The Inhibition of Caeruloplasmin by Azide

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1. The inhibition of the oxidase activity of caeruloplasmin by azide was investigated at 25° and 7.5°. 2. The inhibition is reversible on dilution or Sephadex treatment, indicating a caeruloplasmin–azide complex. 3. The enzyme is protected against azide inhibition by chloride, acetate or EDTA, the last-named acting not by chelation but by a non-specific effect similar to that of acetate. 4. Lineweaver–Burk plots with different concentrations of azide are parallel. This may occur either when the enzyme–substrate complex or when a subsequent intermediate structure of the enzyme forms the inhibited complex. 5. At 7.5° inhibition may be shown not to occur until after the initial reaction of enzyme with substrate. 6. At 7.5°, the inhibition is of the mutual-depletion type, inhibitory concentrations of azide being comparable with the concentration of caeruloplasmin. It is shown that the binding of a single azide group completely inhibits a caeruloplasmin molecule. 7. An arrangement of the four valence-changing copper atoms of caeruloplasmin is proposed in which they are so close together in the cuprous form that reoxidation may occur by the simultaneous transfer of four electrons from the copper atoms to a single oxygen molecule.

Caeruloplasmin, the blue copper-containing protein of plasma, has weak oxidase activity against a variety of polyphenols and aromatic polyamines. The investigation of the oxidase properties can lead to information on the binding of the copper atoms as they are an essential part of the active centre (Morell & Scheinberg, 1958), and the specific nature of their binding is related to oxidase activity (Broman, Malmström, Aasa & Vånnagard, 1962). In the present paper a study of the inhibition of the oxidase activity of caeruloplasmin by azide is described. The inhibition has two notable characteristics. First, concentrations of azide necessary for inhibition are comparable with concentrations of caeruloplasmin and thus a mutual-depletion system occurs (Webb, 1963). Secondly, parallel Lineweaver–Burk plots are obtained.

Caeruloplasmin has eight copper atoms/molecule, of which four are permanently in the cuprous state and four are in the cupric state in the resting enzyme (Broman *et al.* 1962) but are reduced to cuprous copper when the enzyme acts on substrate (Broman, Malmström, Aasa & Vånnagard, 1963). The cuprous atoms are then reoxidized to the cupric form by molecular oxygen according to the formal equation:

It has been found that azide inhibition is explicable in terms of a caeruloplasmin structure in which the four valence-changing copper atoms are close

together when in the cuprous state and are simultaneously reoxidized by an oxygen molecule.

A preliminary report on part of this work has appeared (Curzon, 1965a).

THEORY

Significance of parallel Lineweaver–Burk plots. In the investigation described below it is shown that parallel Lineweaver–Burk plots are obtained for the oxidation of *N,N*-dimethyl-*p*-phenylenediamine by caeruloplasmin in the presence of different amounts of azide. Parallelism has previously been shown to be consistent with anti-competitive inhibition in which the inhibitor combines with the enzyme–substrate complex (D. Burk, unpublished work cited by Ebersole, Guttentag & Wilson, 1944; Friedenwald & Maengwyn-Davies, 1954). In the following treatment parallel plots are interpreted in terms of the inhibitor's combining either with the enzyme–substrate complex or with a form of the enzyme produced by its interaction with substrate. The reaction between caeruloplasmin and substrate has been considered by Broman *et al.* (1963) and by Peisach & Levine (1963) to occur as follows:

where E^{2+} and E^+ represent the oxidized and reduced forms of the enzyme, S the substrate and S^+ the monoradical oxidation product. If it is assumed that the one-electron change from E^{2+}S to E^+S^+ is very rapid (Broman *et al.* 1963) and that a change in the binding of the valence-changing copper atoms is necessary before the azide may form an inhibited complex, then a simplified version of the above

reaction scheme and regeneration of oxidized enzyme may be considered:

It is not necessary to consider $[\text{O}_2]$ as Broman *et al.* (1963) showed that the reoxidation rate is nearly independent of this except under limiting conditions.

In these circumstances it may be shown that when the inhibitor binds $\text{E}^+ \text{S}^+$, forming a complex with dissociation constant K_i , then:

$$\frac{1}{v} = \frac{K_m}{[\text{S}]} \frac{1}{V} + \frac{1}{V} + \frac{[\text{I}]}{K_i V} + \frac{k_{+2}}{KV} \quad (1)$$

When the inhibitor binds E^+ , forming a complex with dissociation constant K'_i , then:

$$\frac{1}{v} = \frac{K_m}{[\text{S}]} \frac{1}{V} + \frac{1}{V} + \frac{k_{+2}}{KV} \left(1 + \frac{[\text{I}]}{K'_i} \right) \quad (2)$$

Both of these equations give rise to parallel Lineweaver-Burk plots.

Mutual-depletion inhibition kinetics. In the derivation of the above rate equations it has been assumed that $[\text{I}]$, the concentration of free inhibitor, equals $[\text{I}_T]$ where $[\text{I}_T]$ is the total concentration of inhibitor. When inhibition occurs with $[\text{I}_T]$ of the same order as enzyme concentration then $[\text{I}]$ is significantly lower than $[\text{I}_T]$ and what Webb (1963) has called a mutual-depletion system occurs. In this case it may be shown that the rate equations are modified, the terms $[\text{I}]/K_i$ and $[\text{I}]/K'_i$ being replaced by $[\text{I}_T]/(K_i + [\text{E}^+ \text{S}^+])$ and $[\text{I}_T]/(K'_i + [\text{E}^+])$ respectively. Thus, in a mutual-depletion system, the above inhibition mechanisms no longer necessarily imply parallel Lineweaver-Burk graphs as at constant total inhibitor concentration $1/V$ is equal to $1/[\text{S}]$ plus a term that is dependent on both $[\text{I}_T]$ and $[\text{S}]$. Lineweaver-Burk graphs may therefore deviate from parallelism in a manner dependent on the relative importance of the various terms in the rate equation.

MATERIALS

Caeruloplasmin. Human caeruloplasmin from the American Red Cross was used (batch no. 1995). This was stored in 10 ml. portions at -25° . Before use, these were rapidly thawed in a water bath at 45° and dialysed against 0.1 M- or 0.01 M-NaCl at 4° . In the absence of chloride caeruloplasmin is unstable. The very light white precipitate that formed during storage could be removed only with difficulty by centrifugation. It was therefore removed by ultrafiltration under pressure in a stainless-steel ultrafiltration apparatus with a group-6 membrane (Membranfiltergesellschaft G.m.b.H., Göttingen, Germany). Any non-caeruloplasmin copper was removed by passage through a column of 100–200-mesh Chelex 100 chelating resin previously equilibrated with 0.1 M- or 0.01 M-NaCl and the resultant clear blue solution stored at 4° and used within a few days. $E_{550}^{1\text{cm}}$ was 1.2–1.6 and the E_{605}/E_{280} ratio was 0.040, indicating about 90% purity (Scheinberg & Morell, 1957; Curzon & Vallet, 1960; Deutsch, Kasper & Walsh, 1962).

NN-Dimethyl-p-phenylenediamine dihydrochloride. This

was obtained as a cream-coloured powder from Kodak Ltd. (Kirby, Liverpool). Working solutions were made freshly from a 0.1 M solution stored at -25° .

NN-Dimethyl-p-phenylenediamine sulphate. This compound was obtained from Kodak Ltd. and purified by dissolving it in methanol containing a little 5% SO_2 , removing the insoluble residue by filtration and precipitating the compound with ether. Alternatively, the hydrochloride of the base was dissolved in water, made alkaline and the free base extracted by ether, crystallized twice, converted into a 0.1 M solution of the sulphate by addition of the calculated amount of dil. H_2SO_4 and stored at -25° .

Sodium azide. This was recrystallized from water, washed with ethanol and ether and air-dried. The purity according to the method of Fiegl & Chargaff (1928) was 99–100%. Solutions were made up freshly as required.

METHODS

Copper determination. The method of Rice (1960) was used.

Caeruloplasmin oxidase-activity determination. NN-Dimethyl-p-phenylenediamine was oxidized by caeruloplasmin and E_{550} of the red reaction product measured. Except where stated otherwise all determinations were done in the presence of EDTA to avoid difficulties of interpretation due to the enhancement of oxidase activity by trace amounts of iron (Curzon & O'Reilly, 1960; Curzon, 1961; Levine & Peisach, 1963). In general, the following method was used for the determination of oxidase activity at 25° . A 1.0 ml. sample of 25 mM-acetate buffer, pH 5.5, containing EDTA ($10\mu\text{M}$) was pipetted into a standard 1 cm. cell held at 25° in a Unicam SP.500 constant-temperature cell housing. Then 0.5 ml. of either water or a sodium azide solution was added and also 0.5 ml. of NN-dimethyl-p-phenylenediamine dihydrochloride solution previously brought to pH 5.5 with 10 M-NaOH. After temperature equilibration 0.5 ml. of a caeruloplasmin solution in 0.1 M-NaCl diluted with water and held at 25° was added. The E_{550} was measured against a blank at 1 min. intervals for 10 min. and activity expressed as $E_{550}^{1\text{cm}}/\text{min}$.

The following modifications were necessary when determining activity at 7.5° . As the dilution of the stock caeruloplasmin solution used was much less at 7.5° than at 25° , stock caeruloplasmin solution was dialysed against 0.01 M-NaCl instead of 0.1 M-NaCl, so that the final chloride concentration was not high enough to inhibit the oxidase activity. If the hydrochloride of the substrate had been used, as at 25° , the chloride so introduced would have caused significant inhibition (Table 1). Therefore NN-dimethyl-p-phenylenediamine sulphate was used, sulphate previously having been found under different conditions to affect caeruloplasmin activity much less than chloride (Curzon, 1960). This was confirmed at 7.5° (Table 1). All determinations at 7.5° were made with 1.0 ml. of 25 mM-acetate buffer, pH 5.5, containing EDTA ($35\mu\text{M}$). The concentration of EDTA used in experiments at 7.5° was greater than in the experiments at 25° to form complexes of the greater amounts of contaminant metal introduced with the higher substrate concentrations. E_{550} was measured at 30 sec. or 1 min. intervals with a Unicam SP.500 spectrophotometer, except for the experiment indicated in Fig. 5 in which an SP.800 spectrophotometer was used with continuous recording.

Table 1. *Effect of chloride and sulphate on the oxidase activity of caeruloplasmin against NN-dimethyl-p-phenylenediamine sulphate at 7.5°*

The incubation mixture contained 0.5 ml. of 5 mM-substrate, 0.5 ml. of caeruloplasmin ($E_{505}^{1\text{cm}}$ 0.40) in 3.3 mM-NaCl and 1.0 ml. of 25 mM-acetate buffer, pH 5.5, containing EDTA (35 μM), in a final volume of 2.5 ml.

Addition to reaction mixture	$E_{505}^{1\text{cm}}/\text{min.}$
None	0.254
NaCl (2.0 mM)	0.248
NaCl (4.0 mM)	0.208
Na ₂ SO ₄ (0.4 mM)	0.274
Na ₂ SO ₄ (4.0 mM)	0.274
Na ₂ SO ₄ (20.0 mM)	0.212

In preliminary experiments at 7.5° it was found that the activity of the caeruloplasmin solution increased for about 30 min. after dilution of the stock solution of caeruloplasmin in 0.01 M-NaCl with water at 7.5°. Therefore the diluted caeruloplasmin solution was preincubated for 30–60 min. at 7.5° before addition to the incubation mixture.

Variation of duplicate from the mean averaged $\pm 1\%$.

RESULTS

Inhibition of caeruloplasmin by azide and its reversal. With an incubation mixture containing 2.0 ml. of 50 mM-acetate buffer, pH 5.5, containing EDTA (10 μM), 1 ml. of 5 mM-NN-dimethyl-p-phenylenediamine dihydrochloride, 1 ml. of water or 250 μM -azide and 1 ml. of a solution made by diluting caeruloplasmin in 0.1 M-sodium chloride (undiluted solution having $E_{505}^{1\text{cm}}$ 1.2) 30-fold in water, it was found on 10 min. incubation at 25° that the azide caused 88% inhibition of the oxidase activity. Inhibition was measured by total product formation. This gave slightly erroneous values as equilibration was not instantaneous (see Fig. 2). Each incubation mixture was then immediately diluted fivefold with the acetate-EDTA buffer, and 1.0 ml. taken for the determination of oxidase activity and added to an azide-free incubation mixture so that the azide concentration was 2 μM and the substrate concentration was almost identical with that in the initial experiment. The buffer concentration was 29 mM, which, though higher than that in the first incubation mixture, only decreased the azide inhibition by 2–3% (Fig. 1). After incubation for 30 min. to obtain an accurately measurable amount of product only 35% inhibition was found. This was identical with the inhibition obtained in the presence of 2 μM -azide. The presence of some product formed during the initial incubation would not be expected to affect activity (G. Curzon, unpublished work). Hence the inhibition of caeruloplasmin by azide is reversible by dilution.

Fig. 1. Effect of variation of carboxyl and chloride concentrations on the inhibition of caeruloplasmin oxidase activity by azide at 25°. The reaction mixtures contained caeruloplasmin (0.33 μM), NN-dimethyl-p-phenylenediamine hydrochloride (1 mM), sodium azide (4 μM) and chloride contributed by substrate and enzyme solution (2.3 mM). The following experiments are included: (a) $\circ$, Acetate buffer kept constant at 10 mM and EDTA varied between 0 and 40 mM; (b) Δ , EDTA kept constant at 4 μM and acetate buffer varied between 10 mM and 0.2 M; (c) $\square$, acetate buffer and EDTA kept constant at 10 mM and 4 μM respectively and NaCl varied between 2.3 mM and 60 mM. In (a) and (b) percentage inhibition was plotted against total ionized carboxyl, calculated by using pK values from Bjerrum, Schwarzenbach & Sillen (1957). In (c) percentage inhibition was plotted against chloride. General conditions were as described in the Methods section.

In another experiment, instead of dilution of the first incubation mixtures, 0.5 ml. samples were applied to identical columns (8.4 cm. $\times$ 0.7 cm. diam.) of Sephadex G-25 previously equilibrated with the acetate-EDTA buffer and were eluted with this buffer, 2.5 ml. of each eluate being collected. The red substrate-oxidation product was visibly retarded behind the collected fraction. Oxidase activity was again determined in 1.0 ml. portions of eluate, the final dilution being the same as in the previous experiment. Azide inhibition was reversible, 80% of the original oxidase activity being recovered whether azide was originally present or not. If azide inhibited the activity of caeruloplasmin by the removal of copper as copper azide then this would have been retained on Sephadex and the inhibition would not have been reversed. Reversibility under these conditions indicates that

when azide inhibits caeruloplasmin it is bound to the protein, presumably via its copper atoms.

Effect of ionic environment on the inhibition of caeruloplasmin by azide at 25°. The effect of altering the ionic environment on the inhibition of caeruloplasmin by azide was studied. Fig. 1 shows the effects on inhibition by $4\mu\text{M}$ -azide of the variation of acetate buffer, EDTA and chloride concentrations. Inhibition decreased as the concentrations of the above substances were increased. When the concentrations of acetate and EDTA were expressed as total ionized carboxyl all the points obtained by varying either lay on the same curve. This indicates that EDTA influences azide inhibition by a non-specific effect similar to that of acetate rather than by chelation. At the lowest buffer concentration used azide inhibition was only slightly greater in the absence of EDTA than in the presence of $4\mu\text{M}$ -EDTA. In subsequent experiments the incubation mixture contained 10mM-acetate buffer and $4\mu\text{M}$ -EDTA, these being the lowest concentrations for adequate buffering and chelation of contaminant traces of metal. The effect of raising chloride concentration was to decrease azide inhibition to a greater extent than the same concentration of carboxyl ion.

Effect of time on the inhibition of caeruloplasmin by azide at 25°. Fig. 2 shows that inhibition by azide does not occur instantaneously but that it is necessary for about 30sec.–1 min. to elapse before it

commences. This time lag was observed consistently.

Kinetics of inhibition at 25°. The effect of varying the concentration of azide on activity at various substrate concentrations was determined and expressed in the Lineweaver–Burk form (Fig. 3). Parallel Lineweaver–Burk graphs were obtained. Parallel Lineweaver–Burk graphs have previously been shown for the inhibition of caeruloplasmin by acetate or chloride (Curzon, 1960). A 50% inhibition (after extrapolation to infinite substrate concentration) occurs with $3\mu\text{M}$ -azide and $0.38\mu\text{M}$ -caeruloplasmin.

Effect of time on the oxidation of NN-dimethyl-p-phenylenediamine by caeruloplasmin at 7.5°. At 25° the formation of the red oxidation product of NN-dimethyl-p-phenylenediamine in the absence of azide is linear with respect to time after a lag period of less than 1 min. However, at 7.5° a well-defined biphasic effect occurs (Fig. 4), an initial very rapid colour formation being followed by a slower linear increase of colour with time. The amount of product formed in the initial period may be determined by extrapolating the linear part of the graph back to zero time. The initial colour increased linearly with caeruloplasmin concentration and also increased as the substrate concentration was increased.

Effect of time on azide inhibition at 7.5°. Fig. 4 shows that up to 4 min. is necessary for the cycling caeruloplasmin and azide to equilibrate with each

Fig. 2. Time-course of inhibition of caeruloplasmin oxidase activity by azide at 25°. The reaction mixtures contained caeruloplasmin ($0.38\mu\text{M}$) and NN-dimethyl-p-phenylenediamine hydrochloride (1mM), together with the following concentrations of azide: $\circ$, none; Δ , $1\mu\text{M}$; $\square$, $3\mu\text{M}$; $\bullet$, $6\mu\text{M}$. General conditions were as described in the Methods section.

Fig. 3. Effect of azide on caeruloplasmin oxidase activity at various substrate concentrations at 25°. The reaction mixtures contained caeruloplasmin ($0.38\mu\text{M}$), together with the following concentrations of azide: $\circ$, none; Δ , $1\mu\text{M}$; $\square$, $3\mu\text{M}$; $\bullet$, $6\mu\text{M}$; $\blacktriangle$, $12\mu\text{M}$. General conditions were as described in the Methods section.

Fig. 4. Time-course of inhibition of caeruloplasmin oxidase activity by azide at 7.5°. The reaction mixtures contained caeruloplasmin (6 μ M) and *NN*-dimethyl-*p*-phenylenediamine sulphate (1mM), together with the following concentrations of azide: ○, none; Δ, 2 μ M; □, 4 μ M; ●, 6 μ M. General conditions were as described in the Methods section.

Fig. 5. Lack of effect of preincubation of caeruloplasmin with azide on the time-course of inhibition at 7.5°. The following mixtures were preincubated for 10 min.: (A) and (B) 0.5 ml. of 31.5 μ M-caeruloplasmin, 1.0 ml. of 25mM-acetate buffer, pH 5.5, containing EDTA (35 μ M) and 0.5 ml. of water; (C) as above, but containing 0.5 ml. of 30 μ M-azide instead of 0.5 ml. of water. Then 0.5 ml. of 20mM-*NN*-dimethyl-*p*-phenylenediamine sulphate was added to (A) and (C), and 0.5 ml. of 20mM-*NN*-dimethyl-*p*-phenylenediamine sulphate containing azide (30 μ M) was added to (B). E'_{550} was recorded continuously, starting 15 sec. after the addition of substrate.

Fig. 6. Effect of azide on caeruloplasmin oxidase activity at various substrate concentrations at 7.5°. The reaction mixtures contained caeruloplasmin (6.8 μ M), together with the following concentrations of azide: ○, none; Δ, 2 μ M; □, 4 μ M; ●, 6 μ M. General conditions were as described in the Methods section.

other and strongly suggests that inhibition of the initial rapid colour formation does not occur. Preincubation of a mixture of caeruloplasmin, buffer and azide for 10 min. at 7.5° before the addition of substrate did not significantly affect the time-course of inhibition, thus indicating that formation of the inhibitory azide-caeruloplasmin complex does not commence until reaction of enzyme with substrate has occurred (Fig. 5).

Effect of substrate concentration on azide inhibition at 7.5°. Fig. 6 shows Lineweaver-Burk plots for inhibition at 7.5°. Inhibition has been calculated by using the linear portions of activity-time graphs. The graphs are approximately parallel, as at 25°.

Determination of the number of azide inhibition sites on each caeruloplasmin molecule. Fig. 4 shows that at 7.5° inhibition occurs at an azide concentration comparable with that of caeruloplasmin itself. Therefore a significant fraction of the azide present must be bound to the enzyme and mutual-depletion kinetics (Webb, 1963) should occur, the concentrations of both free enzyme and free inhibitor being mutually decreased. Myers (1952) showed that under these circumstances with non-competitive inhibition:

$$[I_{50}] = K_i + 0.5e \quad (3)$$

where $[I_{50}]$ is the inhibitor concentration for 50% inhibition and e is the concentration of inhibitor-binding sites. This equation is applicable to the azide inhibition of caeruloplasmin if results obtained at a substrate concentration sufficiently high to saturate effectively the enzyme are used and if under these conditions all the caeruloplasmin molecules are available to azide. Table 2 shows that

reasonably constant and positive values for K_i are obtained only when a single inhibitor-binding site/mol. is assumed.

By application of the graphical method of Easson & Stedman (1936) (Fig. 7) a single inhibitor-binding site/mol. can also be shown.

Table 2. Calculation of apparent K_i for azide inhibition of caeruloplasmin at 7.5° by using eqn. (3)

The substrate concentration was 4 mM. The caeruloplasmin concentration was calculated from the copper content, assuming 8 atoms of Cu/mol. The method of assay was as described in the Methods section. $[I_{50}]$ is the azide concentration for 50% inhibition. K_i values were calculated assuming one (A), two (B) and four (C) inhibitor-binding sites/mol.

Concn. of caeruloplasmin (μM)	$[I_{50}]$ (μM)	K_i (μM)		
		A	B	C
6.75	4.65	1.27	-2.10	-9.15
3.38	3.45	1.74	0.07	-3.31
1.69	2.30	1.44	0.61	-1.08
0.68	1.70	1.36	1.02	0.34

Fig. 7. Determination of number of azide inhibition sites/caeruloplasmin molecule. Inhibition was determined with various azide concentrations and *NN*-dimethyl-*p*-phenylenediamine sulphate (4 mM) at 7.5°, and with the following concentrations of caeruloplasmin: O, 6.7 μM ; Δ , 5.9 μM . General conditions were as described in the text. Results were plotted by the method of Easson & Stedman (1936). $[I_T]$ is the total concentration of azide and α the fractional activity in its presence. The concentrations of inhibitor-binding sites are given by the intercepts and correspond to 0.94 and 0.93 site/caeruloplasmin molecule. The apparent K_i values are given by the slopes and are 1.6 μM and 1.46 μM , in reasonable agreement with Table 2.

DISCUSSION

A series of parallel Lineweaver-Burk graphs with different inhibitor concentrations has been interpreted by D. Burk (unpublished work cited by Ebersole *et al.* 1944) and by Friedenwald & Maengwyn-Davies (1954) as indicating the combination of the inhibitor with the enzyme-substrate complex but not with the free enzyme. Parallel graphs may also be obtained if the inhibitor reacts with an intermediate form of the enzyme formed after the enzyme-substrate complex. Dixon & Webb (1964) have suggested an explanation of parallelism in terms of a special case of Briggs-Haldane kinetics in which the velocity constant for the formation of products is much larger than that for dissociation of the enzyme-substrate complex. In these circumstances a non-competitive inhibitor causes proportional changes in velocity and in K_m , and thus Lineweaver-Burk graphs are parallel. In the inhibition of caeruloplasmin oxidase activity by azide the initial step of product formation is unaffected by azide. Hence it seems likely that the parallel Lineweaver-Burk graphs here are not due to the mechanism of Dixon & Webb (1964) and that the enzyme-substrate reaction proceeds at least to enzyme-product formation before the inhibited complex is formed. It was previously found that parallel Lineweaver-Burk graphs were obtained when caeruloplasmin was inhibited by chloride (Curzon, 1960) and it was suggested that the Cl^- ion was attached to the copper of the caeruloplasmin molecule. As azide is a classical complex-forming agent for metals and as inhibition occurs at an azide concentration so low that other effects are unlikely, it is very probable that azide is bound to the caeruloplasmin copper. Now four of the eight caeruloplasmin copper atoms are available for exchange with ^{64}Cu in the presence of substrate (Scheinberg & Morell, 1957), these being probably the four copper atoms that become accessible on reduction to the cuprous state. It is suggested that these valence-changing copper atoms are also made accessible or bind strongly to azide after reduction by substrate.

Mutual-depletion kinetics, which have been shown for azide inhibition at 7.5°, may in principle be demonstrated for any enzyme inhibition but in almost all cases the necessary enzyme concentration is so high that this is not technically possible. Most reported examples of mutual-depletion inhibition, such as that occurring with certain cholinesterase inhibitors, have involved substances with a very high enzyme affinity so that a conveniently low enzyme concentration could be used.

Caeruloplasmin is a relatively easy enzyme with which to show mutual-depletion inhibition as its low substrate turnover (Broman, 1964; Peisach &

Levine, 1965) enables kinetic study at high enzyme concentration. Thus the exhibition by caeruloplasmin of parallel Lineweaver-Burk plots and mutual-depletion inhibition may be related to two properties of caeruloplasmin, namely the availability of some of its copper atoms in the presence of substrate and the low rate of substrate turnover.

The finding that only one azide group is needed to completely inhibit a caeruloplasmin molecule is significant if the assumption is made that all the azide present is available to enzymically active caeruloplasmin molecules and vice versa. Though the pK of hydrazoic acid is about 4.8 and thus at pH 5.5 azide is only partially dissociated, this has no effect on its eventual availability for formation of the inhibited complex since azide inhibition was unaffected by changing the pH from 5.2 to 6.0 (Holmberg & Laurell, 1951). Also, azide is not likely to be made unavailable to caeruloplasmin by binding with extraneous metal, as EDTA, which is present in the system, may be expected to compete successfully with azide for non-caeruloplasmin metal. Conversely, proton-relaxation rate studies show that EDTA is not bound to caeruloplasmin copper (Blumberg, Eisinger, Aisen, Morell & Scheinberg, 1963), the partial apparent inhibition of caeruloplasmin by EDTA being caused by chelation not of copper but of activating contaminant iron (Curzon, 1961; Levine & Peisach, 1963). Further, it is unlikely that any of the copper present is derived from denatured caeruloplasmin molecules as this would have been removed by treatment with chelating resin. Therefore the experimental findings strongly indicate that one azide group completely inhibits a caeruloplasmin molecule by binding with valence-changing copper. This provides evidence for a structure of caeruloplasmin in which the four valence-changing copper atoms are close together so that binding of one azide group could affect all four copper atoms.

Mason (1957) has discussed the mechanism of regeneration of the oxidized form of four-electron-transfer copper oxidases by the reduction of molecular oxygen. The unlikelihood of the separate reoxidation of the cuprous atoms was pointed out as this would lead to the formation of active free radicals from the oxygen. These would attack the system at random, thereby being ineffectively utilized and possibly attacking enzyme molecules. Free radicals of oxygen have not been detected during caeruloplasmin action (Broman *et al.* 1963). Therefore Broman (1964) and Frieden, Osaki & Kobayashi (1965) have suggested that reoxidation may occur by the simultaneous transfer of four electrons to an oxygen molecule from four cuprous atoms. Fig. 8 indicates that if the cuprous atoms are close enough together for simultaneous electron transfer then the binding of an azide group to one

Fig. 8. Proposed arrangement of valence-changing copper atoms of caeruloplasmin when in the cuprous state and about to be oxidized by an oxygen molecule (ionic radius of Cu^+ from Pauling, 1945). The azide ion is shown in plan and elevation (dimensions from Llewellyn & Whitmore, 1947).

copper atom (or perhaps bridging two copper atoms) would be able to prevent reoxidation of the whole group. Broman (1966) has pointed out that, though caeruloplasmin is very similar to fungal laccase, the latter enzyme cannot simultaneously transfer four electrons to oxygen by the above mechanism, as of its four copper atoms (Mosbach, 1963) only two are valence-changing (Broman *et al.* 1962). Broman therefore suggests that reduced caeruloplasmin reacts with oxygen in two steps, which implies that the four valence-changing copper atoms are not necessarily as close together as shown in Fig. 8 but are in two pairs. On the other hand, though caeruloplasmin and fungal laccase are similar they are not identical either in spectrum (Mosbach, 1963) or in substrate specificity (Mosbach, 1963; Peisach & Levine, 1965) and have very different substrate turnover rates (Broman, 1964; Peisach & Levine, 1965). Thus the active centres of the two enzymes are not necessarily identical. However, if Broman's (1966) second hypothesis is correct, the complete inhibition of caeruloplasmin by a single azide group indicates that the two pairs of valence-changing copper atoms are themselves close together.

It is likely that the four permanently cuprous copper atoms of caeruloplasmin also play a part in the electron transfers that occur during its enzyme action and therefore are close to the valence-changing copper atoms, probably being connected to them by ligand bridges (Blumberg *et al.* 1963). Thus all eight copper atoms of caeruloplasmin are probably localized in a small area of the protein molecule, there being an active centre able to transfer four electrons. Aisen & Morell (1965) showed that when apocaeeruloplasmin was reconstituted by the addition of sub-optimum amounts of copper, only caeruloplasmin molecules containing

eight copper atoms and unchanged apoprotein were obtained. This is consistent with a single active centre and with all of the cluster of eight copper atoms being necessary for stabilization of the tertiary structure of the protein (Curzon, 1959, 1965b).

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