decanoic (margaric) acid (Hansen, Shorland & Cooke, 1957) and the branched-chain (+)-14-methylhexadecanoic acid and 15-methylhexadecanoic acid (Hansen & Shorland, 1951).

SUMMARY

1. *cis*- Δ^9 -Heptadecenoic acid has been isolated from butterfat and conclusively identified.
2. The weight of *cis*- Δ^9 -heptadecenoic acid isolated from the sample of butterfat under investigation represented approximately 0.06 % of the total weight of fatty acids.
3. Odd-numbered unsaturated fatty acids have not formerly been found in butterfat.

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The Effects of some Ions and Chelating Agents on the Oxidase Activity of Caeruloplasmin

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Caeruloplasmin, the blue cuproprotein of plasma, has oxidase activity against a variety of substrates. Holmberg & Laurell (1951) showed that the activity was markedly affected by anions. Complex effects were obtained, activity being increased by anions in low concentration and decreased by the same anions in higher concentration. Broman (1958) reported inhibition of crude caeruloplasmin by dicarboxylic acids and ethylenediaminetetraacetic acid. It was suggested that the effect of the latter was due not to a direct interaction with caeruloplasmin, but to the removal of catalytically active metal-protein complexes present as contaminants.

In the work described below, the effects of some

anions, cations and chelating agents on the oxidase activity of purified caeruloplasmin were investigated.

METHODS AND MATERIALS

The apparatus was washed and the solutions were made up as described by Curzon & Vallet (1960).

Chemicals. A.R. grades were used whenever obtainable. *NN*-Dimethyl-*p*-phenylenediamine dihydrochloride (British Drug Houses Ltd., Poole, Dorset, England), supplied in sealed ampoules, was subdivided into 100 mg. lots and resealed into ampoules under vacuum and stored at -25° . Purity was 99 % with respect to free base. Trace-metal contamination was 10 p.p.m. of Ba, 4 p.p.m. of Al, Ca, Fe, Mg, Na and Ni, and 0.4 p.p.m. of Cr and Mn. These figures are rough estimates obtained from the estimated

upper limits by spectrographic analysis and the sulphated ash content provided by the manufacturers. Tests for Fe were also carried out with $\alpha\alpha'$ -dipyridyl (Vogel, 1946) with ammonium sulphide to reduce ferric iron. A negative reaction was obtained. The lowest concentration of Fe detectable by this method in the presence of substrate was equivalent to 10 p.p.m. of substrate. Solutions of substrate were stored at -25° .

Measurement of pH. A pH meter with an accuracy of ± 0.02 pH unit was used. Usually no change in pH was detectable during determinations of oxidase activity, though occasionally changes of ± 0.03 pH unit were observed.

Caeruloplasmin. This was prepared from the G2 fraction of human plasma by the method of Curzon & Vallet (1960), which includes a final purification on a diethylaminoethyl-cellulose column. Selected eluate fractions were dialysed against water at 4° and stored at 4° . Oxidase activity of the caeruloplasmin so obtained was 44–48 units/ml. A unit was defined as the activity of an amount of caeruloplasmin which on incubation with *NN*-dimethyl-*p*-phenylenediamine oxalate, under conditions described by Curzon & Vallet (1960), resulted in an $E_{1\text{ cm.}}$ at $550\text{ m}\mu$ of 0.10 (21 units $\equiv 1\text{ }\mu\text{g.}$ of caeruloplasmin copper). Activity of the preparations fell 2–4% during 4–6 weeks of storage at 4° . Curzon & Vallet (1960) found that comparable preparations lost activity more rapidly at -25° . The reason for this is not known.

Determination of caeruloplasmin activity. *NN*-Dimethyl-*p*-phenylenediamine was oxidized by caeruloplasmin and the extinction (E) of the red reaction product measured at $550\text{ m}\mu$.

The method described by Curzon & Vallet (1960) was worked out specifically for following caeruloplasmin activity through the stages of its purification from plasma. However, this method involves a too complex ionic environment to be of use in the study of the effects of ions on activity, for both acetate buffer and sodium chloride are present. Moreover, the substrate was used as the oxalate salt. This salt is more stable in the solid state and thus has certain advantages over the more frequently used hydrochloride. Also the oxalate ion inhibits non-enzymic oxidation of the substrate by contaminant metal. A serious disadvantage, however, in kinetic work is that oxalate causes significant inhibition of enzyme activity, resulting in an obscuring of the effects of other inhibitors and in a deviation from the Michaelis curve at high substrate concentration. Therefore *NN*-dimethyl-*p*-phenylenediamine dihydrochloride was used. With this substrate, results fitted Michaelis curves, and linear Lineweaver–Burk graphs were obtained.

To 2.0 ml. of acetate buffer, pH 5.5, of chosen molarity were added 1.0 ml. of either water or a solution of the substance under investigation and 1.0 ml. of substrate solution. After equilibration for 5 min. in a water bath at 25° or 37° , 1.0 ml. of caeruloplasmin solution at the same temperature was added. Appropriate blanks were run simultaneously. After 30 min. at 25° or 15 min. at 37° the reaction was stopped by adding 2.0 ml. of 0.3 mM-azide and $E_{1\text{ cm.}}$ was read at $550\text{ m}\mu$ as described by Curzon & Vallet (1960), the only difference being that when determinations were performed at 25° , the azide was added at room temperature. It was found convenient to run 15–20 tubes at a time and to read extinctions within 7 ± 2 min. of

adding the azide so that errors, due to any slow changes in extinction, were negligible. All determinations were duplicated and variation of duplicates from the mean averaged $\pm 1\%$.

RESULTS

Preliminary experiments. When caeruloplasmin was incubated with substrate at 25° or 37° as described above, $E_{550\text{ m}\mu}$ was directly proportional to the amount of caeruloplasmin within the range, $E_{1\text{ cm.}}$ 0.10–0.60. Caeruloplasmin concentrations in subsequent experiments were chosen so that this range was not exceeded. With incubation for 15 min. at 37° , $E_{1\text{ cm.}} = 2.3 \times$ units of caeruloplasmin $= 48 \times \mu\text{g.}$ of caeruloplasmin copper. $E_{1\text{ cm.}}$ was also directly proportional to the time of incubation with substrate. In the presence of 0.03 M-sodium chloride or 0.2 mM-sodium oxalate inhibition occurred, but a linear relationship of $E_{1\text{ cm.}}$ with time was still maintained. Thus $E_{1\text{ cm.}}$ after 15 min. incubation at 37° or 30 min. incubation at 25° may be used as a measure of initial velocity of the enzymic oxidation. There was no evidence of any

Fig. 1. Effect of pH on caeruloplasmin activity. Buffers were 0.08 M. Acetate buffers were used between pH 4.0 and 5.75 and phosphate buffers between pH 5.68 and 6.7. Incubation mixtures contained substrate, 0.34 mM-*NN*-dimethyl-*p*-phenylenediamine dihydrochloride; caeruloplasmin, 2.6 units by the method of Curzon & Vallet (1960), in a total volume of 5 ml. Incubation was at 37° for 15 min. The reaction was stopped by addition of 2.0 ml. of 0.3 mM-sodium azide and $E_{1\text{ cm.}}$ at $550\text{ m}\mu$ was measured.

induction period. All kinetic investigations were done at pH 5.5, which appears to be optimum for caeruloplasmin activity under the present experimental conditions (Fig. 1). There are difficulties in the determination of pH-activity curves, as acetate buffers have sufficient buffering capacity only in the range pH 4.0–5.8, and above pH 5.8, effective buffers are themselves relatively inhibitory. It is apparent from Fig. 1 that phosphate is strongly inhibitory relative to acetate. The pH-activity curve with *NN*-dimethyl-*p*-phenylenediamine as substrate is similar to that found by Holmberg & Laurell (1951), who used *p*-phenylenediamine.

Comparison of the inhibitory effects of some anions. The anions, thiocyanate, fluoride, chloride, bromide and sulphate (in decreasing order of effect) inhibit the oxidase activity of caeruloplasmin. Plots of $1/E$ against salt concentration are reasonably linear (Fig. 2). The effect of chloride was investigated more fully. The degree of inhibition was the same whether lithium, sodium or potassium chloride was used; it was not influenced by pre-treatment of the caeruloplasmin with chloride, inhibition being dependent only on the chloride

concentration present during incubation with substrate.

Acetate buffer also has some inhibitory effect but in this case the graph of $1/E$ against acetate concentration is concave (Fig. 5).

Kinetics of inhibition

Acetate buffer. The effect of varying the concentration of acetate buffer, pH 5.5, on activity at various substrate concentrations was determined and expressed in the Lineweaver-Burk form. A series of lines was obtained with a definite suggestion of parallelism (Fig. 3). It was only possible to produce limited activity changes by varying acetate concentration and thus there was some doubt about the significance of this. Parallel Lineweaver-Burk graphs indicate an anti-competitive mechanism in which the inhibitor combines with the enzyme-substrate complex but not with the free enzyme (Burk, 1944).

Chloride. When the buffer concentration was kept constant and inhibition by chloride determined at various substrate concentrations, Lineweaver-Burk graphs were clearly parallel (Fig. 4). Thus chloride is an anti-competitive inhibitor. The general equation for anti-competitive inhibition,

Fig. 2. Effect of anions on caeruloplasmin activity. The buffer was 0.08 M-acetate, pH 5.5; other conditions were as in Fig. 1. $\circ$, SO_4^{2-} ; $\triangle$, Br^- ; $\square$, Cl^- ; $\bullet$, F^- ; $\blacktriangle$, SCN^- . Sodium salts were used, except for SCN^- , which was the potassium salt; the change of cation had no effect (see text).

Fig. 3. Effect of variation of the concentration of acetate buffer, pH 5.5, on caeruloplasmin activity at various substrate concentrations. Results are plotted as a Lineweaver-Burk graph; general conditions were as in Fig. 1 but the caeruloplasmin present was 2.9 units. $\circ$, 0.01 M-Acetate; $\triangle$, 0.2 M-acetate; $\square$, 0.4 M-acetate.

assuming Michaelis kinetics (Friedenwald & Maengwyn-Davies, 1954), is:

$$1/V = 1/V_{\max.} + K_s/V_{\max.}S + I/V_{\max.} K_i,$$

where V is the reaction velocity at substrate concentration S ; $V_{\max.}$, the maximum velocity in the presence of inhibitor; K_s , the dissociation constant of the enzyme-substrate complex; K_i , the dissociation constant of enzyme-substrate-inhibitor complex to enzyme-substrate complex + inhibitor; I , the concentration of inhibitor

From this it may be shown that the negative intercepts on the $1/S$ axis (the reciprocals of the apparent Michaelis constants) are directly proportional to inhibitor concentration:

$$1/K_m = 1/K_m^0(1 + I/K_i),$$

where K_m is the Michaelis constant, at inhibitor concentration I and K_m^0 , the Michaelis constant at zero concentration of the inhibitor.

The intercept and gradient of the $(1/K_m)-I$ graph (not shown) give $1/K_m^0$ and $1/(K_m^0 K_i)$ respectively: Thus K_i can be evaluated graphically and for chloride a value of 28 mm was obtained. In acetate inhibition the curve of $1/E$ against acetate concentration is concave, there is thus no linear relationship between $1/K_m$ and acetate concentration, and K_i is not directly obtainable.

Fig. 4. Effect of sodium chloride on caeruloplasmin activity at various substrate concentrations. Results are plotted as a Lineweaver-Burk graph. General conditions were as in Fig. 2. $\circ$, Sodium chloride absent; $\triangle$, 0.02 M-sodium chloride; $\square$, 0.04 M-sodium chloride; $\bullet$, 0.05 M-sodium chloride. The caeruloplasmin present was 2.9 units.

Fig. 5. Effect of carboxylic acids on caeruloplasmin activity. Conditions were as in Fig. 2, except that the mixture was incubated at 25° for 30 min. and 3.3 units of caeruloplasmin were present. The acids were in the form of sodium salts. The effect of variation of concentration of acetate buffer, pH 5.5, is also shown, zero inhibition referring to activity in 0.01 M-buffer. Conditions were as in Fig. 2 except that the caeruloplasmin present was 2.25 units. $\circ$, EDTA; $\triangle$, oxalic acid; $\square$, malonic acid; $\bullet$, acetate buffer, pH 5.5.

Table 1. Effect of some carboxylic acids and chelating agents on caeruloplasmin activity

The reaction mixture contained 0.08 M-acetate, buffer, pH 5.5, 0.34 mm-*NN*-dimethyl-*p*-phenylenediamine dihydrochloride, 3.3 units of caeruloplasmin in a total volume of 5 ml. Incubation was at 25° for 30 min. The reaction was stopped by 2.0 ml. of 0.3 mm-sodium azide and $E_{1\text{ cm}}$ read at 550 m μ . The carboxylic acids and diamines were used as the disodium salts and the sulphates respectively.

Inhibitor	Concn. (mm)	Inhibition (%)
Succinic acid	2	0
Glutaric acid	2	-2
Maleic acid	0.05	34
Maleic acid	0.01	23
Fumaric acid	0.2	0
Phthalic acid	10	12
isoPhthalic acid	10	-3
Terephthalic acid	10	4
1:2-Diaminoethane	10	20
1:3-Diaminopropane	10	11
1:4-Diaminobutane	10	6

Chelating agents. The inhibitory effects of acetic acid, oxalic acid, malonic acid and ethylenediaminetetra-acetic acid (EDTA) are shown in Fig. 5 and the results of tests on some related substances are summarized in Table 1. Oxalic acid was a more powerful inhibitor than malonic acid and higher saturated aliphatic dicarboxylic acids had no effect. Maleic acid was an inhibitor but fumaric acid at comparable concentration was without effect. The phthalic acids had insignificant effects whereas 1:2-diaminoethane, which is a powerful chelator, and its higher homologues were only weakly inhibitory. The effect of EDTA is of some interest as there is a sharp discontinuity in the graph of inhibition against log (inhibitor concentration). This has been observed with crude pre-

Fig. 6. Effect of EDTA on caeruloplasmin activity at various substrate concentrations. Results are plotted as a Lineweaver-Burk graph. General conditions were as in Fig. 5; caeruloplasmin present was 3.1 units. $\circ$, EDTA absent; $\triangle$, $1 \mu\text{M}$ -EDTA.

parations by Broman (1958), who attributed it as probably due to part of the oxidase activity being associated with loose protein-metal complexes, which are present as impurities but from which the metal is removable by EDTA. This is unlikely in the present experiment as purified caeruloplasmin was used. However it has been shown (see below) that trace metals, particularly iron, have an apparent activating effect on caeruloplasmin. The possibility of significant activating metal being

Fig. 7. Protection by acetate buffer and sodium oxalate of caeruloplasmin activity against inhibition by sodium chloride. General conditions were as in Fig. 1. $\circ$, 0.02M-Sodium chloride in the presence of acetate buffer; caeruloplasmin, 2.2 units; $\triangle$, 0.02M-sodium chloride in the presence of sodium oxalate; $\square$, 0.01M-sodium chloride in the presence of sodium oxalate. The sodium oxalate experiments were all done in 0.01M-acetate buffer with 2.9 units of caeruloplasmin.

Fig. 8. Apparent activation of caeruloplasmin by metals. Conditions were as in Fig. 5. $\circ$, Fe^{2+} ; $\triangle$, Mn^{2+} ; $\square$, Co^{2+} ; $\bullet$, Ni^{2+} ; $\blacktriangle$, Zn^{2+} ; $\blacksquare$, Fe^{3+} . The metals were used as sulphates except for Ni^{2+} and Fe^{3+} which were the nitrate and chloride respectively. The caeruloplasmin present was 1.4 units.

present as a contaminant in the enzyme, substrate or buffer was therefore investigated; particular attention was paid to iron. Caeruloplasmin preparations were tested for iron with $\alpha\alpha'$ -dipyridyl as described above, and gave no detectable colour. This means that in the mixture for determination of activity, iron from the caeruloplasmin was $< 10^{-7}M$. The linear relationship between activity and caeruloplasmin concentration is evidence

against activating metal being present in the enzyme preparation unless so much is present that the enzyme is saturated with it (Dixon & Webb, 1958). Also, the addition of enzyme that had been inactivated by acid and subsequently neutralized, in equivalent amount to the intact caeruloplasmin, had no effect on the activity. This is evidence against activating metals being present as a contaminant in the caeruloplasmin.

There was also no evidence of significant activating metal being present in the substrate. Neither purification by vacuum sublimation nor a tenfold increase of the trace metals known to be present had significant effect on caeruloplasmin activity or its inhibition by EDTA. In addition the linear Lineweaver-Burk graphs obtained even at high substrate concentrations are evidence against the presence of activating metal in the substrate.

Finally, activating metal was not detectable in the buffer. Redistillation of the acetic acid and removal of trace metals from the sodium acetate by dithizone and 8-hydroxyquinoline extraction had no significant effect on caeruloplasmin activity or EDTA inhibition under conditions as in Fig. 5. Thus in general it appeared reasonable that the partial inhibition of caeruloplasmin by EDTA was due not to removal of 'complexable' activating metal but to a direct effect of EDTA on the caeruloplasmin (see Discussion).

The effect of substrate concentration on the inhibition of caeruloplasmin by EDTA is shown in Fig. 6. The inhibition was non-competitive and the partially inhibited enzyme lowered the Michaelis constant.

Protection of caeruloplasmin against chloride inhibition by acetate or oxalate. Inhibition of oxidase activity by chloride ions decreased as the concentration of the acetate buffer increased (Fig. 7). Similarly, at a fixed concentration of acetate buffer the inhibitory effect of chloride decreased in the presence of oxalate. Thus both acetate and oxalate protect caeruloplasmin against inhibition by chloride.

Effect of heavy metals

As it has been suggested that inhibition by EDTA was due to removal of metal ions, the effects on activity of various metallic salts was investigated.

Inhibition. The inhibitory effects of some heavy-metal ions are shown in Table 2. Fe^{2+} ions caused considerably increased activity in the first few minutes of incubation followed by inhibition. Higher concentrations of Fe^{2+} ions, but not of other metal ions tested, caused fading of the red oxidation product and Fe^{3+} itself oxidized the substrate. Thus in addition to enzymic effects the following equilibrium reaction may occur:

It was not convenient to study the effect of adding copper at concentrations comparable to those of related metals because of the direct catalytic effect of Cu^{2+} on the oxidation of the substrate. Ni^{2+} ions, Hg^{2+} ions and the trivalent ions Al^{3+} and In^{3+} showed considerable inhibitory power. The effect of In^{3+} ions was particularly marked, $2\mu M$ - In^{3+} causing a 50% inhibition at a caeruloplasmin-copper concentration of $0.6\mu M$.

Activation. Activation by low concentrations of Fe^{2+} ions (see above) was confirmed and compared with the effects of other metals (Fig. 8). Comparison is made difficult by the inhibitory effects, which become obvious at higher concentrations. However, at $2\mu M$ -metal the order of activating power of the ions is $Fe^{2+} > Ni^{2+} > Zn^{2+} > Co^{2+}$. Fe^{3+} ions caused less activation than Fe^{2+} ions. $2\mu M$ - Ca^{2+} , $-Mg^{2+}$, $-Mn^{2+}$ and $-Cu^{2+}$ ions gave no activation and $2\mu M$ - Cr^{3+} ions inhibited slightly. Cu^{2+} and Fe^{3+} ions were the only metal ions tested which caused any direct oxidation of the substrate.

DISCUSSION

Holmberg & Laurell (1951) showed that low concentrations of anions activated caeruloplasmin, whereas higher concentrations caused inhibition. In the present study, anion inhibition only was found, presumably because the acetate buffer present was sufficient to saturate the relevant

Table 2. *Inhibition of caeruloplasmin activity by metal ions*

General conditions were as given in Table 1. The metals were used as sulphates with the exceptions of Hg^{2+} and Fe^{3+} , which were chlorides, and Ni^{2+} , which was used as the nitrate. Concentrations marked with an asterisk are approximate.

Metal ions	Concn. (μM)	Inhibition (%)
Fe^{2+}	200	43
Co^{3+}	200	-4
Ni^{2+}	200	57
Zn^{2+}	200	30
Hg^{2+}	10*	50
Fe^{3+}	200	23
Al^{3+}	25*	50
In^{3+}	2.5*	50

activation centres. Ions may cause enzyme inhibition by a non-specific suppression of ionization of the centres in the enzyme which are necessary for activity. With this mechanism, an example of which is the inhibition of urease by salts (Kistakowsky & Shaw, 1953), inhibition is entirely related to ionic strength and is independent of the nature of the ions present. It is apparent from the differences in inhibitory power shown by the ions investigated here that this non-specific effect is not a major factor in the inhibition of caeruloplasmin activity by anions. The order of inhibitory power of the anions thiocyanate > fluoride > chloride > bromide > sulphate is in agreement with Holmberg & Laurell's (1951) finding thiocyanate > chloride > nitrate.

Caeruloplasmin partly inhibited by acetate and oxalate is protected against chloride inhibition. This indicates that these ions bind either at the same inhibitory sites as chloride ions or at sites so close that binding of chloride ions at one group of sites is prevented by the presence of acetate or oxalate at adjacent sites. A practical conclusion that can be drawn is that in the determination of plasma-caeruloplasmin activity it is advantageous to use a concentrated acetate buffer. Although this results in decreased sensitivity, it also decreases interference by anions.

Chloride, and probably acetate, apparently inhibit caeruloplasmin by the anti-competitive or uncompetitive mechanism (Burk, 1944; Friedenwald & Maengwyn-Davies, 1954) in which the inhibitor combines with the enzyme-substrate complex but not with the free enzyme. Few examples of anti-competitive inhibition are known. Dodgson, Spencer & Williams (1956) showed that inhibition of the arylsulphatase of *Alcaligenes metalcaligenes* by cyanide and hydrazine was an example and suggested that the inhibition of pepsin by hydrazine (Schales, Suthon, Roux, Lloyd & Schales, 1948) was explicable by the same mechanism. Lineweaver-Burk graphs of the inhibition of fumarase by sodium chloride (Alberty & Bock, 1953) suggest that this may be a further example. It is not known if the inhibitory anions are bound to the caeruloplasmin-substrate complex in the region of the point of substrate attachment. However, this is a reasonable assumption since modification of the enzyme by the substrate is most likely in this area. Anti-competitive inhibition by anions implies either that the enzyme-substrate complex possesses a site which is not present in the free enzyme and to which the inhibitor can attach itself (e.g. the arylsulphatase-*p*-nitrophenyl sulphate complex studied by Dodgson, Spencer & Williams, 1955) or that substrate binding induces a change in enzyme geometry so that sites become available for anion binding.

Chloride and acetate ions could possibly inhibit activity by forming caeruloplasmin-copper-anion complexes, substituting singly in the co-ordination shell of copper. Mapson (1941) found catalysis of ascorbic acid oxidation by copper ions to be inhibited by chloride and suggested that this was due to combination of copper with chloride ions. The availability of the copper of the enzyme-substrate complex but not of the resting enzyme, to certain ligands such as chloride ions, finds a parallel in the work of Scheinberg & Morell (1957), who showed that caeruloplasmin copper is available for exchange with ^{64}Cu only in the presence of substrate.

The relative inhibitory effects of the saturated dicarboxylic acids is, to some extent, explicable by the relative stabilities of the copper complexes. Peacock & James (1951) showed that as the number of carbon atoms between the carboxyl groups increased, the complex stability decreased. The reasons why maleic acid and oxalic acid are of comparable inhibitory power and the reasons for the lack of effect of fumaric acid and phthalic acid and ethylenediamine are not clear. Vallee (1955) points out that stabilities of complexes obtained with simple ionic systems are not necessarily related to the more complex metal-protein-inhibitor complexes. The relative inhibitory effects of carboxylic acids found here are considerably different from the order maleic acid > fumaric acid > salicylic acid, phthalic acid > oxalic acid, found by Broman (1958) in the presence of EDTA. This is probably due to differences in pretreatment of the caeruloplasmin and of pH, buffer and incubation temperature. Scheinberg, Aisen & Morell (1959) obtained evidence from titration curves that copper-carboxyl bonds are present in caeruloplasmin. Thus inhibition by carboxylic acids may involve breaking of these bonds and formation of new bonds with the inhibitor. As the copper-carboxyl bond is largely ionic (Sen, Mizushima, Curran & Quagliano, 1955), this would probably require little activation energy.

Low concentrations of metal ions, in particular Fe^{2+} , apparently increase caeruloplasmin activity. Therefore the discontinuous graph of inhibition against $\log [\text{EDTA}]$ and the concave $1/v$ -inhibitor-concentration graphs for oxalate and malonate could well have been due to removal of contaminant activatory metal. Effects of EDTA on activities of non-metallic enzymes are usually explained as being due to chelation of contaminant metal. Interpretation is obviously more difficult when the enzyme itself contains metal. Considerable evidence was obtained mainly from kinetic measurements, and from the lack of effect of purification procedures on inhibition by EDTA, against significant contaminant activating metal being present in the caeruloplasmin, buffer and

substrate used. However, the findings that 0.01 mg. of purified iron-binding β -globulin/ml. inhibits activity 40%, and that 2 mg. of iron-binding β -globulin/ml. only inhibits to a slightly greater extent, strongly indicate that iron is present at very approximately $10^{-7}M$ in the system (G. Curzon, added 13 July 1960) as the iron-binding capacity of the β -globulin was about 0.5 mg. of Fe/g. of protein at pH 5.5. Thus the inhibition by EDTA is very possibly largely due to chelation of trace amounts of iron. The sharp break in the curve of inhibition against log [EDTA] was not found when the oxalate salt of the substrate was used (Curzon & Vallet, 1960). Under these conditions it is obscured by the chelating effect of oxalate on trace iron.

Activation by metal ions at low concentration with inhibition at higher concentration has frequently been shown in enzyme studies (Dixon & Webb, 1958). Caeruloplasmin appears particularly susceptible to metal ions, both activation and inhibition occurring at very low concentration. The marked activatory effect of ferrous ions is explicable by a coupled iron-caeruloplasmin oxidation system (Curzon & O'Reilly, 1960) and may perhaps be of some physiological significance.

SUMMARY

1. The oxidase activity of purified human caeruloplasmin was studied with *NN*-dimethyl-*p*-phenylenediamine as substrate by measuring the extinction of the red reaction product.

2. Anions caused inhibition in the order thiocyanate > fluoride > chloride > bromide > sulphate. The acetate buffer used also had some inhibitory effect.

3. Caeruloplasmin, which is partly inhibited by acetate or oxalate, is protected against inhibition by chloride.

4. Inhibition by chloride, and probably by acetate, is consistent with an anti-competitive mechanism.

5. The effects on activity of some chelating agents were studied. Ethylenediaminetetraacetate (EDTA) inhibits non-competitively. The inhibition-log (EDTA concentration) curve, unlike

the corresponding curves of oxalic acid and malonic acid, is sharply discontinuous.

6. Activity is increased by Fe^{2+} ions and to a lesser extent by Ni^{2+} , Co^{2+} , Zn^{2+} and Fe^{3+} ions at low concentrations. The same metals decrease activity at higher concentrations. Activity is also decreased by low concentrations of Hg^{2+} , Al^{3+} and Ln^{3+} ions.

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