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ETHYLENEDIAMINETETRAACETATE, IRON AND CERULOPLASMIN ACTIVITY

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SUMMARY

1. The influence of iron and EDTA on the oxidase activity of ceruloplasmin was studied using psilocin (*N*,*N*-dimethyl-4-hydroxytryptamine) and *p*-phenylenediamine as substrates.

2. Fe^{2+} greatly stimulated *p*-phenylenediamine oxidation at low concentration and inhibited at high concentration. No stimulation was seen with Fe^{3+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+} or Mn^{2+}. Both Fe^{2+} and Fe^{3+} stimulated psilocin oxidation. Fe^{2+} has been detected in samples of highly purified *p*-phenylenediamine.

3. Micromolar quantities of EDTA and other chelating agents inhibited *p*-phenylenediamine oxidation, while millimolar quantities of these reagents were required to inhibit psilocin oxidation. Millimolar quantities of chloride inhibited both *p*-phenylenediamine and psilocin oxidation.

4. It is concluded that EDTA inhibits ceruloplasmin oxidation of *p*-phenylenediamine by chelation of contaminating Fe^{2+} while inhibiting psilocin oxidation by an ionic-strength effect.

5. Dialysis of ceruloplasmin against buffered EDTA resulted in a minor loss of copper and enzymic activity which was no greater than that seen upon dialysis against buffer alone.

INTRODUCTION

The oxidase activity of mammalian serum, although first described over fifty years ago[1], was studied in a thorough and systematic manner only much later by HOLMBERG AND LAURELL[2–7]. In a series of communications beginning in 1947, these investigators reported the isolation and purification from pig serum of a blue, copper-containing protein which they called ceruloplasmin[5]. They showed that this enzyme could account for the previously described serum oxidase activity. In these and other studies a number of other substances (*e.g.* hydroquinone, catechol, DOPA, andrenaline) have been shown to be oxidized by ceruloplasmin[8–13], but none has a greater reactivity than PPD. LEVINE AND PEISACH[14] recently demonstrated a structure–activity relationship among compounds related to PPD. They found that increased reactivity

Abbreviations: PPD, *p*-phenylenediamine; DPD, *N*,*N*-dimethyl-*p*-phenylenediamine.

was associated with those structures possessing ring substituents which had highly negative Hammett sigma values. In this series also, PPD possessed the greatest reactivity with ceruloplasmin.

In recent studies using DPD as a substrate, two important observations have been described bearing on the nature of ceruloplasmin-catalyzed oxidations. These obversations were (a) stimulation of activity by low concentrations of metal ions, (b) inhibition by EDTA and other chelating agents. CURZON[15,16] reported that Fe^{2+} and Fe^{3+} at concentrations as low as $4 \cdot 10^{-7}$ M enhanced the ceruloplasmin-catalyzed oxidation of DPD. Other metal ions also showed some stimulation but Cu^{2+} was conspicuously inactive. BROMAN[17], CURZON[18], and HUMOLLER *et al.*[9] reported inhibition of ceruloplasmin activity by low concentrations of EDTA. BROMAN attributed this effect to the chelation of contaminating Cu^{2+} which he claimed was stimulatory to the enzymic reaction. CURZON, on the other hand, found no enhancement by Cu^{2+} and felt that Cu^{2+} chelation was not involved in the inhibition. He proposed a coupled ceruloplasmin–iron system in which PPD would be rapidly oxidized and explained the EDTA inhibition in terms of iron chelation. However, he detected no iron in his system and all attempts to remove metals from the reagents did not alter the inhibition shown by EDTA. HUMOLLER[9] dismissed metal ion chelation as the mechanism of EDTA inhibition, but felt that the effect was directly upon the ceruloplasmin molecule itself. He furthermore demonstrated a loss of 50% of ceruloplasmin copper upon dialysis against EDTA.

The present paper is an extension of the observations regarding heavy metal effects on ceruloplasmin catalyzed oxidations. Evidence is presented which suggests that EDTA and other chelating agents may inhibit ceruloplasmin activity by at least two different mechanisms, the first by removal of contaminating metal ions probably found in the substrate, and the second by virtue of a non-specific ionic effect.

METHODS

Ceruloplasmin was prepared from pig serum by modifying the techniques described by HOLMBERG AND LAURELL[5] and by CURZON AND VALLET[13]. A 35–50% ammonium sulfate fraction was prepared, dialyzed against running tap water overnight and then adjusted to pH 5.5. After centrifugation, the ceruloplasmin was precipitated from the supernatent with 15% ethanol and again dialyzed against running tap water. The protein solution was then treated with ethanol–chloroform (9:1) at pH 6.5 and the ceruloplasmin extracted several times from the resulting precipitate by triturating with 0.9% sodium chloride. The combined extracts were concentrated by ammonium sulfate precipitation, dialyzed, and adsorbed onto a DEAE-cellulose column equilibrated with 0.05 M sodium acetate buffer (pH 5.70). The material was eluted with a gradient from this buffer to 0.05 M sodium acetate buffer (pH 5.70) containing 0.2 M sodium chloride. Those fractions having the highest ratio of blue color to protein, *i.e.* $A_{610\ m\mu}/A_{280\ m\mu}$, were combined, precipitated with 65% ammonium sulfate and dialyzed. The final deep blue solution, as well as all buffers used, were passed through a Dowex A-1 Chelating Resin to remove all traces of free metal ions. Copper was determined using the 2,2′-biquinoline reagent[19].

Reactions were carried out spectrophotometrically in a Beckman DU spectrophotometer using a 3-ml cuvette with a 1-cm light path. Measurements were begun

by the addition of enzyme, unless otherwise indicated. The reaction medium always contained 0.1 M sodium acetate buffer (pH 5.5) and the temperature was 37°. The reaction was followed by observing the increase in absorbancy at 490 mμ for PPD and 620 mμ for psilocin (*N*,*N*-dimethyl-4-hydroxytryptamine). Rates were calculated as ΔA / min / ml of ceruloplasmin using the linear portion of the reaction rate curve. Concentrations of ceruloplasmin copper are indicated under the appropriate experiment. PPD was obtained from Matheson, Coleman and Bell as the free base and purified by subliming twice. The resulting snow-white crystals remained colorless if stored in an evacuated desiccator. Solutions of PPD were prepared by dissolving in enough acetic acid to bring the pH to 5.5. Exact pH adjustments were necessary because PPD itself has a high buffering capacity at this pH. Other substances also were adjusted to pH 5.5 with acetic acid or sodium hydroxide before addition to the assay system.

Psilocin was obtained from Sandoz Pharmaceuticals, Hanover, N. J. (U.S.A.). Other chemicals were of the highest purity grade commercially available. Both Fe^{2+} and Fe^{3+} were used as the ammonium sulfate salts. Glass-distilled, deionized water was used in the preparation of all solutions in the assay procedures.

RESULTS

Effects of iron on oxidation rate

Fig. 1 is an illustration of the effects of varying quantities of iron on the oxidation of PPD by ceruloplasmin. Confirming the work of CURZON, who used DPD, we found substantial stimulation at low Fe^{2+} concentrations and inhibition at high concentrations. This curve indicates that stimulation and inhibition occurred simultaneously over a wide concentration range and therefore under our experimental conditions it

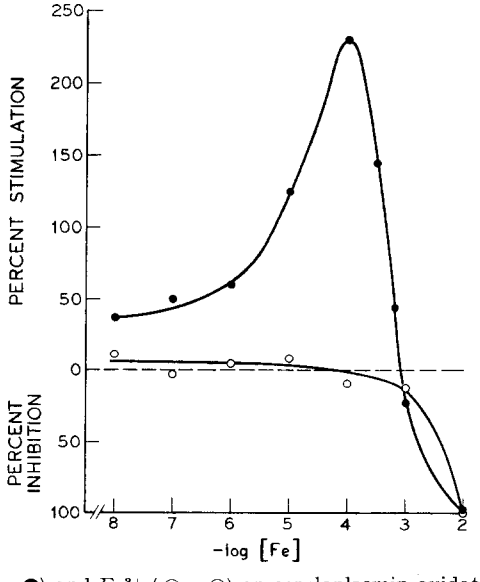

Fig. 1. Effect of Fe^{2+} (●—●) and Fe^{3+} (○—○) on ceruloplasmin oxidation of PPD. $1.7 \cdot 10^{-3}$ M PPD, 5 μl ceruloplasmin (34 μg Cu/ml) in 3 ml total volume.

was impossible to determine the concentrations which gave either maximal stimulation or minimal inhibition, the two effects obscuring each other.

Freshly prepared Fe^{3+} solutions exhibited a considerable stimulatory effect presumably due to the presence of Fe^{2+}, which is an inevitable contaminant of ferric salts (Table I). For the experiment shown in Fig. 1 oxygen had been bubbled through the ferric ammonium sulfate solution for 30 min to oxidize contaminating Fe^{2+}. Here, Fe^{3+} solutions showed no stimulation whatsoever.

TABLE I

EFFECT OF Fe^{2+} AND Fe^{3+} ON THE OXIDATION OF PPD BY CERULOPLASMIN

PPD concentration $3.3 \cdot 10^{-3}$ M, 2 μl ceruloplasmin (34 μg Cu/ml) in a total volume of 3 ml.

Additions	*Rate*	*Percent stimulation*
None	14.8	—
10^{-4} M Fe^{2+}	68.5	360
10^{-4} M Fe^{3+}	29.4	98
10^{-4} M Fe^{3+}*	11.7	0

* O_2 was bubbled through Fe^{3+} solution for 30 min prior to addition to assay system.

At none of the iron concentrations showing strong enhancement of enzymic activity was there a significant degree of non-enzymic oxidation of substrate.

The order of mixing reagents is of great importance in the ceruloplasmin systems. In the experiments described above Fe^{2+} was mixed with PPD prior to ceruloplasmin addition.

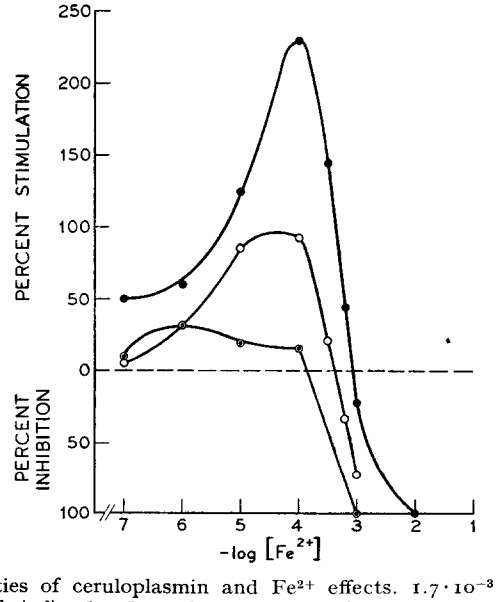

Fig. 2. Varying quantities of ceruloplasmin and Fe^{2+} effects. $1.7 \cdot 10^{-3}$ M PPD, quantities of ceruloplasmin (34 μg Cu/ml): ●—●, 5 μl; ○—○, 10 μl; ○—○, 20 μl. Total volume 3 ml.

TABLE II

INFLUENCE OF INCREASING PROTEIN CONCENTRATION ON Fe^{2+} STIMULATION

PPD concentration $1.7 \cdot 10^{-3}$ M. The ceruloplasmin contained 34 μg Cu/ml.

Ceruloplasmin (μl)	*1% albumin* (μl)	*Percent stimulation* (Fe^{2+})		
		10^{-4} M	10^{-5} M	$7 \cdot 10^{-7}$ M
1	0	226	185	120
2	0	112	62	10
1	0.03	125	110	0

In confirmation of previous results[16], we found that mixing ceruloplasmin with Fe^{2+} before the addition of substrate completely eliminated stimulation. No preliminary incubation was required.

Fig. 2 shows the effect of varying the ceruloplasmin concentration on Fe^{2+} stimulation. As indicated, increased ceruloplasmin was associated with a lessening of Fe^{2+} stimulation while the Fe^{2+} inhibitory effect was increased. This loss of stimulatory capacity may be due in part to a non-specific effect of protein as is shown in Table II. The addition of albumin to the reaction mixture was as effective in diminishing Fe^{2+} stimulation as doubling the ceruloplasmin concentration.

In another set of experiments the effect of varying concentrations of PPD was measured. As PPD concentrations were increased, higher Fe^{2+} concentrations were necessary for maximum stimulation and inhibition (Fig. 3). For example, using $3.3 \cdot 10^{-4}$ M PPD, approx. $2 \cdot 10^{-5}$ M Fe^{2+} gave maximum stimulation, while at 10^{-3} M

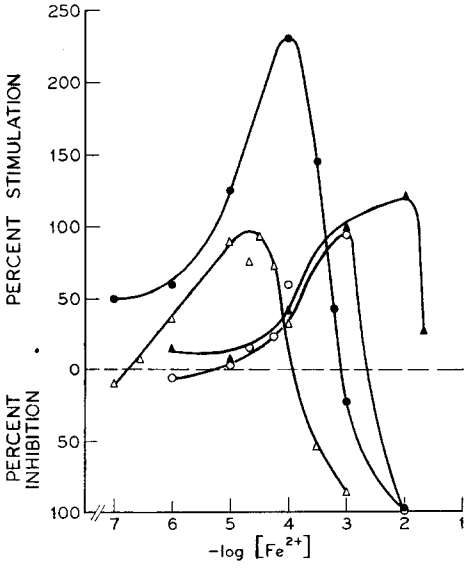

Fig. 3. Varying quantities of PPD and Fe^{2+} effects. PPD concentrations: ●—●, $1.7 \cdot 10^{-3}$ M; ○—○, $1.7 \cdot 10^{-2}$ M; ▲—▲, $6.7 \cdot 10^{-2}$ M; △—△, $3.3 \cdot 10^{-4}$ M, other conditions same as Fig. 1.

Fe^{2+} the reaction was almost entirely inhibited; at $1.7 \cdot 10^{-2}$ M PPD, on the other hand, 10^{-3} M Fe^{2+} was required for maximal stimulation, while maximal inhibition was not reached until 10^{-2} M Fe^{2+}. Within the range of PPD reported here, at maximal stimulation the ratio of Fe^{2+} to PPD remained constant.

BLASCHKO AND LEVINE[8] have shown that psilocin is rapidly oxidized by ceruloplasmin. The above experiments were repeated using this substance as substrate and the results are summarized in Fig. 4 and 5. As in the case of PPD, there was again a substantial rate enhancement at low Fe^{2+} concentrations and inhibition at high Fe^{2+} concentrations. In contrast to PPD, we found that Fe^{3+} markedly enhanced the reaction rate with psilocin. Again, as the substrate concentration was increased, there was a proportional increase in the amount of Fe^{2+} required for maximal stimulation and inhibition (Fig. 5). Psilocin was not oxidized non-enzymically to a significant

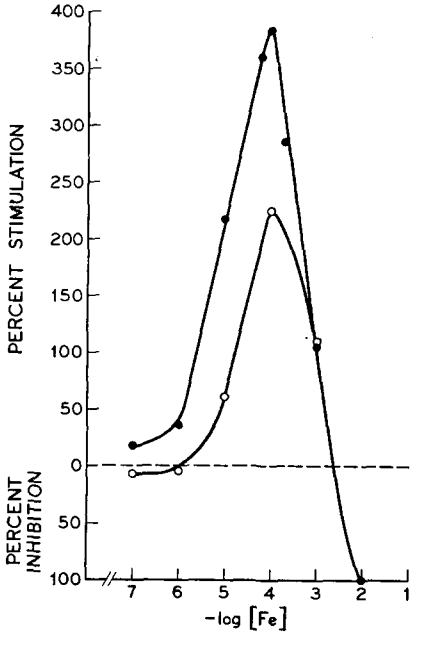

Fig. 4. Effect of Fe^{2+} (●—●) and Fe^{3+} (○—○) on ceruloplasmin oxidation of psilocin. 10^{-3} M psilocin, other conditions same as Fig. 1.

extent by those concentrations of Fe^{2+} and Fe^{3+} which caused strong stimulation in the enzymic system.

When Cu^{2+} was substituted for Fe^{2+} in the enzymic system, there was a similar enhancement of the rate of psilocin oxidation, although Cu^{2+}, like Fe^{2+}, had little effect on the non-enzymic oxidation of psilocin.

Effect of chelating agents

The oxidation of PPD by ceruloplasmin was inhibited by low concentrations of various chelating agents, *e.g.* 8-hydroxyquinoline, 2,2′-dipyridyl, *o*-phenanthroline,

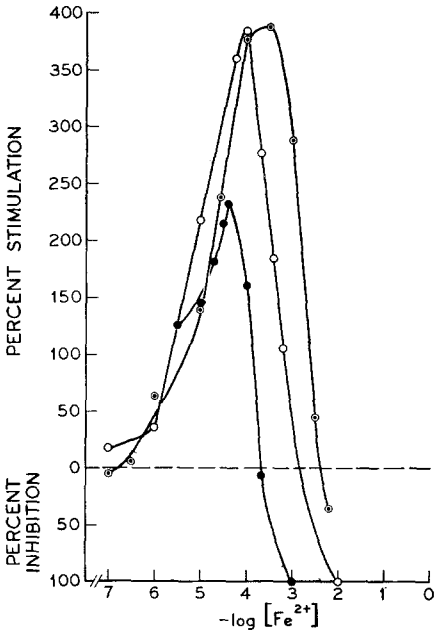

Fig. 5. Varying quantities of psilocin and Fe^{2+} effects. Psilocin concentrations: ●—●, $2.5 \cdot 10^{-4}$ M; ○—○, $5 \cdot 10^{-4}$ M; ○—○, 10^{-3} M. Other conditions same as Fig. 1.

4,5-dihydroxy-1,2-benzenedisulfonic acid, as well as EDTA[9,17,18]. In general, each agent showed a sharp rise in inhibition at low concentrations, which then remained constant, even though the concentrations of inhibitor were increased manyfold.

When using psilocin as substrate, the inhibitory picture was somewhat different. The sharp inhibition previously seen at low concentration of chelating agents was no longer present. Instead it was found that relatively high concentrations were required for inhibition (Fig. 7). A comparison of Fig. 6 and Fig. 7 reveals that the

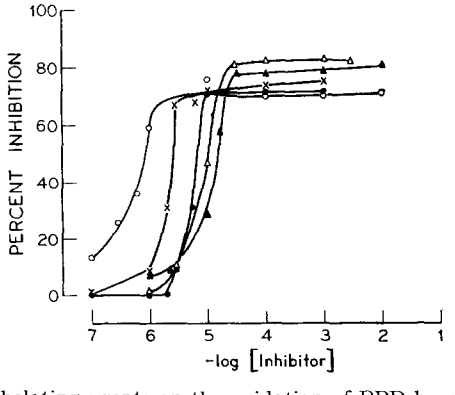

Fig. 6. Effect of various chelating agents on the oxidation of PPD by ceruloplasmin. Conditions same as Fig. 1. ×—×, EDTA; ▲—▲, 8-hydroxyquinoline; △—△, 2,2′-dipyridyl; ●—●, *o*-phenanthroline; ○—○, 4,5-dihydroxy-1,3-benzenedisulfonic acid.

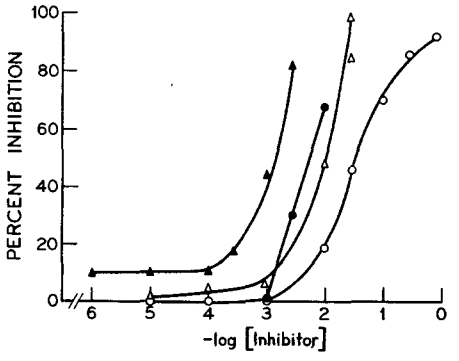

Fig. 7. Effects of various inhibitors on the oxidation of psilocin by ceruloplasmin. Conditions same as Fig. 4. ▲—▲, 4,5-dihydroxy-1,3-benzenedisulfonic acid; △—△, EDTA; ●—●, 8-hydroxyquinoline; ○—○, chloride.

psilocin system required 300–1000 times as much chelating agent for 50% inhibition as did the PPD system. Furthermore, there is demonstrated in Fig. 7 the inhibitory effect of chloride ion on psilocin oxidation. The concentration of chloride required for significant inhibition was approximately the same order of magnitude as that for the chelating agents. Although not illustrated here, inhibition by chloride in the PPD system occurred at the same concentration. The significance of these observations will be discussed below.

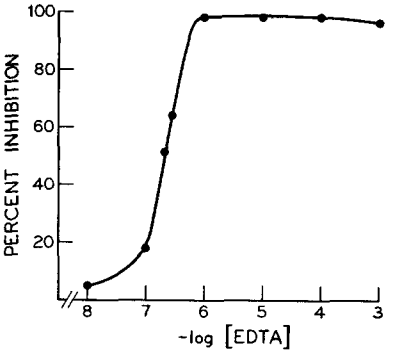

Fig. 8. Effect of EDTA on the oxidation of PPD by H_2O_2. $3.3 \cdot 10^{-3}$ M PPD, 0.2 ml of a 0.3% H_2O_2 solution in a total volume of 3 ml.

Oxidation by H_2O_2

When small amounts of H_2O_2 were added to a buffered solution of PPD, there was a rapid oxidation of the substrate similar to that observed with ceruloplasmin, which was easily followed spectrophotometrically. When EDTA was added to such a system, it inhibited nearly 100% at low concentrations (Fig. 8) which is in contrast to the enzymic system where inhibition was never complete.

A possible explanation for the different results with psilocin and PPD is that either there are two enzymes involved which cannot be separated even by the extensive

procedures described in this paper, or that there are two distinct sites on one enzyme, the first involving PPD, the second, psilocin. To test these hypotheses, the effect of psilocin on ceruloplasmin activity was measured manometrically in the presence of saturating concentrations of PPD. The course of reaction in each experiment is shown in Fig. 9. Oxygen uptake was very rapid with PPD alone relative to the rather weak activity with psilocin. In the flask containing both PPD and psilocin there was considerable inhibition of PPD oxidation. Thus the evidence supports the view that both PPD and psilocin are involved with the same active site.

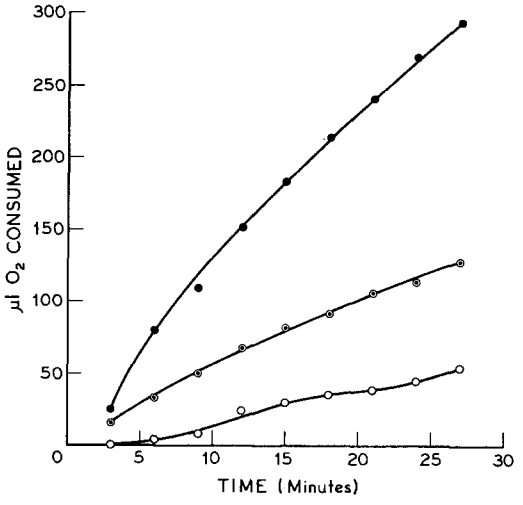

Fig. 9. Effect of psilocin on the oxidation of PPD by ceruloplasmin. $1.7 \cdot 10^{-2}$ M PPD and $3.5 \cdot 10^{-3}$ M psilocin, 0.04 ml ceruloplasmin (59 μg Cu/ml) in each flask. O_2 uptake was measured in a Warburg manometric apparatus at 37°. Reactions were started by tipping in the substrate from the side arm. ●—●, PPD; ○—○, psilocin; ○—○, PPD + psilocin.

These observations led us to suspect that inhibition of PPD oxidation by low concentrations of EDTA and other chelating agents does not involve a direct effect upon ceruloplasmin, but rather that the phenomenon can be explained by removal of contaminating metal ions. We were therefore led to consider which metal(s) might be involved and from what source the metal(s) could be entering the assay system. The observations on Fe^{2+} stimulation as described above made this ion highly suspect. From Fig. 1 it is readily seen that Fe^{2+} contamination amounting to only 10^{-6} or 10^{-7} M could exhibit significant stimulation, and removal of this contamination might very well account for the sharp inhibition seen with low concentrations of chelating agents. As previously mentioned, Fe^{3+} solutions which had been previously aerated with O_2 to oxidize contaminating Fe^{2+} showed no enhanced activity with PPD whatsoever. Other metal ion solutions (Cu^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+}) likewise showed no stimulation if aerated with O_2.

Both ceruloplasmin and the acetate buffer were ruled out as possible sources of

contaminating Fe^{2+} since they had been passed through Dowex A-1 chelating resin columns*.

Using 2,2′-dipyridyl, no iron was detectable in the H_2O_2 solution, but a light red color was seen with the PPD preparation. Negative tests were given by dithizone, which gives no color with iron but does form highly colored complexes with zinc, copper and lead.

These observations made it necessary to examine the report of HUMOLLER *et al.*[9]. These investigators found a 50% decrease in oxidase activity in the presence of EDTA and in addition demonstrated a 50% loss of activity upon prolonged dialysis against EDTA. In attempting to reproduce the latter finding, we dialyzed a sample of ceruloplasmin for 42 h against 0.1 M acetate buffer (pH 5.5) containing 10^{-2} M EDTA. The sample was then tested for oxidase activity with PPD. The activity as measured in this system was inhibited 79% relative to a control sample which had been dialyzed against buffer alone. Both samples were then dialyzed against buffer for three days and then again tested with PPD. This time the two samples had nearly identical activity. The other measurements made on the samples were protein ($A_{280\ m\mu}$) blue color ($A_{610\ m\mu}$) and copper. All of the results, as summarized in Table III, indicate

TABLE III

THE EFFECT OF DIALYZING CERULOPLASMIN AGAINST EDTA

One sample was dialyzed against 10^{-2} M EDTA in 0.1 M sodium acetate buffer (pH 5.5); the control sample was dialyzed against buffer only.

	EDTA		*Control*	
	Before dialysis	*After dialysis*	*Before dialysis*	*After dialysis*
Total activity units	735	518	735	463
Total protein ($A_{280\ m\mu}$)	28.6	21.7	28.6	25.0
Total blue color ($A_{610\ m\mu}$)	1.105	0.745	1.105	0.770
Total Cu (μg)	40	34	40	34
$A_{610\ m\mu}/A_{280\ m\mu}$	0.039	0.034	0.039	0.031

that the loss in oxidase activity, protein, blue color, copper, and blue color to protein ratio was no greater in the sample dialyzed against EDTA than in the control which was dialyzed against buffer only.

DISCUSSION

The experiments described in this paper suggest that inhibition of ceruloplasmin oxidase activity by EDTA and other chelating agents involves at least two distinct mechanisms. The first mechanism is though to be involved when PPD is used as substrate and inhibition occurs in the presence of micromolar quantities of EDTA. In

* The importance of this procedure cannot be underestimated. The lot of Baker reagent grade sodium acetate which we used contained 0.0001% iron and 0.005% other heavy metals. This means that a 1 M solution contains $2 \cdot 10^{-4}$ M iron as well as a considerable quantity of other metals. These obviously could alter significantly the experiments described in this paper.

these experiments, when percent inhibition is plotted against inhibitor concentration, the curve rises sharply to the point of maximal inhibition and then remains nearly level despite a manyfold increase in inhibitor concentration (Fig. 6). This agrees well with the finding of CURZON[18] who used DPD as substrate. We agree with the interpretation that this is a chelation effect in which there is removal of a contaminating metal ion which strongly stimulates enzymic activity at low concentrations. This argument is strengthened by the observation that nearly identical results were obtained using several other chelating agents, *i.e.* 2,2′-dipyridyl, 8-hydroxyquinoline, *o*-phenanthroline and 4,5-dihydroxy-1,3-benzenedisulfonic acid (Fig. 6).

One must next consider the identity of the metal ion involved. From the experiments reported here, only Fe^{2+} need be seriously considered. This ion fulfills the requirement of strong enhancement of activity as demonstrated in Fig. 1. Even at less than micromolar concentration, some stimulation is seen. All other solutions of metal ions tested, including Fe^{3+}, produced no enhancement whatsoever. Thus, even if considerable quantities of other metals were found in one or more of the reagents, it is doubtful if they could be involved in the inhibition demonstrated with chelating agents. Attempts to demonstrate the presence of heavy metal in reagents using 2,2′-dipyridyl and dithizone yielded positive tests for Fe^{2+} in the PPD. No other metals including Fe^{2+} could be demonstrated in any other reagents. This evidence, along with the finding that the oxidation of PPD by H_2O_2 was totally inhibited by chelating agents (Fig. 6) can best be explained by removal of Fe^{2+} present as a contaminant in the PPD.

A second mechanism of inhibition may be involved when psilocin is used as a substrate. Here one finds that the amount of EDTA required for inhibition is in the millimolar range, despite the fact that the conditions of pH, buffer and ceruloplasmin concentration are unchanged. In order to postulate that the first mechanism, that is, metal ion chelation, is also in effect here, one of the following would have to be true. Either a millimolar solution of psilocin (the concentration used in Fig. 7) contains an equimolar concentration of a metal ion which strongly enhances ceruloplasmin activity, or the affinity of this metal ion for some component in the psilocin system is at least 1000 times as great as in the PPD system. The former contention seems very unlikely since we found the melting point of the psilocin we used to be 173–174°, which agrees with the published value of 173–176° (see ref. 20). Since the chelating agents are ionized to some degree at pH 5.5 and inhibit psilocin oxidation in approximately the same concentration range as chloride ions, we wish to suggest an alternative explanation that it is the ionic properties of these compounds which are responsible for inhibition in the psilocin system. There is precedent for this interpretation. HOLMBERG AND LAURELL[7] described the inhibition of ceruloplasmin activity by various anions. They presented evidence that the inhibitory effect is associated specifically with the enzyme rather than with the substrate. CURZON[18] extended these studies using DPD as substrate and the list of anions which show inhibition now includes chloride, acetate, nitrate, bromide, thiocyanate, sulfate and fluoride. Thus, in a properly buffered system at optimal pH, it is almost certain that there be at least one potentially inhibitory anion present. The study of anion effects in ceruloplasmin systems is further complicated by the fact that the presence of one anion can influence the inhibitory effects of another. For example, in the presence of chloride ion, citrate loses much of its inhibitory capacity[7], while high concentrations of acetate which are ordinarily

inhibitory show apparent stimulatory properties in the presence of chloride ion[21].

Although the evidence thus far obtained strongly supports the theories of inhibition just discussed, alternative explanations may be considered. One is that psilocin could have a much stronger affinity than PPD for the active site on the enzyme (presumably at or near the copper atoms), but at the same time being less able to give up electrons and thereby being less readily oxidized. This suggestion is supported by experiments shown in Fig. 9. Here the rate of O_2 uptake by psilocin and ceruloplasmin was very small compared to the rate with PPD. When psilocin was added to the PPD system, the O_2 uptake rate was considerably less than if PPD were present alone. Thus, psilocin seems to inhibit the PPD reaction by acting as a competitive though less reactive substrate. Furthermore, a comparison of the K_m values for psilocin and PPD suggests that psilocin does have a greater affinity for the enzyme than PPD. The K_m value for psilocin obtained in this laboratory is $1.35 \cdot 10^{-4}$ M. K_m values which have been previously obtained for PPD are $1.9 \cdot 10^{-3}$ M (see ref. 22), $2.5 \cdot 10^{-3}$ M (see ref. 6) and $2.2 \cdot 10^{-3}$ M (see ref. 21). It follows, then, that a greater affinity of psilocin for ceruloplasmin may possibly explain the difference in EDTA concentrations required to inhibit psilocin and PPD, if one is willing to assume that inhibition in both instances directly involves the active site on the enzyme.

Another suggestion is that ceruloplasmin may have two active sites, an idea already suggested by FRIEDEN[23] and HUMOLLER *et al.*[9]. Both investigators have proposed that ascorbic acid is oxidized at Site I, while substrates such as PPD are oxidized at both Site I and Site II. EDTA would be effective against Site I only, thus explaining the partial inhibition of PPD oxidation, but complete inhibition of ascorbate oxidation. If psilocin had a greater affinity for Site I, this might explain both the high EDTA concentrations required for psilocin inhibition, and the inhibition of PPD oxidation by psilocin. However, strong evidence against an EDTA effect directly on ceruloplasmin copper has recently been obtained by BLUMBERG *et al.*[24]. Studying free and ceruloplasmin copper by a proton relaxation rate method, these investigators found that the proton relaxation rate due to free copper was virtually abolished upon addition of EDTA, while the proton relaxation rate due to ceruloplasmin copper was unchanged by EDTA.

The enhancement of PPD oxidase activity by Fe^{2+} is dependent upon ceruloplasmin levels (Fig. 2), a greater effect being seen at low enzyme concentration. There are at least two reasons for this phenomenon. One involves a non-specific adsorption of the ion by protein as shown in Table II. Here albumin addition was as effective as increased ceruloplasmin concentration in partially blocking the rate-enhancing property of Fe^{2+}. The second reason is that Fe^{2+} is rapidly oxidized to Fe^{3+} by ceruloplasmin[15], Fe^{3+} having no stimulatory effect in this system. With increased ceruloplasmin concentration, this would be more likely to occur.

The reason for such a strong enhancement of ceruloplasmin activity by iron but no other metal is entirely unknown at this time, and one can only speculate as to the mechanism involved. Because Fe^{2+} is itself rapidly oxidized by ceruloplasmin, CURZON[15,16] suggested the existence of a coupled iron–ceruloplasmin system in which Fe^{2+} was oxidized by ceruloplasmin and Fe^{3+} was presumably reduced by participating in substrate oxidation. It would be expected that if Fe^{3+} is a participant in this system, it too would considerably stimulate PPD oxidation. Our results indicate that this does not occur at pH 5.5 either enzymically or non-enzymically; therefore we find such a

coupled system to be an improbable explanation. In the case of psilocin, on the other hand, both Fe^{2+} and Fe^{3+} stimulate oxidation in the presence of ceruloplasmin and one might consider such a system to operate here. However, this theory is still doubtful because under conditions similar to those described for the ensyme system, Fe^{3+} alone will oxidize psilocin at only a small fraction of the rate obtained with ceruloplasmin.

Another possible explanation for iron stimulation is that Fe^{2+} forms a complex with PPD[25] which is far more readily attacked by ceruloplasmin than is PPD alone. Complex formation is supported by several observations. Firstly, mixing Fe^{2+} with PPD before the addition of ceruloplasmin protects the Fe^{2+} against oxidation by ceruloplasmin. Secondly, even after purifying PPD by subliming twice, iron is still detectable, indicating a volatile, tightly bound complex. Thirdly, upon increasing the PPD concentration, the quantity of Fe^{2+} required for apparent maximal stimulation is also increased. Thus the PPD to iron ratio at the point of maximal stimulation is nearly constant at all PPD concentrations used in these experiments.

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REFERENCES

1 F. BATELLI AND L. S. STERN, *Biochem. Z.*, 46 (1912) 317.
2 C. G. HOLMBERG, *Acta Physiol. Scand.*, 8 (1944) 227.
3 C. G. HOLMBERG AND C. B. LAURELL, *Scand. J. Lab. Clin. Med.*, 3 (1951) 103.
4 C. G. HOLMBERG AND C. B. LAURELL, *Acta Chem. Scand.*, 1 (1947) 944.
5 C. G. HOLMBERG AND C. B. LAURELL, *Acta Chem. Scand.*, 2 (1948) 550.
6 C. G. HOLMBERG AND C. B. LAURELL, *Acta Chem. Scand.*, 5 (1951) 476.
7 C. G. HOLMBERG AND C. B. LAURELL, *Acta Chem. Scand.*, 5 (1951) 921.
8 H. BLASCHKO AND W. G. LEVINE, *Brit. J. Pharmacol.*, 15 (1960) 625.
9 F. L. HUMOLLER, M. P. MOCKLER, J. P. HOLTHAUS AND D. J. MAHLER, *J. Lab. Clin. Med.*, 56 (1960) 222.
10 S. AKERFELDT, *Science*, 125 (1957) 117.
11 C. C. PORTER, D. C. TITUS, B. E. SANDERS AND E. V. C. SMITH, *Science*, 126 (1957) 1014.
12 G. M. MARTIN, E. P. BENDITT AND N. ERIKSEN, *Arch. Biochem. Biophys.*, 90 (1960) 208.
13 G. CURZON AND L. VALLET, *Biochem. J.*, 74 (1960) 279.
14 W. G. LEVINE AND J. PEISACH, *Biochim. Biophys. Acta*, 63 (1962) 528.
15 G. CURZON AND S. O'REILLY, *Biochem. Biophys. Res. Commun.*, 2 (1960) 284.
16 G. CURZON, *Biochem. J.*, 79 (1961) 656.
17 L. BROMAN, *Nature*, 182 (1958) 1655.
18 G. CURZON, *Biochem. J.*, 77 (1960) 66.
19 G. FELSENFELD, *Arch. Biochem. Biophys.*, 87 (1960) 247.
20 F. TROXLER, F. SEEMANN AND A. HOFFMAN, *Helv. Chim. Acta*, 42 (1959) 2073.
21 W. G. LEVINE, unpublished experiments.
22 E. GELLER, S. EIDUSON AND A. YUWILER, *J. Neurochem.*, 5 (1959) 73.
23 E. FRIEDEN, *Horizons in Biochemistry*, Academic Press. New York, 1962, p. 488.
24 W. E. BLUMBERG, J. EISINGER, P. AISEN, A. MORELL AND I. H. SCHEINBERG, *J. Biol. Chem.*, 238 (1963) 1675.
25 R. HARA, *J. Pharm. Soc. Japan*, 71 (1951) 1134.