BBA 12259

ON THE MECHANISM OF CERULOPLASMIN-CATALYZED OXIDATIONS

J. PEISACH AND WALTER G. LEVINE

Departments of Biochemistry and Pharmacology, Albert Einstein College of Medicine, Yeshiva University, Bronx N.Y. (U.S.A.)

(Received February 19th, 1963)

SUMMARY

1. The ceruloplasmin-catalyzed oxidation of *p*-phenylenediamine and other related compounds was studied spectrophotometrically and manometrically.

2. For *p*-phenylenediamine, the reaction sequence proceeded through three stages. The first probably involved the formation of a charge-transfer complex between substrate and ceruloplasmin Cu^{2+}. The second involved the transfer of a single electron from substrate to Cu^{2+} to form a yellow free radical, as determined using electron paramagnetic resonance techniques. The third step involved the loss of another electron from the yellow free radical, either through a disproportionation process or by reaction with enzyme, to form a short-lived diradical species that reacted with more *p*-phenylenediamine to form a stable purple product.

3. The purple product formed in *p*-phenylenediamine oxidation can act as an inhibitor for the oxidation of *p*-phenylenediamine and other substrates.

4. The oxidative sequence of durenediamine was similar to that for *p*-phenylenediamine, except here the diradical that formed rearranged and hydrolyzed in water to form duroquinone and ammonia.

INTRODUCTION

Maximal oxidase activity of the copper-containing protein, ceruloplasmin, is found with N-substituted or N-unsubstituted aryl amine substrates[1,2]. In a recent study from our laboratories, PPD[3] was found to be the best substrate *in vitro* for ceruloplasmin. In order to gain insight into the mode of action of this copper protein, we have undertaken the detailed study of the enzymic oxidation of PPD and related compounds.

MATERIAL

Ceruloplasmin was prepared from pig serum using the techniques of HOLMBERG AND LAURELL[4] and CURZON AND VALLET[5], as modified by LEVINE AND PEISACH[6].

Abbreviations: PPD, *p*-phenylenediamine; TPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; DDA, durenediamine; DPP, *N,N*-dimethyl-*p*-phenylenediamine; MPPD, *N*-methyl-*p*-phenylenediamine; PAP, *p*-aminophenol; PMP, *N*-methyl-*p*-aminophenol; PPP, *N*-phenyl-*p*-phenylenediamine.

Purified enzyme solutions were passed through Dowex A-1 chelating resin and stored at —10° in 3-ml portions.

PPD (Matheson, Coleman and Bell) was sublimed twice and stored in a vacuum desiccator. DPP and PPP were also obtained from the same source. PPD dihydrochloride and MPPD dihydrochloride were purchased from Eastman and were recrystallized from ethanol–water. TPD dihydrochloride and PMP *purum* grade, were purchased from Fluka Chemische Fabrik and were not further purified. DDA, duroquinone and PAP were purchased from K and K Laboratories. The last was sublimed and stored in a dark glass bottle in a vacuum desiccator.

All copper solutions were prepared from Fisher Certified hydrated cupric sulfate. All aqueous solutions were prepared in deionized glass-distilled water. Buffer solutions were passed through Dowex A-1 chelating resin prior to use in these experiments. All other reagents were the best commercial grade.

METHODS

Substrate solutions were prepared fresh daily and stored in an ice bath. The pH was adjusted with acetic acid or NaOH solutions. Kinetic studies were performed manometrically in a Warburg apparatus or spectrophotometrically with a Beckman DU or a Zeiss PMQ II spectrophotometer at 37° using a 1-cm light path and a 3-ml cell. Visible and ultraviolet spectra were studied using a Cary Model 14 recording spectrophotometer.

In manometric experiments, the substrate was delivered from the side arm into a buffered solution of enzyme. Unless otherwise stated, spectrophotometric reactions were started by addition of enzyme to a buffered substrate solution. The copper content of the enzyme was determined with 2,2'-biquinoline reagent[7] (K and K Laboratories).

Electron paramagnetic resonance studies were performed by Dr. W. BLUMBERG, Bell Telephone Laboratories, using a superheterodyne microwave spectrometer described elsewhere[8].

Infrared spectra were obtained using a Perkin Elmer Model 21 double beam spectrophotometer.

RESULTS

Oxidation products of PPD

The addition of crystals of potassium ferricyanide to an aqueous solution of PPD in acetate buffer (pH 5.5) under either aerobic or anaerobic conditions, resulted in immediate formation of an intensely blue-green material which turned yellow in a few seconds and, after 30–60 sec, purple. The last color is that usually associated with oxidation of PPD at pH 5.5. Similar results were obtained using as oxidant either *p*-benzoquinone, bromine, Fenton's reagent, or a mixture of Cu^{2+} and H_2O_2. A concentrated solution of ceruloplasmin, added to PPD in the presence of air, produced similarly colored products. When PPD was oxidized by bromine in a non-aqueous medium such as $CHCl_3$ or CCl_4 there was immediate formation of an intensely blue-green precipitate. Solution of this colored product in water resulted in the yellow to purple transformation described previously.

The yellow color had a sharp maximum at 320 mμ (Fig. 1). In systems containing free copper the quantity of this yellow product was a function of both PPD and Cu^{2+} concentrations. The blue color associated with free Cu^{2+} disappeared in the presence of substrate. The formation of material absorbing at 320 mμ was rapid for the first few minutes and thereafter considerably slower. When the oxidation of PPD ($3.3 \cdot 10^{-3}$ M) was performed with low concentrations of Cu^{2+} (0.8 μg/ml) at

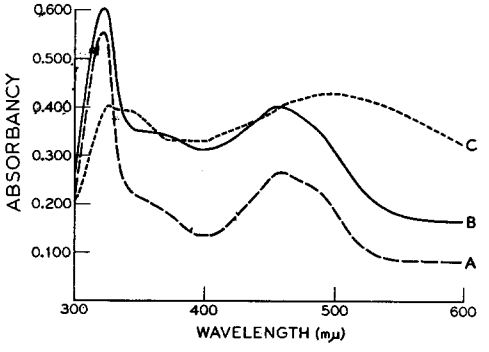

Fig. 1. Spectrum of PPD oxidized with Cu^{2+} and H_2O_2. PPD, $1.2 \cdot 10^{-3}$ M; Cu^{2+}, 10^{-4} M; with 0.05 ml 3% H_2O_2 in a total volume of 3 ml. Curves A and B represent different times after addition of H_2O_2. Curve C after the addition of 0.1 ml of 10^{-1} M EDTA.

pH 5.5, the solution turned yellow, and within 5 min purple. If the concentration of Cu^{2+} was great (80 μg/ml) the formation of purple product was greatly delayed and the yellow product remained. The addition of EDTA to a yellow solution formed with PPD and Cu^{2+}, under aerobic as well as anaerobic conditions, resulted in the instantaneous formation of purple color, in the disappearance of the 320 mμ spectral peak and the formation of a new peak at 340 mμ and a broad plateau around 500 mμ

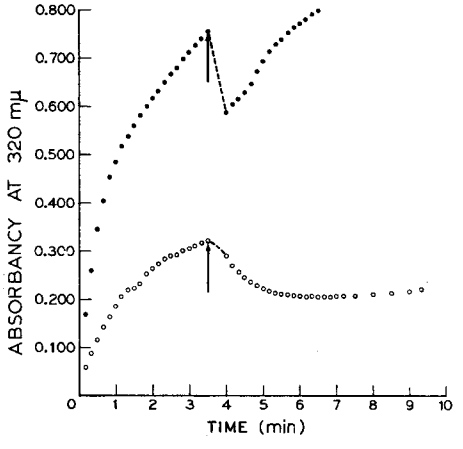

Fig. 2. Oxidation of PPD by ceruloplasmin as measured at 320 mμ. PPD, $3.3 \cdot 10^{-3}$ M; 5 μl ceruloplasmin (34 μg Cu/ml), 0.1 M acetate buffer (pH 5.5) in a total volume of 3 ml. ●—●, with 10^{-4} M Fe^{2+}; ○—○, with no Fe^{2+} added. 0.03 ml of 0.1 M EDTA added at the time indicated by arrows.

(Curve C, Fig. 1). These results suggest first, that copper ion stabilizes the yellow product, and second, that the transformation of yellow to purple product is not an oxygen-dependent reaction.

Upon addition of ceruloplasmin to a solution of PPD buffered at pH 5.5, the formation of yellow product was again observed as was the sharp drop in the absorption at 320 mμ upon subsequent addition of EDTA (Fig. 2). This change in absorption was even more apparent when the reaction was carried out in the presence of Fe^{2+} which has been shown to strongly stimulate the oxidation of PPD by ceruloplasmin[6,9–11]. Addition of large quantities of ceruloplasmin to a buffered PPD solution, under anaerobic conditions, also produced a yellow color.

The next step in the oxidation of PPD was the formation of a purple product. This purple material exhibited no specific spectral peak but did have a broad plateau around 525 mμ and a smaller peak at 340 mμ. At pH 7 or above the color of the product changed from purple to yellow, the latter having a sharp peak at 300 mμ and a low, broad plateau around 525 mμ. At pH lower than 4 the product was orange with a peak at 380 mμ.

Oxidation products of other substrates

The oxidation of PAP proceeded in at least two steps. When potassium ferricyanide, or Cu^{2+} and H_2O_2, were added to a solution of PAP in acetate buffer (pH 5.5), a fleeting purple color formed, which was followed by the formation of a yellow product exhibiting a broad spectral peak at 440 mμ. The addition of EDTA to the solution of Cu^{2+} and H_2O_2 inhibited the oxidation of PAP almost 100% (see ref. 12). Ceruloplasmin-catalyzed oxidations did not perceptively go through a purple intermediate, but seemed to produce the yellow product immediately.

DDA oxidation also proceeded via at least two steps—the initial being the formation of a green intermediate having spectral maxima at 477 and 448 mμ, followed by the formation of a light yellow solution and simultaneously a precipitate which varied from green to off-white, depending on the concentration of substrate and pH of the medium. These results were seen either with ceruloplasmin or with H_2O_2 and Cu^{2+}. The oxidation of DDA was much faster with Cu^{2+} plus H_2O_2 than with either component alone.

Kinetics and copper ion inhibition

At pH 5.5, using $3 \cdot 10^{-2}$ M PPD concentrations, the rate of oxidation of PPD as measured by increase in absorbancy at 490 mμ, was proportional to the ceruloplasmin concentration over a forty-fold range of ceruloplasmin copper (0.011–0.44 μg/ml). Rates of formation of yellow and purple products were pH-dependent. The pH optimum was 4.9 when measured at 320 mμ (yellow product), and 5.9 when studied at 550 mμ (purple product). Fig. 3 shows the rate of oxidation of PPD measured at 320 mμ and 550 mμ. Although the purple product exhibited a maximum at 525 mμ, measurements were performed at 550 mμ in order to avoid interference by absorption due to yellow product (Fig. 1). Further increase in absorbancy at this wavelength is due to contribution by purple product (Curve C of Fig. 1). The shape of these curves indicates a time lag in the formation of the 550-mμ absorbing

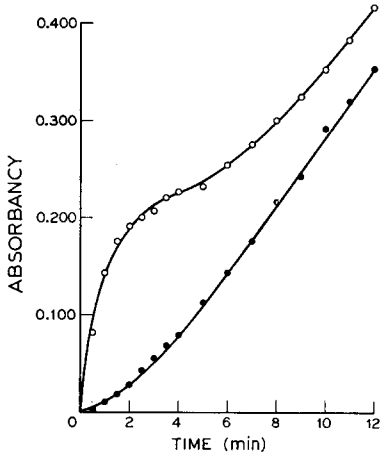

Fig. 3. Comparison of the rate of oxidation of PPD as measured at 320 mμ (○—○) and 550 mμ (●—●). PPD, $3.3 \cdot 10^{-3}$ M; 10 μl ceruloplasmin (15.5 μg Cu/ml), 0.1 M acetate buffer (pH 5.5) in a total volume of 3 ml.

material, and that the 320-mμ absorbing material precedes the formation of 550-mμ absorbing material.

When the oxidation of PPD was studied manometrically there was a direct relation between enzyme concentration and rate of oxygen uptake. When the reaction was studied in the presence of added Cu^{2+} there was a marked inhibition of O_2 uptake (Table I). A comparable spectrophotometric experiment (Table II) showed that when the concentrations of ceruloplasmin and ionic Cu^{2+} were equal, no inhibition was seen, while increasing the ionic Cu^{2+} tenfold resulted in a 30% inhibition. In neither case did stimulation result from adding Cu^{2+}. Raising the concentration

TABLE I

THE Cu^{2+} INHIBITION OF OXYGEN UPTAKE BY PPD WITH CERULOPLASMIN

Free Cu^{2+} and ceruloplasmin copper concentrations were 0.24 μg/ml. Reactions were run in a Warburg apparatus in 0.1 M acetate buffer (pH 5.5) with 10^{-2} moles PPD dihydrochloride in a total volume of 3 ml.

Conditions	*μl O_2/h/μg Cu*
Ceruloplasmin	162
Cu^{2+}	0
Ceruloplasmin + Cu^{2+}	110

of ionic copper 1000-fold resulted in the formation of a yellow product independent of the presence of ceruloplasmin. In contrast to PPD, the enzymic oxidation of DPD and PAP were neither stimulated nor inhibited by added Cu^{2+}.

Stoichiometry

The ratio of oxygen uptake to substrate concentration in reactions catalyzed

TABLE II

THE Cu^{2+} INHIBITION OF PPD OXIDATION WITH CERULOPLASMIN STUDIED AT 550 mμ

PPD dihydrochloride, $3.3 \cdot 10^{-3}$ M, in 0.1 M acetate buffer (pH 5.5). Total volume 3 ml; ceruloplasmin Cu concentration, 0.08 μg/ml.

Conditions	*ΔA/min/ml*	*Color of product*
Ceruloplasmin	8.64	Purple
Cu^{2+} (0.8 μg/ml)	0.200	Purple
Ceruloplasmin + Cu^{2+} (0.8 μg/ml)	6.26	Purple
Cu^{2+} (80 μg/ml)	3.29	Yellow
Ceruloplasmin + Cu^{2+} (80 μg/ml)	3.64	Yellow

by ceruloplasmin were measured. The results are shown in Table III. Considerably different stoichiometric relationships exist for the several substrates. For example, TPD consumed approx. two molecules of O_2 per molecule of substrate, while for DDA, only one atom of oxygen was consumed per molecule of substrate.

Inhibitor formation during PPD oxidation

In another experiment, PPD was oxidized by ceruloplasmin in a manometric apparatus until there was no longer any uptake of oxygen. Then, an equal amount of fresh PPD was added and oxygen uptake again followed. This time the total

TABLE III

THE RATIO OF OXYGEN TO SUBSTRATE CONCENTRATION

For each experiment the reaction was performed in 0.1 M acetate buffer (pH 5.5) in a total volume of 3 ml, with a concentration of ceruloplasmin great enough for the enzymic reaction to be completed in 3 h.

Substrate	*μmoles*	*μmoles O_2/μmole substrate*
PPD	5	0.74
PPD	10	0.76
PPD	20	0.80
TPD	3	2.36
TPD	6	2.15
TPD	9	2.26
DDA	5	0.60
DDA	10	0.48
DDA	20	0.50
DPP	10	1.06
MPPD	10	0.95
PAP	10	0.70
PMP	10	1.15
PPP	10	0.49

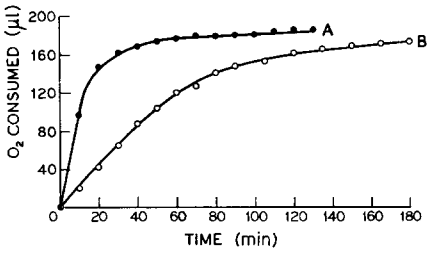

Fig. 4. Inhibition of oxygen uptake by PPD and ceruloplasmin due to oxidation product of PPD. Curve A: PPD, 10 µmoles; 0.2 ml ceruloplasmin (15.5 µg Cu/ml), 0.1 M acetate buffer (pH 5.5) in a total volume of 3 ml. Curve B: contents of flask from Curve A after reaction completed plus fresh sample of PPD (10 µmoles).

oxygen uptake was the same but the reaction rate was much slower (Fig. 4), indicating the probable formation of an inhibitor during PPD oxidation. If an identical experiment was performed using TPD, both samples of substrate were oxidized at the same rate, and the total oxygen uptake was the same. In a third experiment a sample of fresh TPD was added to oxidized PPD. The rate of TPD oxidation was much slower than TPD alone and the total oxygen uptake was decreased (Fig. 5).

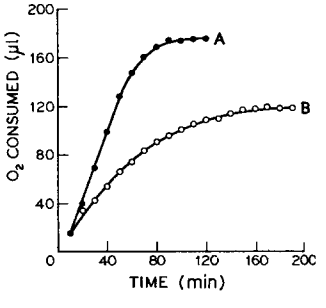

Fig. 5. Inhibition of oxygen uptake by TPD and ceruloplasmin due to oxidation product of PPD. Curve A: TPD, 5 µmoles; 0.2 ml ceruloplasmin (15.5 µg Cu/ml), 0.1 M acetate buffer (pH 5.5) in a total volume of 3 ml. Curve B: contents of flask from Curve A of Fig. 4 plus fresh sample of TPD (5 µmoles).

Oxidation products

We have been able to identify one of the oxidation products of DDA as duroquinone. It could be obtained crystalline, could be easily sublimed and had a sharp melting point at 111°; authentic duroquinone melts at 112° (see ref. 13). The infrared spectrum of this material is identical with that for duroquinone (Fig. 6). Ultraviolet spectra for this oxidation product and an authentic sample of duroquinone were also identical, with maxima at 262 and 270 mµ. Solutions of oxidized DDA tested with Nessler's reagent gave a positive NH_3 reaction. Incubating 0.1 M, 0.01 M and 0.001 M solutions of DDA with ceruloplasmin in 0.1 M acetate buffer (pH 4.0) overnight at room temperature yielded in each case a clear solution, with a

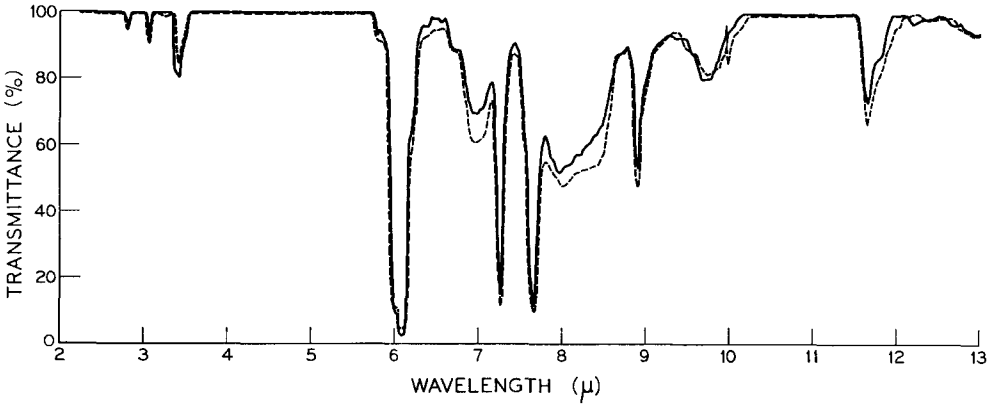

Fig. 6. Infrared spectrum of purified oxidation product of durenediamine. Sample dissolved in $CHCl_3$ and studied with a 1.0-mm NaCl cell.

settled precipitate which was off-white in the 0.001 M DDA incubation and tinged dark green in the others.

The amounts of NH_3 produced during the oxidation, as determined using Nessler's reagent, are shown in Table IV. Approximately two molecules of NH_3 were produced per molecule of DDA only in very dilute solutions. With more concentrated solutions of DDA, less NH_3 and less duroquinone were formed, and a dark blue material could be isolated from the green precipitate. This blue material exhibited a spectral maximum at 575 mμ in $CHCl_3$ and could be decolorized by $Na_2S_2O_4$ but

TABLE IV

NH_3 PRODUCED DURING DDA OXIDATION

Incubation with ceruloplasmin overnight at room temperature in 0.1 M acetate buffer (pH 4.0)

Initial concentration of DDA	*Molecules of NH_3/molecule DDA*
0.1	0.9
0.01	1.6
0.001	2.1

not by H_2O_2 or hot water. No NH_3 was produced during the oxidation of PPD by ceruloplasmin. In fact, unbuffered solutions of PPD showed a distinct lowering of pH during the course of reaction.

Electron paramagnetic resonance studies

Electron paramagnetic resonance experiments are in progress and the quantitative data obtained from these studies will be presented more fully elsewhere. Preliminary findings showed that the addition of PPD either to Cu^{2+} or cerulo-

plasmin diminished both the color and the electron paramagnetic resonance signal associated with divalent copper, with the concomitant formation of a free radical species. Fully oxidized PPD exhibited no radical signal. The addition of PPD to a mixture of Cu^{2+} and H_2O_2 resulted in an altered Cu^{2+} signal and also showed the formation of a free radical species in higher concentration than was seen without the addition of peroxide. Addition of H_2O_2 to Cu^{2+} did not alter the normal Cu^{2+} electron paramagnetic resonance signal. EDTA added to the Cu^{2+}, H_2O_2, PPD mixture precipitously diminished the free radical concentration. The blue-green material formed by addition of bromine to a solution of PPD in $CHCl_3$ also gave a free radical signal in the electron paramagnetic resonance apparatus.

H_2O_2 added to a concentrated DDA solution produced a very strong electron paramagnetic resonance signal which became more intense as the green color associated with this mixture darkened, and diminished as the color of the solution lightened. Concentrated solutions of DPP produced similar electron paramagnetic resonance effects with H_2O_2. In this case the radical produced had a longer life, but was not stable indefinitely and diminished in concentration with time. A mixture of PAP, H_2O_2 and Cu^{2+} also exhibited a radical species.

DISCUSSION

It has been suggested that the blue color in ceruloplasmin is due to a charge transfer phenomenon involving at least a single Cu^{1+}–Cu^{2+} pair, where an electron from Cu^{1+} becomes promoted and fills an empty hole in the 3d orbital of Cu^{2+} (see ref. 14). This type of structure is similar to that described by ROBIN[15] for the Fe^{2+}–Fe^{3+} couple in ferric ferrocyanide. BROMAN *et al.*[16] exclude the concept that an electron is actually shared by two coppers in close proximity because this type of structure would exhibit an electron paramagnetic resonance signal much different from that observed for ceruloplasmin.

The addition of $K_3Fe(CN)_6$ or bromine to aqueous PPD solutions resulted in a transient blue-green coloration. From its intensity and fleeting nature we should like to postulate that, at least for the oxidation of PPD, the first step involves formation of a charge transfer complex between substrate and oxidizing agent. The formation of color may result from bonding of this type. Electron paramagnetic resonance studies showed the presence of a free radical species in this blue-green material when it was formed from PPD and bromine in $CHCl_3$. This radical may be due to the presence of partially oxidized PPD (320-mμ material), or possibly the blue-green material may itself be a radical species totally different from the yellow one described below.

In the ceruloplasmin-catalyzed oxidation of aromatic diamines, the addition of substrate to enzyme is always accompanied by the disappearance of blue color from the enzyme. It has been shown by electron paramagnetic resonance measurements that the blue color of ceruloplasmin is associated with divalent copper in the molecule[14]. Since the electron paramagnetic resonance signal of this copper is diminished but never lost in the presence of substrate, one may suspect that the residual Cu^{2+} participates in a charge transfer pair with the substrate in an enzyme–substrate complex. In effect, the original charge transfer complex is destroyed and a new

Cu^{2+}-substrate charge transfer complex is produced. This phenomenon has only been observed for PPD.

The next stage may be the actual transfer of an electron from reductant to oxidant with ceruloplasmin Cu^{2+} being reduced to Cu^{1+}. This reaction involves the formation of a free radical derived from the substrate, an associated loss of blue color, and a diminution of the electron paramagnetic resonance signal. Similar studies with the copper-containing enzyme, laccase, have shown that the addition of substrate causes a decrease in the Cu^{2+} electron paramagnetic resonance signal which returns to its original value once all of the substrate is oxidized[17].

The radicals that are produced by the oxidation of aromatic diamines have already been described in a classic paper by MICHAELIS[18]. Methyl substituents on the amino groups apparently stabilize such radicals. We have shown that the radical formed from PPD is also stabilized by copper. The addition of Cu^{2+} to PPD solutions in the presence of either H_2O_2 or ceruloplasmin produced an intense yellow solution (which has been demonstrated to contain a free radical by electron paramagnetic resonance) which slowly turned purple. Removing the metal ions by chelation with EDTA caused the immediate formation of purple product and a sharp decrease of electron-paramagnetic resonance radical signal. Therefore, the purple product of PPD oxidation is not a radical but represents a different oxidation state from the yellow product.

Under anaerobic conditions the addition of EDTA to the yellow free radical produced by adding Cu^{2+} to PPD immediately produced a purple solution. Since this conversion did not require oxygen, this step is best described by a radical disproportionation process:

$$\text{PPD} \rightarrow \text{PPD}\cdot \tag{1}$$

$$\text{PPD}\cdot \rightarrow \tfrac{1}{2}\,\text{PPD:} + \tfrac{1}{2}\,\text{PPD} \tag{2}$$

$$\text{PPD:} \rightarrow \text{purple product} \tag{3}$$

Step 1 is slow. The product in Step 1, PPD·, a yellow radical, is stabilized by copper ion. Step 2, formation of a diradical, PPD: proceeds rapidly. In the presence of high concentrations of copper ion, Step 2 is inhibited due to the stabilizing effect of the metal on PPD·. Removal of the metal by EDTA chelation allows the reaction to proceed rapidly through Steps 2 and 3.

In ceruloplasmin-catalyzed systems the concentration of yellow radical produced is very small, since the steady state concentration of this species appears to depend on the concentration of copper in the system. Unlike the free-Cu^{2+}-catalyzed oxidations of PPD, where the addition of EDTA binds the metal ion so that it is not available for reduction by PPD, EDTA does not bind the Cu^{2+} in ceruloplasmin[6]. Even in the presence of EDTA, the addition of PPD to ceruloplasmin will show loss of blue color due to the reduction of Cu^{2+}. In this case the oxidation is only partially inhibited[6] compared to the free Cu^{2+} system where EDTA inhibits nearly 100%.

It would appear that ring electrons play an important role in the reaction because the substitution of an electron-withdrawing group (such as $-NO_2$) on the aryl ring of a ceruloplasmin substrate has been shown to produce a profound diminution of the rate of oxygen uptake. It should also be noted that the Hammett sigma value (a measure of electron supply or removal by an aromatic ring sub-

stituent) for the $-N(CH_3)_2$ group does not differ very much from that for the $-NH_2$ group, indicating that both have approximately the same effect on electron density in the benzene ring. If complexing with enzyme occurred at the nitrogen of the substrate, then one might suspect that there would be partial steric hindrance of this bonding by the addition of two *N*-methyl groups to PPD. Yet the oxidation of DPP proceeds at a rate only slightly less than that for PPD[3]. One could conclude that if the formation of free radical is the rate-controlling step, then the bonding of enzyme might be in some way involved with the π-electrons of the aromatic ring. If, on the other hand, this step is not rate-controlling, which would be quite possible in a stepwise series of reactions, then the implication of π-electrons is less definite.

RICE has suggested that the purple oxidation product of PPD resembles BANDROWSKI's base, which is a trimer of PPD[19]. To form this material, one could postulate the formation of a diradical from PPD which attacks unoxidized PPD to form product. This sort of trimer formation would entail the loss of six electrons and six protons from three molecules of PPD and the expected O_2 to substrate ratio should be 0.5. Since the ratio be observed is consistently more than 0.5 (Table III), in agreement with HOLMBERG AND LAURELL[1], one suspects that the nature of the purple product of PPD oxidation has not been unequivocably established, and what is already known does not preclude the existence of high-molecular weight polymer in the product, or a trimer resembling BANDROWSKI's base which has been further oxidized. Certainly, polymer formation via nitrogen bonding is implied because of the observed lowering of pH during the course of oxidation.

It has been shown that addition of the products from a PPD–ceruloplasmin reaction to a fresh solution of TPD diminishes both oxidation rate and total O_2 uptake when this substance is oxidized by ceruloplasmin (Fig. 4). Yet products from a TPD–ceruloplasmin reaction do not exhibit this effect. It is suggested, therefore, that the final PPD oxidation product decreases the oxygen uptake by

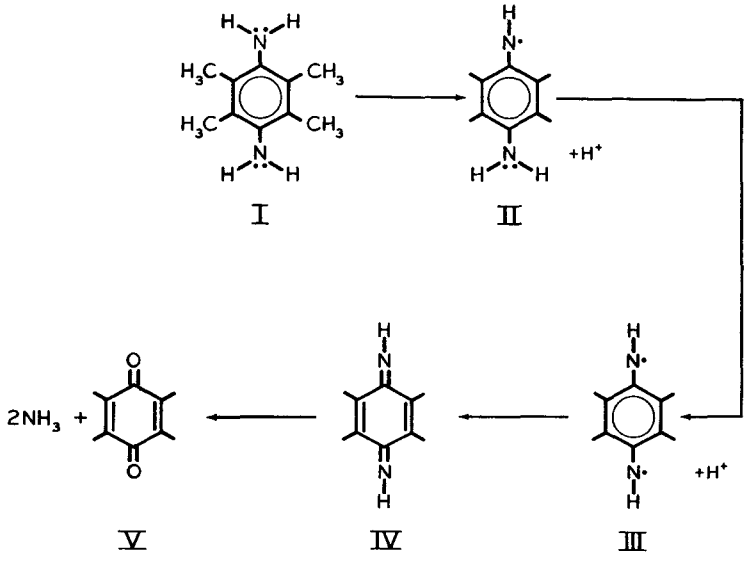

Fig. 7. Steps in the oxidation of DDA.

directly affecting the TPD, or alternatively by blocking a subsequent step in the reaction.

Our results with DDA were somewhat clearer than with PPD. Here, the radical produced by initial electron transfer was bright green and not as short-lived as the one produced by PPD. The proposed sequence for the oxidation of DDA is illustrated in Fig. 7. DDA (I) is oxidized to form a green radical (II) which in turn loses a second electron forming (III). This instantaneously rearranges to form durenediamine (IV) which readily hydrolyzes in water to form duroquinone (V). With high concentrations of substrate a side reaction can occur in which less than stoichiometric quantities of NH_3 and duroquinone are produced. The blue material formed in this reaction may result from an attack of radicals II or III on DDA (I). The structure of this blue material cannot be analogous to that of BANDROWSKI's base because in DDA all the ring *ortho*-positions are blocked by methyl groups. Possibly, then, this blue material may be an indoaniline- or indophenol-like compound.

It has been shown that the blue color of ceruloplasmin does not involve oxygen-binding[20]. Under anaerobic conditions we showed that the addition of PPD to ceruloplasmin resulted in the formation of a yellow solution, and also a decolorized ceruloplasmin. In effect, then, the first step in the oxidation of PPD can take place in the absence of oxygen. This is similar to the anaerobic oxidation of PPD by $K_3Fe(CN)_6$. All that is necessary to oxidize this substrate is an electron acceptor. In Fig. 8, a mechanism for ceruloplasmin-catalyzed oxidations is presented using PPD as a model. The first step as shown involves the formation of a charge transfer complex between enzyme copper and substrate. This is followed by the first oxidative step, the transfer of an electron from PPD to Cu^{2+}–enzyme yielding a free radical. After the copper in ceruloplasmin is reduced it can be reoxidized by oxygen and is

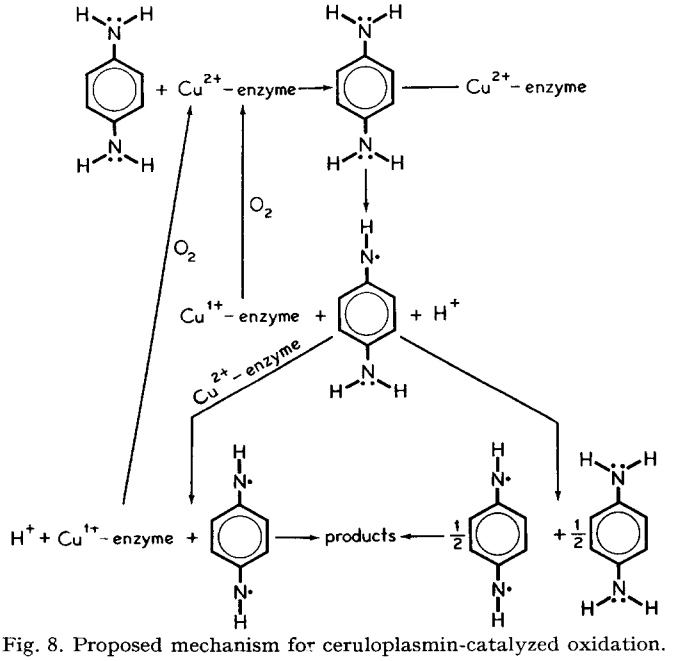

Fig. 8. Proposed mechanism for ceruloplasmin-catalyzed oxidation.

again available for further substrate oxidation. Based on these observations we have concluded that cupryl ion or percupryl ion, suggested intermediates in tyrosinase catalyzed systems[21], need not be implicated for ceruloplasmin-catalyzed reactions, at least not in the first step of the oxidation. The second oxidative step, namely, the formation of a diradical which either reacts quickly with more substrate or rearranges to an imine, may not be enzymic in nature. As was demonstrated anaerobically, addition of EDTA to the copper-stabilized monoradical of PPD resulted in immediate formation of a purple product, implying that this step requires no O_2 and perhaps no enzyme. One may therefore envision an enzymic formation of a radical species which then non-enzymically disproportionates. Recently, NAKAMURA[22] has shown that the radical formed in the laccase-catalyzed oxidation of hydroquinone dismutates in a similar fashion.

Another possibility, illustrated in Fig. 8, is that the diradical is formed by oxidation of the monoradical, either by another enzyme molecule, or by another Cu^{2+} in the same enzyme molecule that performed the initial oxidation. Either of these pathways is consistent with the proposed mechanism.

Attempts to oxidize aromatic monoamines and monophenols (*e.g.* aniline and pentamethylphenol) with ceruloplasmin have been unsuccessful. It has not been possible to reconcile this fact with the ease of oxidizing aromatic diphenols or diamines. It should be noted at this point that a wide spectrum of molecules of various sizes and shapes are substrates for ceruloplasmin oxidation[1-3]. The factors common to all aryl substrates are that each possesses a minimum of two electron-supplying groups, and none possesses strong electron-withdrawing groups. This suggests that in addition to steric requirements for substrate activity there is a definite electronic requirement, that is, the availability of electrons on the aromatic nucleus. Similar findings have been reported for a DPN-linked aromatic aldehyde dehydrogenase[23], for the hydrolysis of β-phenylated glucosides by emulsin[24], and for the hydrolysis of benzoylcholine derivatives by cholinesterase[25],*.

ACKNOWLEDGEMENTS

We are indebted to Dr. W. E. BLUMBERG for his valuable advice and the performance of our electron paramagnetic resonance studies, to Dr. J. PAVLOS for obtaining the infrared spectra, and to Miss R. CARLTON for her excellent technical assistance. This work was supported by a grant from the United States Public Health Service, HE-06450-02S1.

NOTE ADDED IN PROOF

Recent work indicates the possibility that the intense blue color of copper-containing proteins such as ceruloplasmin may be explained by other than a Cu^{1+}—Cu^{2+} pair. SUTHERLAND AND WILKINSON[26] have isolated a blue protein from *Bordetella pertussis* which has a ratio of optical density (625 mμ) to μg of copper of 0.018 and contains one atom of copper per molecule. In addition, recent studies[27] on the blue protein obtained from *Rhus vernicifera*, first described by OMURA[28], revealed a ratio of optical density (604 mμ) to μg of copper of 0.0533. This protein also contains one

atom of copper per molecule[27,28], while EPR studies indicate that this copper is entirely divalent[27].

Received October 28th, 1963.

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