Inhibitors of Caeruloplasmin

By G. CURZON AND BARBARA E. SPEYER

*Department of Chemical Pathology, Institute of Neurology, The National Hospital,
Queen Square, London, W.C. 1*

(Received 30 January 1967)

1. A method is described by which substances inhibiting caeruloplasmin oxidase activity directly may be distinguished from those acting on stimulatory contaminant iron or on the product of enzyme action. 2. Many previously reported inhibitors, including saturated aliphatic carboxylates, hydrazines, 1,10-phenanthroline, borate and various psycho-active drugs, are found either not to act on the enzyme or to inhibit it only weakly. 3. A series of inorganic anions are compared as inhibitors. Anions such as azide and cyanide with strong copper-binding properties are the most effective inhibitors. There is a general inverse relationship between anion size and inhibitory power. Iodide is anomalous, the order of effectiveness of halides being $F^- > I^- \approx Cl^- > Br^-$. 4. Multidentate copper-chelating ligands have little inhibitory effect. 5. A group of substances containing

the structural unit $\begin{array}{c} | \\ >C=C-CO_2H \end{array}$, including fumarate and benzoate, cause inhibition. 6. Relative inhibitions by a series of mono-substituted benzoates are inversely related to molecular size. 7. Results are discussed in relation to earlier work on the disposition and function of the copper atoms of caeruloplasmin.

Caeruloplasmin, the blue copper-containing protein of plasma, shows oxidase activity *in vitro* towards aromatic compounds having two electron-releasing groups (Levine & Peisach, 1962; Peisach & Levine, 1965). This activity is associated with a cyclic valence change between the cupric and cuprous states in approximately half of the total of 8 copper atoms in the molecule. These copper atoms, when in the cupric state, are responsible for the intense blue colour and unusual electron-spin-resonance spectrum of the protein (Broman, Malmström, Aasa & Vänngård, 1962). As the valence-changing copper atoms account for the total paramagnetic signal, the remaining 4 copper atoms have been thought to be permanently in the cuprous state. It has been suggested that these cuprous atoms play a role in substrate binding (Broman, Malmström, Aasa & Vänngård, 1963). Information on the copper atoms and on the mechanism of oxidation may result from studies with inhibitors, especially those with metal-binding properties. For example, a kinetic analysis of azide inhibition has indicated that the 4 valence-changing copper atoms are close together when the enzyme is in the cuprous state (Curzon, 1966, 1967a).

A number of inorganic anions have been reported to inhibit caeruloplasmin, the strongest inhibitions being obtained with azide, cyanide, cyanate and thiocyanate (Holmberg & Laurell, 1951a,b; Curzon, 1960, 1966; Peisach & Levine, 1965). Borate has

been claimed to cause inhibition (Osaki, 1961). Carboxylate ions reported to inhibit include acetate and oxalate (Holmberg & Laurell, 1951b; Curzon, 1960; Humoller, Mockler, Holthaus & Mahler, 1960), maleate (Broman, 1958; Curzon, 1960), citrate (Holmberg & Laurell, 1951b; Osaki, McDermott & Frieden, 1964) and *o*-hydroxybenzoate (Broman, 1958, 1964). Also various metal ions, especially tervalent ones, inhibit possibly by causing protein aggregation (Curzon, 1960; Wald, Szajbel & Murawski, 1962; Peisach & Levine, 1965). Other claimed inhibitors include thiols (Holmberg & Laurell, 1951a; Zarafonetis & Kalas, 1960a), non-thiol chelating agents (Nakajima, 1959; Humoller *et al.* 1960; Frieden, 1962), hydrazines (Abood, Gibbs & Gibbs, 1957; Martin, Eriksen & Benditt, 1958; Hanson, Austin & Aprison, 1959; Zarafonetis & Kalas, 1960a,b), tryptophan metabolites (Aprison, Hanson & Austin, 1959), 3,4-dihydroxyphenylalanine (Hanson *et al.* 1959) and miscellaneous substances (Abood *et al.* 1957; Zarafonetis & Kalas, 1960a). Hanson *et al.* (1959) reported reduction of DPD^{+*} by ascorbic acid, cysteine and penicillamine, and Abood *et al.* (1957) considered that apparent inhibition by hydrazines was probably due to DPD^{+} reduction.

In studying caeruloplasmin inhibition it is important to exclude substances that decrease the

* Abbreviations: DPD , *N,N*-dimethyl-*p*-phenylenediamine; DPD^{+} , the first free-radical oxidation product of DPD .

apparent rate of the oxidase reaction by mechanisms other than those involving combination with the enzyme. Thus (1) removal of stimulatory iron (Curzon, 1961; Levine & Peisach, 1963) by chelation and (2) destruction of the reaction product, if the extinction of this is used to determine the reaction velocity, must be distinguished from true inhibition (i.e. in which the inhibitor reacts with the enzyme). Also when the disappearance of substrate is being used to determine the velocity then regeneration of substrate by reduction of product must be distinguished from true inhibition.

In the present study a large number of substances were tested as inhibitors under the same conditions. DPD was chosen as substrate because the coloured free radical (DPD⁺), to which it is oxidized by caeruloplasmin, is relatively stable to further oxidation or other change (Michaelis, Schubert & Granick, 1939), in particular under the conditions of caeruloplasmin assay (Curzon, 1967a). EDTA was present in the reaction mixtures to eliminate the marked enhancement of activity by contaminant iron. Though EDTA is a chelating agent proton-relaxation studies indicate that it is not bound to caeruloplasmin cupric copper (Blumberg, Eisinger, Aisen, Morell & Scheinberg, 1963). All substances showing apparent inhibition were tested for their effect on DPD⁺.

MATERIALS

Caeruloplasmin. Human caeruloplasmin (American Red Cross, batch no. 1995) was dialysed against 10 mM-NaCl, ultrafiltered and freed from contaminant metal as described by Curzon (1966). Material treated as above was 106–131 μ M (assuming 8 atoms of copper/molecule). E_{605}/E_{280} was 0.039–0.040, which indicates a purity of about 90% and $E_{605}^{1\text{cm}}/[\text{copper } (\mu\text{g./ml.})]$ was 0.021–0.022. Working solutions, prepared by dilution with water, were used within 5 hr.

Substrates. DPD dihydrochloride (Kodak Ltd., Kirkby, Liverpool) was obtained as a cream-coloured powder and was stored as a 0.1 M solution in 10 ml. portions at -25° . Before use, samples were brought to pH 5.5 with 0.1 N-NaOH and suitably diluted with water. These working substrate solutions were kept in ice-water and used within 5 hr. during which time only slight visible auto-oxidation occurred.

Other reagents. Purest available grades were used. Reagents were obtained from British Drug Houses Ltd., Poole, Dorset, with the exception of histidine, isophthalic acid, terephthalic acid, tiglic acid and acetylenedicarboxylic acid (Koch-Light Laboratories Ltd., Colnbrook, Bucks.), 5-aminotetrazole, 3-amino-1,2,4-triazole, 4-amino-1,2,4-triazole, propiolic acid, propargyl alcohol and pyrrole-2-carboxylic acid (Aldrich Chemical Co. Inc., Milwaukee, Wisc., U.S.A.), iproniazid and isoniazid (Roche Products Ltd., Basle, Switzerland) and chlorpromazine (May and Baker Ltd., Dagenham, Essex). Benzotriazole was a gift from Dr J. M. Walshe, rescinamine from Selpharm Laboratories Ltd., London, W. 1, and reserpine from Ciba Laboratories Ltd., Horsham, Sussex. Sodium azide, *o*-aminobenzoic acid and *m*-hydroxybenzoic acid were

recrystallized from water. Potassium cyanate contained less than 0.02% of cyanide by the copper acetate-benzidine acetate test (Feigl, 1960).

Most substances under investigation as inhibitors were dissolved in water with addition of dilute NaOH or H₂SO₄ to bring the pH to 5.5 and used immediately. Cyanide, because of its volatility at pH 5.5, was dissolved in water without adjustment of pH and was added to the cell 1–3 min. before addition of enzyme. A few substances (indicated in the text) required ethanol or benzyl alcohol to effect solution. Final incubation mixtures were pH 5.5.

Spectrophotometers. SP.500 and SP.800 instruments (Unicam, Cambridge) were fitted with constant-temperature cell housings.

Molecular models. These (Catalin Products Ltd., Waltham Abbey, Essex) were space-filling models with dimensions mainly according to Pauling (1960) and Wheland (1944).

METHODS

Apparent inhibition. The reaction mixture was held at 25° in a 10 mm. path cell and contained 1.0 mM-DPD dihydrochloride, 10 mM-sodium acetate-acetic acid buffer, pH 5.5, 4 μ M-EDTA, inhibitor and 0.38 μ M-caeruloplasmin, which was added last, making a volume of 2.5 ml. E_{550} was measured at 1 min. intervals against a blank containing all reagents except the enzyme. $\Delta E/\text{min.}$ during the period of linear increase of E_{550} was measured both in the presence and absence of inhibitors. $\Delta E/\text{min.}$ in the absence of inhibitors was 0.09–0.10. When the inhibitor required an additive to effect solution this was also added to the cell containing enzyme without inhibitor and to the blanks.

In investigations of inhibition by aromatic acids, 40 μ M-EDTA was added since some of these substances were grossly contaminated with metal (presumably iron), and 4 μ M-EDTA was not sufficient to eliminate the resulting enhancement of activity. The higher EDTA concentration had little effect on inhibition. Thus the concentrations of benzoate and fumarate for 50% inhibition with 4 μ M-EDTA gave 47% and 48% inhibition respectively with 40 μ M-EDTA.

Decolorization of reaction product. DPD is rapidly oxidized by bromine or iodine to a free radical DPD⁺, which has an absorption spectrum identical with that of the product of the caeruloplasmin-catalysed oxidation (Curzon, 1967a). The chemically formed product was used to determine whether apparent inhibitors decolorized DPD⁺. The same total quantities of DPD dihydrochloride, acetate buffer and EDTA as used in the enzymic reaction were held at 25° in 1.5 ml. and to this was added 0.5 ml. of approx. 0.45 M-bromine solution adjusted to pH 5.5, and at 25° , followed 1 min. later by 0.5 ml. of a solution of the apparent inhibitor at 25° . $E_{550}^{1\text{cm}}$ was measured as before against a blank containing all the reagents except bromine. When water was used instead of inhibitor there was a slow decrease in E_{550} (0.002–0.003/min.). Some substances increased the rate of decolorization. These gave rise to enzymic progress curves in which the slope decreased with time; i.e. the rate of decolorization of DPD⁺ increased as its concentration increased.

Correction of apparent inhibition for decolorization of product. When decolorization of DPD⁺ was increased by a substance it was determined whether the apparent inhibition was due entirely to such a reaction or if true inhibition

also occurred. Thus apparent inhibition and rate of DPD⁺ decolorization were measured at the same concentrations of substrate, product and inhibitor. Inhibitor concentration was chosen so that the rate of decolorization did not exceed $\Delta E_{550}^{1\text{cm}}/\text{min. } 0.13$. First, decolorization of DPD⁺ was measured as before except that an extra 0.5 ml. of water was added, bringing the final volume of reaction mixture to 3.0 ml. After addition of the substance under investigation, readings of E_{550} were taken every 15 sec. to obtain the initial slope of E_{550} against time. By subtracting the corresponding slope in the absence of inhibitor the change in slope due to decolorization by the substance was obtained.

To measure total apparent inhibition under these conditions the usual total quantities of substrate, acetate buffer and EDTA, in a volume of 2.0 ml., were placed in a cell at 25° and 0.5 ml. of caeruloplasmin solution at 25° was added. Readings of E_{550} were taken at 1 min. intervals against a blank containing no enzyme. To make these readings comparable with those obtained after dilution by 0.5 ml. of inhibitor (see below) they were multiplied by 0.81. This factor was experimentally determined by dilution of the above incubation mixture with 0.5 ml. of water and is largely due to product dilution ($2.5/3 = 0.83$). The corrected readings were plotted against time. Approx. $\frac{1}{2}$ –1 min. before a corrected E_{550} equal to the initial E_{550} of the DPD⁺ decolorization experiment was reached, 0.5 ml. of inhibitor solution at 25° was added to both test and blank solutions. The time of addition was chosen so that the ensuing slope was measured over approximately the same range of E_{550} as the decolorization slope. Readings of E_{550} were taken at 15 sec. intervals and the slope immediately after addition of inhibitor was determined. The difference between this and the slope before addition of inhibitor gives the total change in slope due to the inhibitor. The change in slope due to decolorization of DPD⁺, obtained from the preceding experiment, was subtracted to give a value from which true inhibition was calculated.

Reversibility of inhibition by Sephadex treatment. A 2 g. sample of Sephadex G-25 (coarse grade) (Pharmacia, Uppsala, Sweden) was washed three times by sedimentation and decantation with 1.0 mM-acetate–1.0 mM-NaCl, pH 5.5, poured into a column (28 cm. $\times$ 0.7 cm. diam.) and the acetate–chloride solution was passed through the column at 4°. The rate of substrate oxidation was determined in the presence and absence of inhibitor as before except that a final concentration of $1.83 \mu\text{M}$ -caeruloplasmin was used. The concentration of inhibitor was chosen to give an inhibition of 72–84%. After reaction had proceeded for 4 min. (or 10 min. with cyanide) the incubation mixture was poured into an ice-cooled tube and, after a further 2–3 min. for temperature equilibrium, 0.5 ml. was absorbed on the column and followed by acetate–chloride solution. The first 3.0 ml. of eluate contained no protein and was discarded. The next 4.2–4.3 ml. was collected, but collection was stopped before the red DPD⁺ band reached the base of the column. After brief centrifugation to remove column debris, the oxidase activity of 1.0 ml. of the eluate was determined. Protein recovery was calculated from the E_{280} of the eluate and used to correct activity for loss of protein. Protein recoveries were 110%, 100%, 90% and 94% in four inhibitor experiments and 79%, 103%, 83% and 106% in four control experiments. Equivalent dilution by the acetate–chloride solution but without column fractionation resulted in an apparent loss of $18 \pm 6\%$ of the oxidase activity

(average of seven determinations). Recoveries were corrected for this loss, which may have resulted from the high dilution of the enzyme solutions.

RESULTS AND DISCUSSION

Effects of inorganic anions. Table 1 summarizes the results. Reversibility of the azide inhibition of caeruloplasmin has been described by Curzon (1966). Corrected recoveries of caeruloplasmin activity after reversal of other inhibitions by Sephadex treatment were 84% with cyanide, 91% with thiocyanate and 98% with cyanate. These may be compared with corrected recoveries in the absence of inhibitors (87%, 91%, 98% and 107% in four experiments). The greatest inhibitory effects occur with cyanide and azide. Methyl cyanide at 10 mM concentration had no effect on activity. Frieden (1962) suggests that the small size of the cyanide group is a factor in its effectiveness as a copper-enzyme inhibitor. Cyanate, thiocyanate and selenocyanate are effective in inverse order of ionic size. Similarly, inhibition by halides decreases as size increases with the exception of iodide, which is approximately as inhibitory as chloride. The halide order agrees with the order fluoride > chloride > bromide obtained under different conditions (Curzon, 1960). Peisach & Levine (1965) found approximately equal inhibitions by chloride, bromide or iodide but did not give numerical data. Holmberg & Laurell (1951b) report that iodide was oxidized in the

Table 1. *Inhibition of caeruloplasmin oxidase activity by inorganic anions*

Experimental details are given in the Materials and Methods sections. None of the anions decolorized DPD⁺. Sodium or potassium salts were used. Inhibition is not affected by changing the alkali-metal cation (Curzon, 1960; Peisach & Levine, 1965).

Substance	Concn.	Inhibition (%)
Cyanide	1.7 μM	50*
Azide	3.7 μM	50
Cyanate	120 μM	50
Thiocyanate	580 μM	50
Selenocyanate	800 μM	50
Fluoride	6.0 mM	50
Iodide	14 mM	50
Nitrate	15 mM	50
Chloride	17 mM†	50
Bromide	29 mM	50
Perchlorate	10 mM	19
Tetraborate	10 mM	11
Boric acid, phosphate, sulphate and cacodylate	10 mM	< 5

* Though all other inhibitors in this and subsequent Tables attained equilibrium with the cycling enzyme within 2 min., cyanide required 10–15 min.

† Additional to 2 mM from substrate.

p-phenylenediamine-caeruloplasmin system. However, in the present study, a caeruloplasmin-iodide incubation mixture gave a negative starch-iodine test and did not take up oxygen in the Warburg apparatus. As ferric iron oxidizes iodide it is probable that the results of Holmberg & Laurell were due to the coupled iron-caeruloplasmin oxidation mechanism (Curzon, 1961), iron being an almost inevitable contaminant of *p*-phenylenediamine.

Though the most inhibitory anions are small, univalent and able to bind free copper well, the least inhibitory anions are large, often polyvalent and being oxyanions are unlikely to form complexes with cuprous copper (Pearson, 1963). The inhibition obtained by Osaki (1961) with tetraborate was probably due to chloride introduced during pH adjustment with hydrochloric acid. We have used sulphuric acid since sulphate has little inhibitory effect, but when using hydrochloric acid we have obtained results similar to those of Osaki (1961). The order of inhibitory effectiveness of unidentate anionic ligands shows differences to the order of increasing inhibitory effectiveness of anions against many enzymes (Warren & Cheatum, 1966; Warren, Stowring & Morales, 1966). This latter order ($\text{CH}_3\cdot\text{CO}\cdot\text{O}^- < \text{Cl}^- < \text{Br}^- \cong \text{NO}_3^- < \text{I}^- < \text{ClO}_4^- \cong \text{SCN}^-$)

is thought to be due to an effect of anions upon the general organization of the protein (Warren *et al.* 1966).

Effects of multidentate organic ligands. Though many unidentate anionic ligands cause considerable inhibition, multidentate ligands able to bind free copper powerfully have little influence on caeruloplasmin activity (Table 2). This confirms and extends the observations of Levine & Peisach (1963). The slight effect of citrate differs from the findings of Osaki, McDermott & Frieden (1964), who obtained considerable inhibition in the absence of EDTA. Curzon (1967*b*) suggested that this was due to chelation of activating iron by citrate, and this mechanism has been confirmed by Osaki,

McDermott, Johnson & Frieden (1966). The general lack of effect of the above ligands suggests that those copper atoms of caeruloplasmin that are necessary for activity have insufficient co-ordinate valencies available for the binding of multidentate ligands. Another factor, indicated by the relationships between inorganic anion size and inhibitory power (Table 1), may be that copper atoms are in clefts in the protein and thus inaccessible to these larger ligands. *o*-Hydroxybenzoate, which forms a bidentate complex in solution with ionic copper, chelating it between the carboxyl and hydroxyl groups, has been reported to inhibit caeruloplasmin (Broman, 1958; 1964). We have confirmed this, but have also found that benzoate (Table 3), which is unable to bind copper in such a manner, has an even greater inhibitory effect. Benzoate inhibition was 98% reversible by Sephadex treatment.

Inhibition by substances containing the structural

$\text{>C}=\text{C}\cdot\text{CO}_2\text{H}$ unit. The structural features necessary for inhibition by substituted benzoates are summarized in Table 3. Saturated aliphatic carboxylates and non-carboxylic aromatic substances had very small effects. Unsaturated aliphatic carboxylates inhibited less than benzoate but more than related saturated carboxylates or unsaturated non-carboxylates. Thus fumarate and maleate inhibited more than succinate; propionate and acrylate more than propionate. Therefore both carbon-carbon unsaturated bonds and a carboxyl group are required for inhibition. Inhibition was decreased by separation of the carboxyl group from the double bond. Thus vinylacetate inhibited less than crotonate and phenylacetate less than benzoate. Therefore a distinct class of inhibitors

having the structural unit $\text{>C}=\text{C}\cdot\text{CO}_2\text{H}$ is indicated. Inhibition by maleate and fumarate was less than previously found in the absence of EDTA (Curzon, 1960). The greater inhibition by fumarate than by maleate indicates that metal binding between the carboxyl groups, which could be significant in cupric chelation, is not involved. The suggestion (Webb, 1966) that inhibition of caeruloplasmin by maleate may be due to SH binding is unlikely since *N*-ethylmaleimide has no effect (Table 3*d*). The lack of effect of acetate suggests that previous findings of inhibition (Holmberg & Laurell, 1951*b*; Humoller *et al.* 1960; Curzon, 1960) are due to the formation of a complex of activating iron as ferric acetate.

The inhibition of tyrosinase by benzoate and related substances was studied by Kuttner & Wagreich (1953) and by Krueger (1955). Tyrosinase contains cuprous copper only (Kertesz & Zito, 1965) and it is noteworthy that the relationships between

Table 2. *Multidentate copper-binding ligands without effect on caeruloplasmin oxidase activity*

Experimental details are given in the Materials and Methods sections. Numbers in parentheses refer to apparent percentage inhibition due to decolorization of DPD⁺ where significant. The following had negligible effect (<5% inhibition).

Substance	Concn.
Glycine, serine, histidine, 1,10-phenanthroline	10 mM
Dimethylglyoxime, 1-nitroso-2-naphthol* (13), bis-cyclohexanoneoxalylidihydrazone*, benzotriazole	1 mM
8-Hydroxyquinoline (37)	50 μM

* 4% ethanol present.

Table 3. *Inhibition of caeruloplasmin oxidase activity by compounds with structural features in common with benzoate*

Experimental data are given in the Materials and Methods sections. Inhibitors were 10mM except where otherwise indicated. Numbers in parentheses indicate apparent percentage inhibition due to decolorization of DPD+ when significant. 10mM- and 3mM-benzoate gave 71% and 50% inhibition respectively.

Substance	Inhibition (%)	Substance	Inhibition (%)
(a) Aromatic carboxylates		(c) Aromatic non-carboxylates	
Pyrrole-2-carboxylate* (32)	24	5-Aminotetrazole*	23
1-Naphthoate*	16	Benzenesulphonate*	11
2-Furoate*, cinnamate*	12	Benzene, phenol (36), benzamide*, aniline (5mM) (39), indole, imidazole, 3- and 4-amino-1,2,4-triazole, methyl sali- cylate	< 5
Phenylacetate	< 5		
(b) Unsaturated aliphatic carboxylates		(d) Unsaturated aliphatic non-carboxylates	
Fumarate	56	Allyl alcohol, propargyl alcohol, N-ethylmaleimide	< 5
Crotonate	46		
Tiglate, propiolate (7)	33	(e) Saturated aliphatic carboxylates	
Maleate	31	Oxalate	20
Sorbate	29	Citrate	15
Acrylate	27	Formate, trichloroacetate	9-12
Vinylacetate, itaconate	21	Acetate (200mM additional to 10mM buffer), propionate, malonate, succinate, glutarate	< 5
Acetylenedicarboxylate (5)	8		

(f) Monosubstituted benzoates*

Substituent	Relative volumes† of substituents	Inhibition (%)		
		Substituent <i>ortho</i> to carboxyl	Substituent <i>meta</i> to carboxyl	Substituent <i>para</i> to carboxyl
F	6.5	63	48	35
OH	7.5	59	43 (22)	16 (1)
NH ₂	9.5	43 (96)	—	7 (5)
Cl	11.5	8	—	—
CH ₃	12.5	9	11	22
Br	15.5	7 (2)	12	—
NO ₂	15.5	—	8	—
CO ₂ ⁻	16.0	4	9	0
O·CH ₃	17.5	3	—	13
SO ₃ ⁻	25.0	—	5	—

* 40 μM-EDTA present.

† Measured in molecular models.

the structures of benzoate analogues and inhibitory power were very similar to those found in the present study with caeruloplasmin except that unsaturated aliphatic acids did not inhibit tyrosinase (Krueger, 1955).

The existence of cuprous-olefine complexes suggests that unsaturated aliphatic acids and benzoates may bind to caeruloplasmin cuprous copper. Cuprous copper forms complexes with allyl alcohol (Kepner & Andrews, 1948) and with unsaturated carboxylic acids (Keefer, Andrews & Kepner, 1949). Ionized carboxyl groups were not involved in this binding as a pH well below the relevant pK values was used. Andrews & Keefer (1948) considered that interaction between the cuprous copper and the

double bond occurred. Cuprous-benzenoid complexes have not been reported, though univalent silver-benzenoid complexes are well known and thought to involve metal-double bond interaction (Mulliken, 1952). The stability constants for the unsaturated acid-cuprous copper complexes found by Keefer *et al.* (1949) are in the order vinylacetic > fumaric > itaconic > crotonic > maleic > tiglic. This is similar to the order of inhibitory powers of the anions of these substances, with the exception of vinylacetate and itaconate which inhibit less than maleate. Vinylacetate differs from the other carboxylate anions in that the carboxyl group is not adjacent to the double bond, and both vinylacetate and itaconate have a larger substituent

adjacent to the double bond than do other members of the above group.

Relative inhibitions by ring-substituted benzoates are shown in Table 3(f). None of the 21 substances tested is as effective an inhibitor as benzoate at the concentration used. All possess, in common with benzoate, a completely or almost completely ionized carboxyl group at pH 5.5 (Sillen & Martell, 1964; Albert & Serjeant, 1962). There is no apparent relationship between inhibition and the effect of the substituent on electron distribution in the inhibitor molecule. Inhibition clearly decreases sharply as substituent size increases. Thus steric factors have a predominant influence on the formation of the inhibited complex. Keefer *et al.* (1949) similarly found the stabilities of cuprous-unsaturated aliphatic carboxyl complexes were influenced more by steric than by electronic effects of substituents. The steric requirements for binding with cuprous copper, which is part of a protein, are probably stricter than those for access to free cuprous copper and therefore ligand shape and size is likely to be even more important. The critical nature of size in binding into protein clefts is illustrated by the finding (Wishnia & Pinder, 1966) that the hydrophobic binding properties of β -lactoglobulin indicate a cleft able to accommodate substances with gram-molecular volumes up to 210ml. but not of 230ml. An exception to the relationship of size of substituted benzoate molecules with inhibition is *o*-hydroxy-*m*-methylbenzoate, which at 10mM concentration gives 57% inhibition. This is similar to the inhibition caused by *o*-hydroxybenzoate and much more than that caused by *m*-methylbenzoate.

We suggest that caeruloplasmin inhibitors with structures containing $\text{>C}=\text{C}\cdot\text{CO}_2\text{H}$ bind caeruloplasmin cuprous copper weakly by the π electrons of the double bond and that the ionized carboxyl group is bound to the protein at another site. Chelate formation with carboxyl binding to the same copper atoms as the π electrons is unlikely as the carboxyl ion is 'hard' or of low polarizability and therefore has little tendency to bind to cuprous copper (Pearson, 1963; Hemmerich, 1966). Also chelation of this type would probably involve formation of a strained ring.

Other reported inhibitors. A number of substances, some of pharmacological importance, which have previously been reported to affect caeruloplasmin activity were tested as inhibitors (Table 4). None inhibited caeruloplasmin appreciably at the concentrations at which it was possible to test them. Some caused apparent inhibition due to destruction of DPD⁺. Ascorbate, dithionite, cysteine, penicillamine, hydrogen sulphide and pyrrolidyl- and diethyl-dithiocarbamate rapidly decolorized DPD⁺ and were not investigated further. Dihydroxyphenylalanine, like 5-hydroxytryptamine and 5-hydroxyindol-3-ylacetic acid (Curzon & Cumings, 1966), reacts with DPD⁺ to give a coloured product.

General considerations. The application of methods designed to distinguish between true and apparent inhibitors of caeruloplasmin has provided evidence against previous reports that certain substances inhibited caeruloplasmin. The true inhibitors, including the newly distinguished class of inhibitors containing the structural unit

Table 4. *Absence of direct effect on caeruloplasmin oxidase activity by substances previously reported to affect activity*

Experimental details are given in the Materials and Methods sections. Numbers in parentheses refer to apparent percentage inhibition due to decolorization of DPD⁺.

Substance	Concn.	Inhibition (%)
Hydrazine (31), hydroxylamine (39)	2.5 mM	8-10
Methylhydrazine* (43), semicarbazide (32)	2.5 mM	<5
Iproniazid (20)	100 μM	<5
Isoniazid (6)	1.0 mM	<5
Chlorpromazine	15.0 mM	<5
Reserpine†	430 μM	<5
Rescinnamine†	110 μM	<5
3,4-Dihydroxyphenylalanine (143)‡	500 μM	<5

* 40 μM -EDTA present.

† 7% benzyl alcohol and 21% ethanol present.

‡ Apparent inhibition greater than 100% indicates that the slope of the E_{550} /time plot became negative after addition of this substance.

are all unidentate and restricted in size. This indicates a limited accessibility of the copper atoms of caeruloplasmin that are involved in oxidase activity. It is also consistent with previous indications that the copper atoms are 'buried' in the protein part of the caeruloplasmin molecule. Thus they are not removed by dialysis or ion-exchange resins (Curzon, 1959) or by chelating agents, except on prolonged treatment (Kasper & Deutsch, 1963; Marriott & Perkins, 1966) or in the presence of denaturing agents (Curzon, 1962; Marriott & Perkins, 1966). Proton-relaxation studies also show that cupric copper atoms are buried in the protein matrix (Blumberg *et al.* 1963). Also, the lower resistance of apocaeeruloplasmin than of intact caeruloplasmin to denaturation by heat or organic solvents (A. G. Morell, personal communication) indicates that copper atoms stabilize the tertiary structure of caeruloplasmin and hence will tend to be relatively inaccessible situated between peptide chains (Curzon, 1959).

Whereas aromatic compounds with two electron-supplying groups are caeruloplasmin substrates, related compounds with single electron-supplying groups are not substrates (Peisach & Levine, 1965; Walaas, Walaas & Løvstad, 1966), nor are they inhibitors (Table 3c). This indicates that both electron-supplying groups are necessary for complex-formation at substrate-binding sites. This may be either because the complex is formed through these groups or because its formation involves charge transfer which in turn is related to electron donations and only those substances having more than one electron-supplying group may have sufficiently strong electron donor properties.

Whereas the valence-changing copper atoms of caeruloplasmin clearly are a sink for electrons supplied by the substrate, the function of the permanently cuprous copper atoms is less clear. Broman *et al.* (1963) have suggested that substrates are bound at the cuprous copper atoms of the enzyme, mainly because cuprous copper tends to bind substances containing π electrons (Orgel, 1960), and Levine & Peisach (1963) had previously claimed that substrates with a high density of π electrons on their aromatic rings were oxidized faster by caeruloplasmin than substrates with a low density. However, Hammett σ values (Hammett, 1940; Jaffe, 1953) were used to estimate the relative π electron densities. These values, as calculated by Levine & Peisach (1962), relate only to the effect of each electron-supplying group at the other electron-supplying group and at similarly orientated ring positions and do not give a measure of net π electron density in the ring. If enzyme cuprous atoms are substrate-binding sites then it might be expected that substances able to bind cuprous copper would inhibit competitively. However, though four substances have been claimed to inhibit caeruloplasmin competitively, i.e. citrate (Osaki *et al.* 1964), iproniazid (Martin *et al.* 1958; Hanson *et al.* 1959), 5-hydroxytryptamine (Aprison *et al.* 1959) and 3,4-dihydroxyphenylalanine (Hanson *et al.* 1959), the effect of citrate is largely not true inhibition but prevention of enhancement of activity by contaminant iron (Osaki *et al.* 1966) and the effects of the other substances are due in considerable measure to interactions with DPD^+ (Table 4; Curzon & Cumings, 1966). Table 2 and Table 3(c) contain a number of known strong cuprous chelators and substances with high π electron density that do not inhibit caeruloplasmin. This finding is consistent with a limited accessibility of the permanently cuprous copper of the enzyme.

We thank the American National Red Cross for supplies of caeruloplasmin. The work was supported by grants from the Medical Research Council, the Central Research Fund of the University of London and the Research Advisory Committee of the Institute of Neurology.

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