diameter (Eastoe, 1955). The overall error with the semi-micro method is approximately ± 5 % for the larger peaks, compared with ± 3 % claimed for the original method (Moore & Stein, 1951). The increased error arises from the greater effect of variations in volume of test solutions and reagents together with fading. Errors for very small peaks may greatly exceed 5 %, being mainly associated with assessing the base-line.

SUMMARY

1. A semi-micro modification of the Moore & Stein ion-exchange method of amino acid determination is described, which enables a complete analysis to be carried out on 0·3–0·6 mg. of protein.

2. The increased sensitivity obtained results from a reduction of resin-column diameter from 0·9 to 0·4 cm. with a corresponding reduction of the original volume of the fractions and reagents to one-fifth.

3. A simple method of modifying a spectrophotometer for convenient measurements on small volumes of solution in standard optical cells is described.

4. Preliminary separation of basic amino acids may be carried out on a column (2·2 cm. × 0·4 cm.) of Dowex 50 at pH 5·0. Separation of hydroxylysine from histidine is possible by combining the small column with a 15 cm. column and eluting the two in series.

5. Colorimetry on the reduced scale is closely similar to the original procedure, but the rate of fading is increased.

6. The sharpness and resolution of amino acid peaks were not affected by reduction of column diameter.

7. A comparison of amino acid values for a gelatin hydrolysate analysed by the original and semi-micro methods showed good agreement. The overall accuracy of the modified method is approximately 5 %.

I should like to thank Mr J. H. Trendall for his help in making the chromatography tubes, and the British Empire Cancer Campaign for providing a spectrophotometer.

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Some Properties of Coupled Iron–Caeruloplasmin Oxidation Systems

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Caeruloplasmin, the blue copper-containing protein of plasma, has been reported to have oxidase activity *in vitro* towards many substrates. Holmberg & Laurell (1951) found activity towards *p*-phenylenediamine, quinol, catechol, pyrogallol, dihydroxyphenylalanine, adrenaline and ascorbic acid. They used purified caeruloplasmin in the presence of albumin or gelatin, which tend to eliminate any effects due to trace metals. Abood, Gibbs & Gibbs (1957), Porter, Titus, Sanders & Smith (1957), Martin, Eriksen & Benditt (1958), Sankar (1959), Nakajima (1959), Curzon & Vallet (1960) and Levine (1960) found that 5-hydroxytryptamine and some related hydroxyindoles were oxidized *in vitro* by caeruloplasmin. It is not known to what extent caeruloplasmin is involved in the metabolism of any of these substances *in vivo*.

During investigation of the effects of inorganic ions on the oxidase activity of purified human caeruloplasmin towards *NN*-dimethyl-*p*-phenylenediamine, it was found that the system was very sensitive to metals, both inhibition and activation being observed (Curzon, 1960). The most marked activating effect was found with ferrous iron. Curzon & O'Reilly (1960) showed that caeruloplasmin was able to oxidize ferrous to ferric iron and explained the activating effect of iron in terms of a coupled iron–caeruloplasmin oxidation system. This paper describes some properties of the system

and its effects on *NN*-dimethyl-*p*-phenylenediamine and on the substrates of physiological interest, 5-hydroxytryptamine, 5-hydroxyindolylacetic acid and adrenaline.

MATERIALS

A.R. grades of chemicals were used whenever obtainable. Purified human caeruloplasmin was prepared by the method of Curzon & Vallet (1960). A preparation with E_{605}/E_{280} 0·044 was used throughout except in the work described in Table 4, in which a less purified material with E_{605}/E_{280} 0·033 was used. Pure iron sponge was obtained from Johnson Matthey & Co. Ltd., London, E.C. 1. Stock ferric iron was prepared by dissolving 200 g. of ferric ammonium sulphate in 400 ml. of 5 mN-HCl and filtering. Ferrous iron solutions were freshly prepared from ferrous ammonium sulphate. 5-Hydroxytryptamine creatinine sulphate and 5-hydroxyindolylacetic acid were supplied by the Upjohn Co., Kalamazoo, Mich., U.S.A., adrenaline by British Drug Houses Ltd., Poole, Dorset, the human transferrin by Cutter Laboratories, Berkeley, Calif., U.S.A., and bovine plasma albumin by Armour Ltd., Eastbourne, Sussex. Other materials were as described by Curzon (1960). Stock reagents were stored in polythene bottles.

Purification of reagents. Since caeruloplasmin oxidation systems are very sensitive to traces of metal (Curzon, 1960), precautions were taken to avoid introduction of metal impurities. Acetic acid for preparing acetate buffers was distilled from glass and the fraction boiling between 126° and 130° collected. Sodium acetate (M) was extracted twice with 0·05 vol. portions of 0·01% of dithizone in CCl_4 by shaking occasionally during periods of 30 min. The first portion of dithizone became red–brown, indicating the presence of metal ions. The aqueous solution was then extracted four times with 0·05 vol. portions of CCl_4, twice with 0·05 vol. of 1% 8-hydroxyquinoline in $CHCl_3$, repeatedly with $CHCl_3$, then finally twice with CCl_4. 5-Hydroxytryptamine creatinine sulphate, 5-hydroxyindolylacetic acid and adrenaline (free base) were made up as 10 mM solutions in 0·2M-acetate buffer, pH 5·5, and similarly purified. Measurement of E_{278} before and after purification indicated that 4–5% of the 5-hydroxyindole material was lost during extraction procedures.

Buffers. 0·2M-Sodium acetate–acetic acid was used between pH 4·0 and 5·7. 0·2M-Sodium phosphate–sodium dihydrogen phosphate was used at pH 6·05 and 6·8.

METHODS

Apparatus was washed in Pyroneg detergent (Deosan Ltd., 42 Weymouth Street, London, W. 1) followed by water, 4N-HCl, water and deionized water. All solutions were made up in deionized water.

Determination of iron. The *o*-phenanthroline–hydroxylamine method described by Sandell (1959) was used. A primary iron standard was prepared by dissolving 500 mg. of iron sponge in 1·5 ml. of conc. H_2SO_4 and making to 1 l. with water.

Determination of oxidase activity of caeruloplasmin. Activity of caeruloplasmin towards *NN*-dimethyl-*p*-phenylenediamine dihydrochloride was measured under various conditions at 25° as previously described (Curzon, 1960). Activities are also given in terms of previously defined arbitrary units (Curzon & Vallet, 1960), of which 21 represent 1 μg. of caeruloplasmin copper.

Activities towards 5-hydroxyindoles were investigated as follows. To 2·0 ml. of various buffers (see below for details) were added 1·0 ml. of water or other additions and 1·0 ml. of 2 mM-5-hydroxytryptamine or -5-hydroxyindolylacetic acid. After incubation for 5 min. at 37°, 1·0 ml. of a suitable dilution of caeruloplasmin at 37° was added and incubation allowed to proceed at 37°. Identically treated blanks were run containing buffer, substrate and, where relevant, Fe^{2+} ions or ethylenediaminetetra-acetic acid (EDTA). At various times samples (1·0 ml.) were pipetted into conical centrifuge tubes and residual 5-hydroxyindole material was determined essentially as described by Udenfriend, Titus & Weissbach (1955) with the α-nitroso-β-naphthol colour reaction. The reagents were added rapidly to stop enzymic oxidation. The final purplish solution was centrifuged to remove denatured protein and the extinction was measured in a Unicam SP. 500 spectrophotometer with 1 cm. path microcells.

The shape of the spectral curve of the α-nitroso-β-naphthol colour obtained with the 5-hydroxyindolylacetic acid reaction mixture was identical with that obtained with pure 5-hydroxyindolylacetic acid. Residual 5-hydroxyindolylacetic acid was therefore determined from E_{540} as in the method of Udenfriend *et al.* (1955). Estimation of residual 5-hydroxyindolylacetic acid by paper chromatography gave results which agreed well with those obtained colorimetrically. The spectra obtained with 5-hydroxytryptamine digests were different from those obtained with the pure substance, and when E_{540} was used to determine residual 5-hydroxytryptamine results were much higher than when paper chromatography was used. This is because during digestion with caeruloplasmin, 5-hydroxytryptamine is oxidized to material which, on reaction with α-nitroso-β-naphthol, absorbs in the 400–600 mμ region, the absorption between 450 and 550 mμ being almost constant. The spectrum obtained with 5-hydroxytryptamine has a minimum at 450 mμ and a maximum at 546 mμ. When residual 5-hydroxytryptamine was determined from $E_{540} - E_{450}$ the results agreed well with those obtained by paper chromatography.

Activity of caeruloplasmin towards adrenaline was investigated as described above, except that 1·0 ml. of 2 mM-adrenaline was used as substrate. Adrenaline was oxidized to a pink pigment showing absorption maxima at 300 and 480 mμ characteristic of adrenochrome (Ellis & Jones, 1943). After various incubation times samples were cooled in ice–water and E_{300} was determined with 1 cm. microcells.

Paper chromatography. One-way ascending chromatograms of 5-hydroxytryptamine–caeruloplasmin and 5-hydroxyindolylacetic acid–caeruloplasmin reaction mixtures were run in the butan-1-ol–acetic acid–water system of Jepson (1955). The papers were sprayed with Ehrlich's reagent for indoles, and heated to develop colours as described by Curzon (1957). Residual 5-hydroxytryptamine and 5-hydroxyindolylacetic acid were estimated by comparison of spot areas with those of graded amounts of the relevant substances run in parallel. Brentamine fast red G.G. (British Drug Houses Ltd.) was also used as a reagent for phenolic groups (Harvey & Penketh, 1957).

RESULTS

Oxidations catalysed by the caeruloplasmin–iron system

NN-Dimethyl-p-phenylenediamine. When caeruloplasmin was added to a buffered solution of *NN*-dimethyl-*p*-phenylenediamine plus ferrous iron the oxidase activity as measured by the development of red colour was increased, as shown previously (Curzon & O'Reilly, 1960). The extinction at 550 mμ increased in an essentially linear manner with time of incubation, except with 0·1 mM-Fe^{2+} ion, which was the highest concentration of iron used (Fig. 1). If Fe^{2+} ion was preincubated with buffer plus caeruloplasmin at 25° before addition of *NN*-dimethyl-*p*-phenylenediamine, then, as the time of preincubation increased, the activation rapidly decreased (Table 1). This is probably due to the binding by various sites on the caeruloplasmin molecule of the Fe^{3+} ion formed. Under the standard conditions the *NN*-dimethyl-*p*-phenylenediamine reduces Fe^{3+} ion and protects the enzyme from the effects of this binding except at higher concentrations of Fe^{2+} ion.

When the concentration of added Fe^{2+} ion was kept constant and the activating effect measured at different concentrations of *NN*-dimethyl-*p*-phenylenediamine, percentage activation decreased as *NN*-dimethyl-*p*-phenylenediamine concentration increased (Table 2). Activation by Fe^{2+} ion was completely prevented by excess of EDTA, which also halved the activity in the absence of added iron, as shown previously (Curzon, 1960).

While 1 atom of Fe^{2+} ion/atom of caeruloplasmin copper causes reversible decolorizing of the caeruloplasmin during oxidation to Fe^{3+} ion (Curzon & O'Reilly, 1960), up to 10 atoms of Ni^{2+} or Co^{2+} ion/atom of caeruloplasmin copper did not cause decolorizing under identical conditions. Thus the definite, though relatively small, activation by these metals (Curzon, 1960) is apparently due to a different mechanism.

5-Hydroxytryptamine. The oxidation of 5-hydroxytryptamine at pH 5·5 is shown in Table 3. During incubation of 5-hydroxytryptamine with caeruloplasmin a yellow–brown colour developed. Paper chromatography showed that 5-hydroxytryptamine was destroyed and a new substance appeared, with R_F about 0·3, which gave a purple reaction with Ehrlich's reagent similar to that

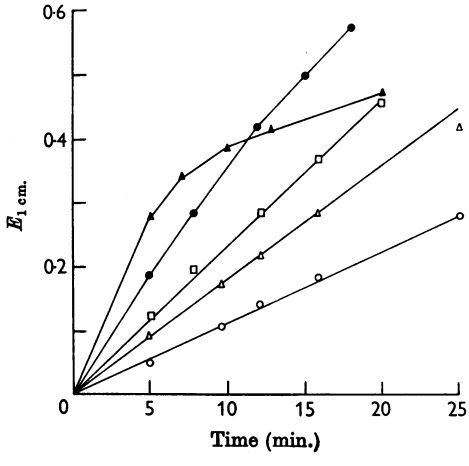


Fig. 1. Effect of ferrous iron on caeruloplasmin activity. The reaction mixture contained 0·08 M-acetate buffer, pH 5·5, 0·34 mM-*NN*-dimethyl-*p*-phenylenediamine dihydrochloride, various amounts of freshly prepared ferrous ammonium sulphate solution and 2·4 units of caeruloplasmin in a total volume of 5 ml. Incubation was at 25°; the reaction was stopped at various times by 2·0 ml. of 0·3 mM-sodium azide and $E_{1\,cm.}$ read at 550 mμ against identically treated blanks containing *NN*-dimethyl-*p*-phenylenediamine, buffer and relevant amounts of Fe^{2+} ion. ○, No Fe^{2+} ion added; △, 0·5 μM-Fe^{2+} ion; □, 2 μM-Fe^{2+} ion; ●, 10 μM-Fe^{2+} ion; ▲, 100 μM-Fe^{2+} ion.

Table 1. *Decrease of ferrous-iron activation by preincubation of ferrous iron with caeruloplasmin*

To 2·0 ml. of 0·2 M-acetate buffer, pH 5·5, at 25° were added 1 ml. of freshly prepared 0·05 mM-ferrous ammonium sulphate and 2·7 units of caeruloplasmin in 1 ml. of water at 25°. After incubation for various times, 1·0 ml. of 1·7 mM-*NN*-dimethyl-*p*-phenylenediamine held at 25° was added. After 15 min. the reaction was stopped by 2·0 ml. of 0·3 mM-sodium azide and $E_{1\,cm.}$ was read at 550 mμ. Activation by iron is expressed as percentage of *E* obtained when determinations were done by adding caeruloplasmin in buffer alone, preincubated as described above, to substrate + iron. Blanks were identically treated and contained *NN*-dimethyl-*p*-phenylenediamine and buffer and also Fe^{3+} ions when relevant.

Concn. of iron in reaction mixture (μM)	Percentage activation Preincubation times (min.)				
	0	3	15	30	60
10	220	155	31	−9	−4

Table 2. *Effect of ferrous iron on caeruloplasmin activity towards different concentrations of* NN-*dimethyl-*p*-phenylenediamine*

General conditions were as given in Fig. 1. Incubation time was 15 min.

Concn. of *NN*-dimethyl-*p*-phenylenediamine (mM)	Percentage increase of caeruloplasmin activity	
	0·38 μM-Fe^{2+} ion	1·0 μM-Fe^{2+} ion
0·67	66	146
1·0	58	137
2·0	48	95
4·0	33	83
16·0	15	53

Table 3. *Oxidation of 5-hydroxyindoles at 37° by caeruloplasmin and iron–caeruloplasmin systems*

Incubation and determinations were performed as described in the Methods section. Concentrations all refer to complete incubation mixture. 5HT, 5-Hydroxytryptamine creatinine sulphate; 5HIAA, 5-hydroxyindolylacetic acid.

Incubation mixture		5-Hydroxyindole destroyed (%) 30 min.	80 min.	170 min.
0·4 mM-5HT, pH 5·5; 8 units of caeruloplasmin	+water	10	48	86
	+0·36 μM-Fe^{2+}	10	46	86
	+1·0 μM-Fe^{2+}	—	52	—
	+9·6 μM-Fe^{2+}	0	23	50
	+0·01 mM-EDTA	—	—	86
	+0·2 mM-EDTA	—	44	86
0·4 mM-5HIAA, pH 4·5; 8 units of caeruloplasmin	+water	2	4	10
	+0·36 μM-Fe^{2+}	13	37	48
	+9·6 μM-Fe^{2+}	42	76	87
0·4 mM-5HIAA, pH 4·5; 32 units of caeruloplasmin	+water	8	20	38
	+0·01 mM-EDTA	4	12	19
	+0·2 mM-EDTA	—	14	20

Table 4. *Effect of* pH *on oxidation of 5-hydroxyindoles by caeruloplasmin*

Incubation and determinations were performed as described in the Methods section. Buffers were all 0·08 M. Acetate buffers were used between pH 4·0 and pH 5·7 and phosphate buffers at pH 6·05 and 6·8. Incubation was for 90 min. at 37°. 5HT, 5-Hydroxytryptamine creatinine sulphate; 5HIAA, 5-hydroxyindolylacetic acid.

	5-Hydroxyindole destroyed (%)							
pH	4·0	4·55	4·70	5·2	5·5	5·7	6·05	6·8
0·4 mM-5HT, 0·2 mM-EDTA; 12 units of caeruloplasmin	0	8	—	64	77	76	26	0
0·4 mM-5HIAA, 0·2 mM-EDTA; 90 units of caeruloplasmin	24	27 (44)*	27	15	6	—	0	—

* Under these conditions some of the caeruloplasmin precipitated. Figure in parentheses is an estimate obtained by calculation from the results in Table 3.

given by 5-hydroxytryptamine itself. 5-Hydroxyindolylacetic acid and 5-hydroxytryptamine had R_F 0·82 and 0·58 respectively. Table 3 indicates that no evidence was obtained for the oxidation of 5-hydroxytryptamine by a coupled iron–caeruloplasmin system. Indeed, at 9·6 μM-Fe^{2+} ion some inhibition occurred. Also, in confirmation of the results obtained by H. Blaschko and W. G. Levine (personal communication), it was shown that destruction of 5-hydroxytryptamine was not significantly affected by EDTA. In agreement with Blum & Ling (1959) some oxidation of 5-hydroxytryptamine by Fe^{3+} ion was found. Chromatography of the product of oxidation by Fe^{3+} ion showed a complex mixture of substances giving a positive reaction with Erlich's reagent and which, like the products of oxidation of 5-hydroxytryptamine by Ag^{+} ion (Eriksen, Martin & Benditt, 1960), varied qualitatively in the presence of different anions.

5-Hydroxyindolylacetic acid. Preliminary paper-chromatographic experiments in which no special precautions were taken to avoid contamination by trace metals showed that the oxidation of 5-hydroxyindolylacetic acid by caeruloplasmin had an unusually low optimum pH of about 4·5 (Curzon & Vallet, 1960). Determination of 5-hydroxyindolylacetic acid destruction by caeruloplasmin in the presence of EDTA confirmed this (Table 4). During incubation with caeruloplasmin a very faint purple developed. It can be seen from Table 3 that oxidation of 5-hydroxyindolylacetic acid by caeruloplasmin, unlike that of 5-hydroxytryptamine, is extremely sensitive to iron, as little as 0·36 μM-Fe^{2+} ion causing a five- to eight-fold increase in activity. The oxidation of 5-hydroxyindolylacetic acid was unaffected by Fe^{2+} ion in the absence of caeruloplasmin. The activity of caeruloplasmin against 5-hydroxyindolylacetic acid is roughly halved in the presence of EDTA. Paper chromatograms of samples taken at various incubation times showed that as the 5-hydroxyindolylacetic acid spot at R_F 0·82 disappeared, a new substance appeared at R_F 0·62, giving a blue colour with Ehrlich's reagent and a weak yellow colour with diazotized Brentamine fast red G.G., which changed to a weak dirty purple on standing, unlike 5-hydroxyindolylacetic acid, which gave an im-

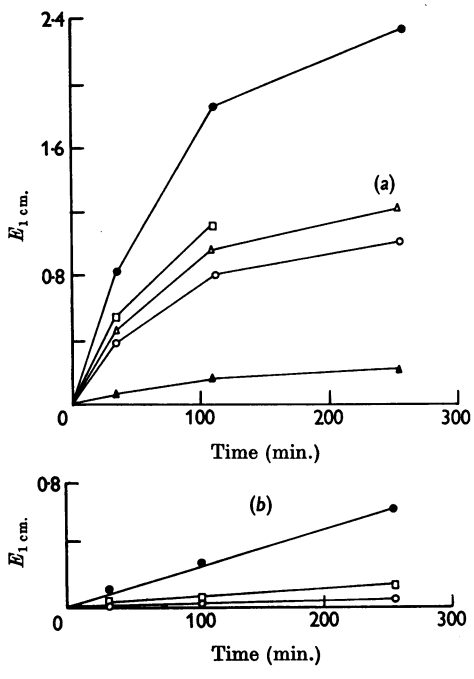


Fig. 2. Oxidation of adrenaline by caeruloplasmin and iron–caeruloplasmin systems. The reaction mixture contained 0·08 M-acetate buffer, pH 5·5, 0·4 mM-adrenaline, various additions and 8 units of caeruloplasmin in a total volume of 5 ml. Incubation was at 37°, samples were removed at various times and cooled in ice–water and $E_{1\,cm.}$ was read at 300 mμ. ○, No additions; △, 0·36 μM-Fe^{2+} ion; □, 2 μM-Fe^{2+} ion; ●, 9·6 μM-Fe^{2+} ion; ▲, 200 μM-EDTA. In (*a*) results have been corrected for blank values obtained in the absence of caeruloplasmin. Some of these are shown in (*b*), the relevant symbols being used.

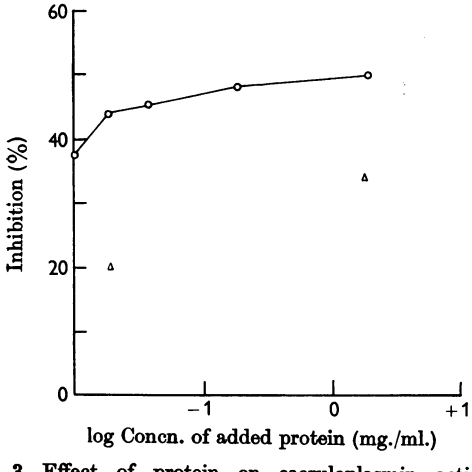


Fig. 3. Effect of protein on caeruloplasmin activity. General conditions were as given in Fig. 1. ○, Human transferrin; △, bovine plasma albumin. Caeruloplasmin present was 6·0 units; incubation time, 15 min.

mediate strong red. As incubation proceeded, the amount of oxidation product increased until it gave a spot on the chromatogram roughly equivalent in area to that of the original 5-hydroxyindolylacetic acid. Further incubation caused the spot to disappear gradually. The oxidation product was chromatographically identical with an indolic oxidation product previously produced in small yield when 5-hydroxyindolylacetic acid buffered at pH 7 was incubated in the presence of air (Curzon, 1957). The indolic oxidation product was also formed when 0·02 M-5-hydroxyindolylacetic acid was oxidized at room temperature by an equal volume of ferric ammonium sulphate containing 1–2 g.atoms of Fe^{3+} ion/mole of 5-hydroxyindolylacetic acid. Higher concentrations of Fe^{3+} ion caused destruction of the indolic oxidation product.

Adrenaline–iron–caeruloplasmin system. The rate of oxidation of adrenaline by caeruloplasmin increased in the presence of Fe^{2+} ion and was much decreased, but not abolished, by EDTA (Fig. 2). Ferrous iron in the absence of caeruloplasmin caused some oxidation of adrenaline. Both a coupled iron–caeruloplasmin oxidation and autocatalysis by accumulated adrenochrome (Falk, 1949) are possible, though it is apparent from the shape of the oxidation–time curve that the latter is not dominant.

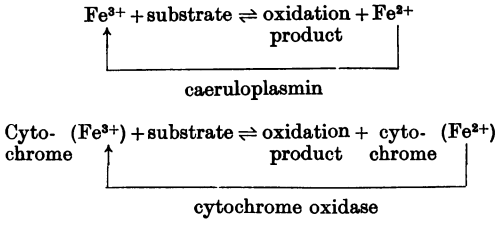
Further investigation of the oxidase activity of caeruloplasmin in the absence of added iron

Curzon (1960) found that EDTA partially inhibited the oxidation of *NN*-dimethyl-*p*-phenylenediamine by caeruloplasmin. Much evidence suggested that this was due to a direct effect of EDTA upon caeruloplasmin. On the other hand, the finding that oxidation of 5-hydroxytryptamine by caeruloplasmin was neither increased by Fe^{2+} ion nor decreased by EDTA suggests that EDTA inhibition of *NN*-dimethyl-*p*-phenylenediamine oxidation may involve chelation of activating trace metal and that in the absence of the latter EDTA would have been without effect. It has also been shown (Fig. 3) that transferrin partially inhibited activity against *NN*-dimethyl-*p*-phenylenediamine and that the curve of inhibition against log concentration of inhibitor was similar to that found previously with EDTA. Bovine plasma albumin inhibited more weakly. Transferrin also strongly inhibited the effect of added iron.

DISCUSSION

It has been known for many years that cupric copper catalyses the oxidation of ferrous iron (Lamb & Elder, 1931), and it is of interest that caeruloplasmin, the plasma copper–protein, can also catalyse this reaction. There is a formal re-

semblance between the caeruloplasmin–iron and cytochrome–cytochrome-oxidase systems which is strengthened by recent evidence for the involvement of copper in the enzymic function of cytochrome oxidase (Sands & Beinert, 1959) and by the fact that *p*-phenylenediamine and its derivatives are substrates of both systems.



While the function of the cytochrome system is well established, that of caeruloplasmin is unknown. Iron is largely absorbed from the gut in the ferrous form (Moore, Arrowsmith, Welch & Minnich, 1939) and is oxidized to ferric iron before combination with the transferrin of plasma (Koechlin, 1952). The finding that the considerable iron-oxidizing power of blood (Starkenstein & Harvalik, 1933) is unaffected by cyanide (Hendrych & Mori, 1933), which inhibits caeruloplasmin, suggests that the latter is unlikely to play much part in this oxidation. On the other hand, Chase, Gubler, Cartwright & Wintrobe (1952) have found that copper-deficient rats absorb less iron than do rats supplied with copper. S. O'Reilly (personal communication) showed that coupled iron–caeruloplasmin oxidation may be demonstrated in the presence of plasma but at higher iron concentrations than those effective with purified caeruloplasmin.

Sensitivity of the oxidase activity of caeruloplasmin to iron is markedly dependent on the nature and concentration of the substrate used. Ferrous iron is essentially a second substrate in competition with the first substrate for the active centre of the enzyme. Thus variation of iron activation with *NN*-dimethyl-*p*-phenylenediamine concentration (Table 2) is explicable in terms of substrates competing for the enzyme, although it is also possible that competition between *NN*-dimethyl-*p*-phenylenediamine and caeruloplasmin for iron may be involved since iron–*p*-phenylenediamine complex formation has been demonstrated (Hara, 1951). Any substance which is oxidizable by ferric iron is potentially oxidizable by the coupled iron–caeruloplasmin system. The effectiveness of this system and its ability to compete with a direct caeruloplasmin–substrate interaction vary widely from substrate to substrate.

Martin *et al.* (1958) found that plasma has no oxidase activity against 5-hydroxyindolylacetic acid at pH 6·0. Caeruloplasmin at pH 4·5 has small but definite activity in the absence of added iron which is about half inhibited by 0·01–0·20 mM-EDTA. The rather acid optimum, pH 4·5, is of interest as an optimum pH 5·5 has been established for many substrates (Holmberg & Laurell, 1951), and also in the present study for 5-hydroxytryptamine. Aprison, Hanson & Austin (1959) showed that, unlike 5-hydroxytryptamine, which was a competitive inhibitor, 5-hydroxyindolylacetic acid inhibited the activity of caeruloplasmin against *NN*-dimethyl-*p*-phenylenediamine non-competitively at pH 6·2 and suggested that the ionized carboxyl group of the acid was involved in the inhibited complex. This is in agreement with the finding that 5-hydroxytryptamine but not 5-hydroxyindolylacetic acid is a substrate at pH 6·05. The markedly acid optimum pH for 5-hydroxyindolylacetic acid breakdown is explicable in terms of two caeruloplasmin–5-hydroxyindolylacetic acid interactions, formation of an enzyme–inhibitor complex involving the ionized carboxyl and also of an enzyme–substrate complex. Results suggest that at pH 6·05 the former entirely predominates but at more acid pH some depression of carboxyl ionization occurs and a slight oxidase activity is manifested.

5-Hydroxyindoles may give rise to indolic autoxidation products (Curzon, 1957). The autoxidation product formed from 5-hydroxyindolylacetic acid has been found in the urine of subjects with carcinoid disease (Dalgliesh, 1956; Curzon, 1957), although it is not clear whether it is a metabolite or an artifact: certainly it tends to increase on storage of the urine. The substance behaves chromatographically like the unidentified indole U2 found by Jepson (1955) in urine of subjects with carcinoid disease. Formation of apparently identical material by oxidation of 5-hydroxyindolylacetic acid with caeruloplasmin and of the mixture of indolic oxidation products obtained by oxidation of 5-hydroxytryptamine with caeruloplasmin, in agreement with Eriksen *et al.* (1960), is of interest as it suggests a possible pathway of metabolism of 5-hydroxyindoles which could lead to a hitherto unknown series of indolic substances. As has been pointed out by these authors, there are many structural possibilities for ring-oxidation products of 5-hydroxyindoles. They give evidence supporting formation of one of the 5-hydroxytryptamine oxidation products by oxidative dimerization. Even much simpler aromatic hydroxy compounds may be oxidized to quite complex substances, e.g. the oxidation by ferricyanide of *p*-cresol to a tricyclic dimerization product analogous to usnic acid (Barton, Deflorin & Edwards, 1956) and containing an aromatic keto group but no free phenolic groups. The weak atypical phenolic reactions shown by both the 5-hydroxyindolyl-

acetic acid oxidation product and by the dimeric 5-hydroxytryptamine oxidation product (Eriksen *et al.* 1960) suggest that perhaps the reactions by which they are formed may be to some extent analogous to the *p*-cresol oxidation.

Interpretation of the partial inhibition by EDTA of *NN*-dimethyl-*p*-phenylenediamine oxidation by caeruloplasmin (Curzon, 1960) presents considerable difficulty. The effect may reasonably be considered as due either to chelation of activating contaminant metal or to a direct interaction between caeruloplasmin copper and EDTA. The first explanation is consistent with the great enhancement of *NN*-dimethyl-*p*-phenylenediamine oxidation by iron, which is inhibited by EDTA, by the lack of effect of both iron and EDTA on the oxidation of 5-hydroxytryptamine by caeruloplasmin and also by the partial inhibition of the *NN*-dimethyl-*p*-phenylenediamine oxidation by transferrin. Use of transferrin for investigation of the significance of iron in enzyme systems has, however, been criticized by Singer & Massey (1957) because of anomalous results obtained with succinic dehydrogenase.

A direct interaction between caeruloplasmin and EDTA is consistent with the lack of effect on inhibition by EDTA of all procedures for the removal of contaminant metal from the system and by the undetectability of significant contaminant metal in the system (Curzon, 1960). In addition, Humoller, Mockler, Holthaus & Mahler (1960) find that EDTA gives 50% inhibition of *NN*-dimethyl-*p*-phenylenediamine oxidation by caeruloplasmin in 0·66M-acetate buffer, pH 6·0, though at the much higher EDTA concentration of 1·5 mM. This corresponds exactly to the discontinuity in the percentage inhibition–log EDTA concentration curve demonstrated by Curzon (1960). This exact quantitative agreement between two independent series of experiments in which different conditions and materials were used suggests that a direct interaction between caeruloplasmin and EDTA may be involved rather than contamination by trace metals. The evidence for 50% accessibility of caeruloplasmin copper to EDTA is paralleled by its 50% exchangeability with ^{64}Cu (Scheinberg & Morell, 1957) and its 50% dialysability after chymotryptic digestion (Curzon, 1958).

The uncertainties inherent in the interpretation of the effect of a metal-binding agent on an enzyme which contains metal bound in two different ways and which is also affected by traces of extraneous metal are, however, considerable and caution is necessary in the interpretation of results.

SUMMARY

1. Some properties of coupled iron–caeruloplasmin oxidation systems have been investigated.

2. Ferrous iron enhances the activity of caeruloplasmin against *NN*-dimethyl-*p*-phenylenediamine, the effect decreasing with increasing *NN*-dimethyl-*p*-phenylenediamine concentration and upon preincubation of iron with caeruloplasmin. Enhancement is prevented by ethylenediaminetetra-acetic acid or excess of protein.

3. 5-Hydroxytryptamine and 5-hydroxyindolylacetic acid are oxidized to indolic products by caeruloplasmin, the optima being about pH 5·5 and 4·5 respectively.

4. Oxidation of 5-hydroxytryptamine by caeruloplasmin is neither inhibited by ethylenediaminetetra-acetic acid nor enhanced by iron under the conditions used. Oxidation of 5-hydroxyindolylacetic acid is partly inhibited by ethylenediaminetetra-acetic acid and greatly enhanced by iron.

5. Oxidation of adrenaline to adrenochrome by caeruloplasmin is partly inhibited by ethylenediaminetetra-acetic acid and is enhanced by iron.

6. The inhibition of caeruloplasmin by ethylenediaminetetra-acetic acid is discussed in the light of these and of earlier findings.

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Inactivation of Arginine Vasopressin by Rat-Kidney Slices

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Previous studies have indicated that the mammalian kidney can inactivate as well as excrete vasopressin. Only 5–10% of a dose of vasopressin injected intravenously into rats can be recovered from the urine (Heller, 1952; Ginsburg, 1954), although the kidneys have been found responsible for removing approximately half of the injected hormone from the circulating blood (Ginsburg & Heller, 1953; Dicker, 1954; Crawford & Pinkham, 1954; Lauson & Bocanegra, 1960). There is suggestive evidence *in vivo* that this unexcreted vasopressin is destroyed by the kidney (Heller & Zaidi, 1957). At the present time, however, little is known about the nature of the cellular or enzymic processes involved in this inactivation of vasopressin. In the present paper we have attempted *in vitro* to develop a system useful for such investigations. Also reported are studies in which vasopressin, labelled *in vivo* with [^{35}S]cystine, has been used in an effort to study the manner in which this polypeptide hormone is inactivated by rat-kidney slices.

EXPERIMENTAL

Materials. Arginine vasopressin was purified as described by Sachs (1960*a*) with an estimated potency of 300–400 i.u./mg. This material showed only a single component when subjected to paper ionophoresis (Sachs, 1960*a*) or paper chromatography (Heller & Lederis, 1958). Oxytocin was highly purified synthetic oxytocin with a stated potency of 450 i.u./ml. from Sandoz Co., Hanover, N.J., U.S.A. Heparin was from Upjohn Co., Kalamazoo, Mich., U.S.A. Phenoxybenzamine hydrochloride (Dibenzyline) was from Smith, Kline and French Laboratories, Philadelphia, Pa., U.S.A.

Preparation of kidney slices. Male Sprague–Dawley rats weighing from 200 to 350 g. were stunned by a blow on the head and killed by decapitation. A midline incision was made and both kidneys, freed from perirenal fat and decapsulated, were placed in ice-cold Cross & Taggart (1950) buffer containing 0·01 M-sodium acetate. Longitudinal sections of 0·5 mm. thickness were cut with a Stadie–Riggs microtome; the first slice on each side of the kidney was rejected and the second slice placed in fresh ice-cold buffer. Each slice weighed about 75 mg. and contained both cortical and medullary tissue. These operations were performed at 6°.

Incubation procedure. Unless otherwise stated, each incubation flask contained four kidney slices in 4 ml. of Cross & Taggart buffer (containing about 300 mg. of tissue). Slices and buffer were initially shaken for 30 min. at 25° under O_2, after which time the total wet weight of the slices was determined and the slices were transferred to 4 ml. of fresh buffer. Vasopressin (3 i.u.) was added and the incubation continued, under the same conditions, for a further time interval ranging from 30 to 120 min. During this second incubation period, measured portions of the incubation fluid were periodically taken for assay; the samples were pipetted into 5% trichloroacetic acid to stop any enzymic reaction. The trichloroacetic acid was subsequently removed by ether extraction (4 × 3 vol.). Ether dissolved in the aqueous phase was evaporated in a stream of N_2 at 40°, and the samples were assayed for pressor activity.

Assay of pressor activity. The method of Dekanski (1952) was used except that Dibenzyline (1 mg./100 g. body wt.) was injected intramuscularly 12–24 hr. before the beginning of assay. Anaesthesia was induced by intraperitoneal

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