Biochem. J. (1977) **168**, 283–288
Printed in Great Britain

The Inhibition of Lipid Autoxidation by Human Caeruloplasmin

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(*Received* 11 *May* 1977)

1. Purified caeruloplasmin was shown to inhibit lipid autoxidation induced by ascorbic acid or inorganic iron in the following systems: (*a*) an emulsion of linolenic acid in water; (*b*) an untreated ox brain homogenate in phosphate buffer; (*c*) a similar homogenate whose susceptibility to autoxidation had been abolished by dialysis or by heating and then restored by the above pro-oxidants. 2. The optimum conditions for this antioxidant activity were studied. 3. Caeruloplasmin did not inhibit autoxidation by u.v. irradiation in dialysed or preheated homogenates. 4. The apoprotein (without copper) had no antioxidant activity, whereas $CuSO_4$ alone was much less effective as an antioxidant. 5. Iron-free transferrin also had some antioxidant activity.

Human blood serum has been known for some years to be a powerful lipid-autoxidation inhibitor (Barber, 1961; Vidlakova *et al.*, 1972; Stocks *et al.*, 1974*a*). An assay system based on the spontaneous autoxidation in a standard ox brain homogenate has been developed partly to measure this inhibition and partly to identify the main antioxidant serum fractions (Stocks *et al.*, 1974*b*). Preliminary studies have pointed to caeruloplasmin as the most potent antioxidant fraction both in normal and in abnormal serum (Cranfield *et al.*, 1977). The aim of the present work was to confirm and characterize this action by using purified human caeruloplasmin in a variety of autoxidizing systems.

Experimental

Human caeruloplasmin (type III), linolenic acid and horse spleen ferritin (type I) were obtained from Sigma (London) Chemical Co., Kingston, Surrey, U.K. Iron-free human transferrin was obtained from Hoechst Pharmaceuticals (Hounslow, Middx., U.K.), and Tween 20 from Koch–Light (Colnbrook, Bucks., U.K.). All other chemicals were of AnalaR grade and obtained from British Drug Houses (Poole, Dorset, U.K.).

An ox brain homogenate was prepared and autoxidation was induced and measured in 5ml samples as described by Stocks *et al.* (1974*a*). The assay is based on the generation of malonyldialdehyde as measured by the thiobarbituric acid reaction (Sinnhuber & Yu, 1958). Antioxidant activity was calculated from the equation:

$$\text{Antioxidant activity (\% inhibition)} = \left(1-\frac{\text{nmol of malonyldialdehyde/ml (T)}-\text{nmol of malonyldialdehyde/ml (Z)}}{\text{nmol of malonyldialdehyde/ml (C)}-\text{nmol of malonyldialdehyde/ml (Z)}}\right)\times 100$$

where T = test, Z = zero time and C = control.

Unless otherwise indicated all additions to the homogenates were made in 50μl volumes, and phosphate/saline buffer (40mM-KH_2PO_4/K_2HPO_4/142mM-NaCl), pH7.4, was used both as the solvent and as the control solution. The Tables and Figures give the final concentrations of added substances in the homogenates. Preliminary experiments established that percentage inhibition (i.e. antioxidant activity) by caeruloplasmin did not change significantly over the first 2h of the assay. Unless otherwise indicated all results in the Tables and Figures are based on measurements after 1h incubation at 37°C. The values are means of three or more independent experiments ± S.D. Malonyldialdehyde-based measurements of autoxidation and of antioxidant activity were compared with polarographic measurements with a Clark electrode system (YSI Biological Oxygen Monitor; Shandon Southern Ltd., Aldershot, Hants., U.K.). All polarographic measurements were performed at 37°C.

Dialysis of the stock brain homogenate was carried out at 4°C against several changes of deionized water over 24h. After dialysis the homogenate was diluted with 3vol. of phosphate/saline buffer, pH7.4. Preheating of the homogenate was for 5min at 95°C. U.v. irradiation of the homogenate was carried out with a short-wavelength light-source (Anderman Ltd., London S.E.1, U.K.). The material was placed in an open Petri dish and exposed as a thin film at room temperature (25°C) at a distance of 6cm from the light-source for 1h.

A linolenic acid emulsion, used as an alternative autoxidizing substrate, was prepared by emulsifying 10μl of the fatty acid in 100ml of deionized water containing 100μl of Tween 20.

Commercially obtained type III human caerulo-

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plasmin was purified on a DEAE-cellulose column (0.6cm × 30cm) from which the protein was eluted with a constant-volume continuous-gradient technique adapted from the method of Deutsch *et al.* (1962). The purity of the protein was assessed from the A_{610}/A_{280} ratio, based on the value of A_{610}/A_{280} = 0.044 for 100% caeruloplasmin (Hirschman *et al.*, 1961). The estimated purity of the final preparation was 89%. Caeruloplasmin apoprotein was prepared as described by Morell *et al.* (1964). Erythrocyte superoxide dismutase was prepared as described by Scudder *et al.* (1976). Unless otherwise indicated all protein preparations were made up in phosphate/saline buffer, pH7.4. Their copper concentration was measured by atomic absorption spectroscopy by using a Perkin–Elmer 303 spectrophotometer.

Results

Inhibition of autoxidation in the ox brain assay was directly proportional to (*a*) the concentration of caeruloplasmin added to the system and (*b*) the degree of purification of the original commercial preparation. With both the original commercial and the purified caeruloplasmins the relationship between caeruloplasmin concentration and inhibition of autoxidation was linear up to 92% inhibition. The caeruloplasmin concentration corresponding to 50% inhibition was 0.82g/litre for the commercial and 0.50g/litre for the purified preparation. The coefficient of variation based on 25 independent measurements of antioxidant activity between 20 and 95% inhibition was 4.1%. The antioxidant effectiveness of caeruloplasmin was further influenced by a number of variables, some of which also affected the rate of autoxidation in the control system. Particular attention was paid to variables known to affect the oxidase-like action of the protein. An increasing NaCl concentration between 50 and 300mM caused a near-linear decline in autoxidation in the control homogenates from 10 to 8.4nmol of malonyldialdehyde/ml and a near-linear increase in the antioxidant activity of caeruloplasmin (1g/litre) from 51 to 60%. An increase in pH from 5.5 to 8 caused a near-linear decline in autoxidation in the control homogenates from 14 to 7.8nmol of malonyldialdehyde/ml. The antioxidant activity of caeruloplasmin (1g/litre) was 54% at pH5.5, 65% at pH6.7 and thereafter declined to 20% at pH8.

NaN_3 was a relatively weak inhibitor of autoxida-

Table 1. *Effect of NaN_3 on the autoxidation in brain homogenate and on the antioxidant activity of caeruloplasmin*
For details see the Experimental section.

Preparation	NaN_3 (mM)	Malonyldialdehyde (nmol/ml)	Inhibition of autoxidation (%)	Inhibition of caeruloplasmin activity (%)
Brain homogenate	—	9.52±0.51	—	—
	1	8.90±0.40	7±1.9	—
	2	7.82±0.50	18±1.0	—
	3	7.48±0.52	22±1.2	—
	4	7.14±0.45	26±1.5	—
Brain homogenate+caeruloplasmin (1g/litre)	—	3.74±0.31	60±2.0	—
	1	4.42±0.40	50±2.1	16±0.6
	2	4.76±0.42	40±1.5	33±1.4
	3	5.10±0.35	33±1.3	45±1.6
	4	5.10±0.34	29±1.0	51±1.6

Table 2. *Effect of inorganic copper, caeruloplasmin and caeruloplasmin apoprotein (copper-free) on the autoxidation in brain homogenate*
For details see the Experimental section.

Preparation	Caeruloplasmin concentration (g/litre)	Copper concentration (μmol/litre)	Malonyldialdehyde (μmol/ml)	Antioxidant activity (% inhibition)
Brain homogenate	—	—	9.6±0.52	—
+caeruloplasmin	0.25	12.5	7.8±0.35	19.0±1.0
	0.50	25.0	6.8±0.55	30.5±1.5
	0.75	37.0	5.4±0.40	45±1.2
	1.00	50.0	4.2±0.30	57±1.0
Brain homogenate	0.25	0.3	9.6±0.52	0
+apoprotein	0.50	0.8	9.6±0.55	0
	0.75	0.1	9.6±0.51	0
Brain homogenate	0	50	9.60±0.51	0
+$CuSO_4$	0	100	9.60±0.52	0
	0	500	9.10±0.60	6±2.0
	0	2000	8.60±0.42	11±3.2
	0	4000	7.65±0.40	21±3.0

tion in the control system, but a powerful inhibitor of the antioxidant action of caeruloplasmin (Table 1). Preheating at 60°C for 15min decreased the antioxidant potency of caeruloplasmin by 40%; preheating at 90°C for 15min abolished it.

Inorganic copper salts representing molar copper concentrations equivalent to 30 times the copper concentration in caeruloplasmin had no antioxidant effect; at higher concentrations they were inhibitory. The copper-free caeruloplasmin apoprotein had no antioxidant action (Table 2). The copper-containing enzyme erythrocyte superoxide dismutase had no antioxidant effect in a copper concentration range comparable with that of caeruloplasmin.

The effect of inorganic iron salts, iron complexes and ascorbic acid were measured in view of their catalytic role in lipid autoxidation. Both ferrous and ferric salts but neither ferritin nor transferrin-bound iron promoted autoxidation. Ascorbic acid at concentrations of less than 10mM also behaved as a prooxidant (autoxidation promoter). (At higher concentrations it became an antioxidant.) Figs. 1 and 2 show the antagonistic (i.e. antioxidant) effect of caeruloplasmin in the presence of added prooxidants. Iron-free transferrin (as distinct from transferrin saturated with iron) also behaved as an antioxidant, the antioxidant effect of the two proteins being additive (Fig. 3).

To clarify the antioxidant mode of action of caeruloplasmin its effect was investigated (*a*) in ox brain homogenates treated in various ways and (*b*) in linolenic acid emulsions. Dialysis virtually abolished autoxidation in the homogenate. Autoxidizability could be restored by the addition of either iron in high concentrations or ascorbic acid to the dialysed

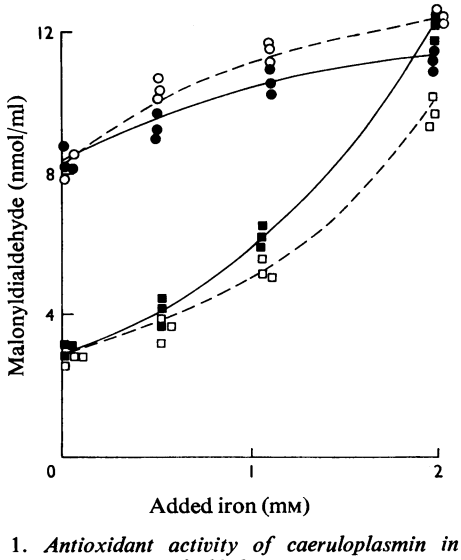

Fig. 1. *Antioxidant activity of caeruloplasmin in the presence of added inorganic iron*
$FeCl_2$ (○, □) or $FeCl_3$ (●, ■) was added to brain homogenates containing caeruloplasmin (1g/litre) (■, □) or buffer (●, ○). Malonyldialdehyde generation was measured after 1h incubation at 37°C.

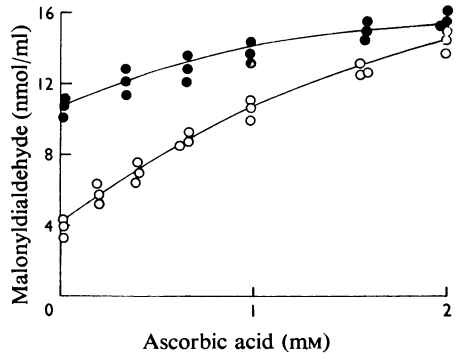

Fig. 2. *Antioxidant activity of caeruloplasmin in the presence of added ascorbic acid*
Ascorbic acid was added to brain homogenates containing caeruloplasmin (1g/litre) (○) or buffer (●). Malonyldialdehyde generation was measured after 1h incubation at 37°C.

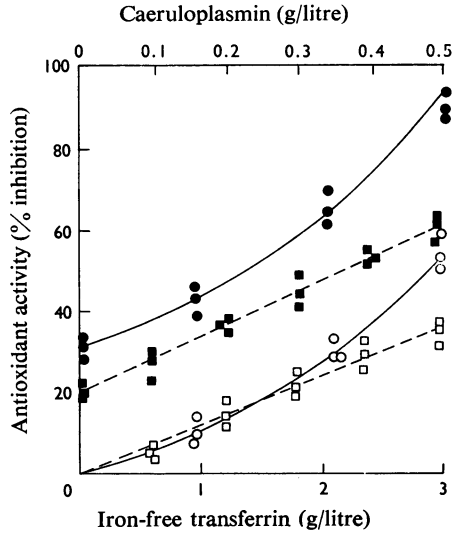

Fig. 3. *Effect of iron-free transferrin on the antioxidant activity of caeruloplasmin* (——) *and the effect of caeruloplasmin on the antioxidant activity of iron-free transferrin* (----)
Iron-free transferrin was added to brain homogenates containing caeruloplasmin (0.5g/litre) (●) or buffer (○), and caeruloplasmin was added to brain homogenates containing iron-free transferrin (1.5g/litre) (■) or buffer (□). After incubation for 1h at 37°C autoxidation was measured and antioxidant activity was calculated and expressed as described in the text.

Table 3. *Antioxidant activity of caeruloplasmin in standard, dialysed and 'reconstituted' brain homogenates*
For details see the Experimental section.

Preparation	Caeruloplasmin (1 g/litre)	Malonyldialdehyde (nmol/ml)	Antioxidant activity (% inhibition)
Brain homogenate	−	9.68±0.52	90±6.6
	+	0.96±0.20	
Dialysed brain homogenate	−	None	—
	+	None	
Dialysed brain homogenate+ascorbic acid (0.1 mM)	−	9.68±0.80	12±1.5
	+	8.71±1.00	
Dialysed brain homogenate+ascorbic acid (0.01 mM)	−	4.35±0.45	33±2.0
	+	2.66±0.32	
Dialysed brain homogenate+$FeCl_2$ (0.4mM)	−	6.77±0.81	76±4.0
	+	2.17±0.50	
Dialysed brain homogenate+$FeCl_3$ (0.4mM)	−	3.63±0.70	33±1.8
	+	2.42±0.51	

Table 4. *Antioxidant activity of caeruloplasmin in standard, preheated and 'reconstituted' brain homogenates*
For details see the Experimental section.

Preparation	Caeruloplasmin (1 g/litre)	Malonyldialdehyde (nmol/min)	Antioxidant activity (% inhibition)
Brain homogenate	−	9.62±0.51	90±6.5
	+	0.88±0.19	
Preheated brain homogenate	−	None	—
	+	None	
Preheated brain homogenate+ascorbic acid (0.1 mM)	−	9.19±1.50	66±5.7
	+	3.40±1.00	
Preheated brain homogenate+$FeCl_2$ (0.4mM)	−	5.08±1.30	83±5.5
	+	0.96±0.30	
Preheated brain homogenate+$FeCl_3$ (0.4mM)	−	2.60±0.70	73±4.8
	+	0.72±0.20	

Table 5. *Antioxidant activity of caeruloplasmin in brain homogenates exposed to u.v. irradiation*
For details see the Experimental section.

Preparation	U.v. irradiation	Caeruloplasmin (1 g/litre)	Malonyldialdehyde (nmol/ml)	Antioxidant activity (% inhibition)
Standard brain homogenate	−	−	9.68±0.52	81±5.8
	+	−	10.16±0.66	
	+	+	1.93±0.25	
Preheated brain homogenate	−	−	1.93±0.56	10±1.8
	+	−	4.48±1.00	
	+	+	4.35±1.10	
Dialysed brain homogenate	−	−	0.24±0.12	7.0±1.7
	+	−	7.98±0.50	
	+	+	7.30±0.52	

homogenate. Table 3 shows the antioxidant effect of caeruloplasmin in the 'restored' system. Preheating of the homogenate similarly abolished autoxidation. This effect too could be reversed by the addition of iron in high concentrations, or of ascorbic acid. Table 4 shows the antioxidant effect of caeruloplasmin in the 'restored' preparation. Autoxidation in a dialysed or preheated homogenate could also be induced by u.v. irradiation. (The same dose of irradiation had a slight enhancing effect on autoxidation in an untreated homogenate.) Caeruloplasmin had no antioxidant effect on irradiation-

Table 6. *Antioxidant activity of caeruloplasmin in linolenic acid emulsions*
For details see the Experimental section

Preparation	Caeruloplasmin (1 g/litre)	Malonyldialdehyde (nmol/ml)	Antioxidant activity (% inhibition)
Basic emulsion	−	None	—
	+	None	
Emulsion+$FeCl_2$ (0.01 mM)	−	8.47±1.60	35±3.8
	+	5.56±0.80	
Emulsion+$FeCl_2$ (0.04 mM)+ascorbic acid (0.1 mM)	−	11.13±1.50	68±5.4
	+	3.36±0.70	
Emulsion+$FeCl_3$ (0.4 mM)	−	2.42±0.50	10±0.8
	+	2.17±0.45	
Emulsion+$FeCl_3$ (0.4 mM)+ascorbic acid (0.1 mM)	−	8.95±1.10	52±4.1
	+	4.35±0.70	

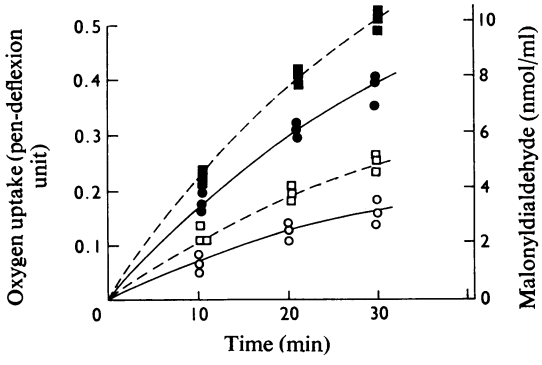

Fig. 4. *Effect of caeruloplasmin on lipid autoxidation in the ox-brain homogenate as measured by malonyldialdehyde generation* (●, ○) *and by oxygen uptake* (■, □)
Caeruloplasmin (1 g/litre) (□, ○) or buffer (■, ●) was added to parallel series.

induced autoxidation (Table 5). In view of earlier and current work on the antioxidant effect of serum (Vidlakova *et al.*, 1972; Al-Timimi, 1977), the action of purified caeruloplasmin was compared with the action of serum. Pooled normal serum (50 µl) had an antioxidant activity of 48%. In terms of increased antioxidant activity, recovery of added caeruloplasmin was linear and more than 95% up to a concentration of 0.70 g/litre corresponding to 90% inhibition.

Immediate autoxidation in a simple linolenic acid emulsion could be induced by the addition of ascorbic acid and inorganic iron. Table 6 shows the antioxidant effect of caeruloplasmin in such a comparatively simple system.

The close parallelism between autoxidation in brain homogenates as measured by malonyldialdehyde generation and as measured polarographically is illustrated in Fig. 4.

Discussion

Lipid autoxidation takes place rapidly when fresh or fresh-frozen tissue homogenates are incubated in air at 37°C (Zalkin & Tappel, 1960; Stocks *et al.*, 1974*a*; McMurray & Dormandy, 1974). Both enzymic and non-enzymic mechanisms are involved, their relative contributions depending on experimental conditions (Hochstein & Ernster, 1963; Wills, 1965, 1969; Dormandy, 1969; Bishayee & Balasubramanian, 1971; Slater, 1972). Blood serum has been shown to be a powerful inhibitor of the process, and the results described in the present paper strongly suggest that this effect is partly at least the function of caeruloplasmin. It may be noted, in particular, that the final concentration of purified caeruloplasmin in the assay system was of the same order as the caeruloplasmin concentration after the addition of serum when the assay is used for measuring serum antioxidant activity. In view of the well-established oxidase-like action of the protein (Holmberg & Laurell, 1951), this antioxidant effect may seem at first sight paradoxical. A reversible redox system is, however, essential for the chemical generation of oxidizing free radicals, and the overall effect of an oxidase that rendered the system irreversible could be that of an antioxidant. U.v. irradiation can generate oxidizing free radicals without a chemical redox system, and caeruloplasmin does not inhibit this type of autoxidation. It may also be noted that the variables that affect the antioxidant potency of caeruloplasmin, e.g. Cl^- concentration, pH, temperature and NaN_3, have been shown to have a similar effect on its oxidase-like action *in vitro* (Osaki *et al.*, 1966). A number of other possible antioxidant mechanisms could be excluded. Copper alone had no antioxidant action, and a copper-containing superoxide dismutase enzyme was also ineffective. In reconciling the oxidase-like and antioxidant properties of caeruloplasmin it may also be recalled that

the former is purely a phenomenon observed *in vitro*; there is no evidence of an oxidase-like role *in vivo*.

Fractionation by Stocks *et al.* (1974*b*) showed that the antioxidant activity of serum is due not only to a caeruloplasmin-containing fraction but also to iron-free transferrin. It was at one time suggested that the mode of action of the two proteins, both with a characteristically high affinity for a transitional trace metal, might be analogous. This suggestion must now be dismissed. The antioxidant activity of serum transferrin has been shown to depend entirely on the iron-free fraction (Al-Timimi, 1977); once the protein is saturated with iron it ceases to be an antioxidant. By contrast, copper is essential for the antioxidant action of caeruloplasmin; it is the copper-free apoprotein that is ineffective.

T. L. D. gratefully acknowledges financial support of the N.E. Thames Regional Health Authority. D. J. A.-T. is a holder of a postgraduate fellowship of the University of Baghdad.

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