH range. This suggests that negatively charged carboxyl groups interfere with substrate binding, possibly by repulsion of a negative charge on the enzyme near the active site, which would be expected to decrease with decreasing pH due to protonation of negatively charged groups.

We thank Mr. C. W. Amery and Dr. L. Williams for various compounds tested as substrates, the Blood Products Laboratory, Lister Institute for outdated plasma from which caeruloplasmin was purified, and Dr. C. R. J. Ruthven and Dr. M. Sandler for neonatal serum. The work was supported by a grant from the Medical Research Council.

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