Copper Proteins and Oxygen

Correlations between structure and function of the copper oxidases

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ABSTRACT A comprehensive survey of the interaction of the copper proteins and oxygen is presented including a correlation of structure, function, and other properties of the known copper oxidases and of hemocyanin. The origin of their blue color and the structure of copper complexes and copper proteins are related to the oxidation state of copper ion and relevant electronic transitions probably arising from the formation of charge transfer complexes. The oxygen reactions of hemocyanin, ceruloplasmin, and cytochrome oxidase show half-saturation values far below the other Cu enzymes. The formation of hydrogen peroxide as a reaction product is associated with the presence of one Cu atom per oxidase molecule or catalytic system. Water is the corresponding product of the other Cu oxidases with four or more Cu atoms per molecule, except for monoamine oxidase. Mechanisms for the oxidase action of the two and four electron transfer Cu oxidases and tyrosinase are proposed. These reactions account for the number, the oxidation-reduction potential, and the oxidation state of Cu in the resting enzyme, the cyclical change from Cu(II) to Cu(I), the diatomic nature of O_2, the sequence of the oxidation and reduction reactions, and other salient features. The catalytic reactions involved in the oxidation of ascorbic acid by plant ascorbate oxidase, ceruloplasmin, and Cu(II) are compared. Finally the substrate specificity, inhibitory control, and the detailed mechanism of the oxidase activity of ceruloplasmin are summarized.

INTRODUCTION

We now turn to another group of colorful proteins. Previous authors have discussed the red proteins including hemoglobin and the cytochromes, and the yellow proteins which usually contain some flavin moiety. It is therefore appropriate now to consider the blue proteins which almost invariably contain copper, although it is true that there are several exceptions among the Cu

TABLE I

SOME PROPERTIES OF THE COPPER ENZYMES AND PROTEINS

Name	Major source	Cu	Molecular weight 10^3 gm	Cu/ mole	Enzymic activity or other possible functions
		per cent			
Tyrosinase					
Mold	*Neurospora*	0.21	33	1	L-Dopa
Plant	Mushrooms	0.21	119	4	Phenol and polyphenol oxidation
Insect phenol oxidase	Blowfly	—	500	—	Oxidation of dopamines
Mammalian	Melanoma, skin	—	—	—	Tyrosine, dopa oxidation, melanin formation
Cytochrome oxidase	Virtually all cells	0.09	70	1	Reduced cytochrome oxidation
Ascorbic acid oxidase	Plants: squash	0.25	146	6	Ascorbate to dehydroascorbate
Laccase	Plants: lacquer tree	0.22	120	4	Oxidation of aromatic amines, phenols
Uricase	Liver	0.06	120	1	Uric acid to allantoin, H_2O_2
β-Mercaptopyruvate transulfurase	Liver	0.17	35	1	β-Mercaptopyruvate to pyruvate
Galactose oxidase	Mold	0.085	75	1	Galactose oxidation to D-galactohexodialdose, H_2O_2
Rhus vernicifera blue protein	Japanese lac tree	0.33	25	1	Electron transport
Pseudomonas blue protein	*Pseudomonas*	0.35	17	1	Electron transport
Plastocyanin	Chloroplasts	0.58	21	2	Photosynthesis (photoreduction)
Azurin	Bacteria; Bordetella (pertussis)	0.45	14.6	1	Oxidation of reducing agents, cysteine GSH; reoxidation by O_2, cytochrome *c*, cytochrome oxidase
Diamine oxidase	Pea, pig kidney	0.09	73	1	Certain diamines and monoamines, H_2O_2
Monoamine oxidase	Animal serum	0.088	225	4	Oxidation of substituted aliphatic amines to the corresponding aldehyde, H_2O_2
Dopamine-β-hydroxylase	Adrenal glands	0.10	290	4–7	Dopamine to epinephrine
Ceruloplasmin	Animal serum	0.34	160	8	Cu transport; ascorbate, epinephrine, *p*-phenylenediamine, arylamine oxidation
Hemocyanin	Lobster plasma	0.16	780	20	Oxygen carrier
	Snail plasma	0.19	6,700	200	Oxygen carrier

TABLE I *concluded*

SOME PROPERTIES OF THE COPPER ENZYMES AND PROTEINS

Name	Major source	Cu	Molecular weight 10^3 gm	Cu/ mole	Enzymic activity or other possible functions
		per cent			
Cerebrocuprein	Brain	0.30	35	2	Possibly copper storage, transfer etc.
Erythrocuprein	Human red blood cells	0.35	34	2	Possibly copper storage, etc.
Hemocuprein	Beef red blood cells	0.35	30	2	Possibly copper storage, etc.
Hepatocuprein	Horse liver, ox liver	0.34	35	2	Possibly copper storage, etc.
Milk copper protein	Milk	0.19	—	—	Possibly copper storage, etc.

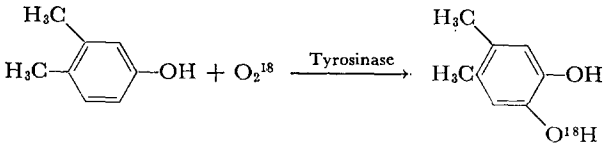
There have been additional reports of less well characterized copper proteins from various sources, including brewer's yeast (0.12 per cent Cu), vaccinia virus (0.05 per cent Cu).

proteins such as plasma monoamine oxidase, which is pink, and tyrosinase, which is colorless.

More than twenty well defined copper proteins have now been recognized, even if the many different tyrosinases and hemocyanins are counted as only one each. As shown in Table I, their distribution is ubiquitous—from a specific copper protein in plants, plastocyanin, to the widely distributed terminal respiratory enzyme, cytochrome oxidase. They range in molecular weight from 14,600 gm for azurin to almost 7,000,000 gm for hemocyanin. Some are essential in their biological function as hemocyanin and cytochrome oxidase, and some play a limited catalytic role as does uricase. The most recent addition to this list is the enzyme, dopamine hydroxylase, which is involved in the biosynthesis of norepinephrine. Catalytic activity has not been associated with all these copper proteins, but in our view the versatility of copper ion as a catalyst makes every copper protein a prime suspect for enzymic activity. So far the catalytic role of these copper proteins usually involves oxidation but participation in hydrolytic or transfer reactions should not be excluded. It is unlikely that the identity and function of all the copper proteins have been recognized (1), especially in view of the existence of a group of copper proteins to which no obvious physiological function other than transfer or storage can be ascribed at present (Table I).

All but several of the copper proteins in Table I react in some demonstrable way with oxygen and they have played an impressive role in the study of biological oxidation and oxygenation. Hemocyanin is one of the oldest known biological pigments, second only to hemoglobin as an oxygen carrier. No enzyme plays a more vital part in the metabolism of plants and animals than cytochrome oxidase, the almost universal terminal oxidase. Since the

role of copper in this enzyme system is still unsettled, we will only note that this enzyme system contains copper ion which appears to change in valence during catalytic activity. The copper enzyme, ascorbate oxidase, is an alternative plant terminal oxidase of some significance. The discovery of the oxygenase property of many oxidizing enzymes may be credited to the pioneering work of Mason *et al.* (2) on the copper enzyme, mushroom tyrosinase. It was shown that in the tyrosinase-catalyzed oxidation of 3,4-dimethylphenol, the oxygen atom incorporated into the product, 4,5-dimethylcatechol, was derived exclusively from molecular oxygen and not from the oxygen of water. This experiment, represented below, was in sharp contrast with the previously widely accepted thesis that oxygen could act only as an ultimate or terminal electron acceptor in biological oxidations. Much of the valuable work on oxygenases and mixed function oxygenases discussed here has evolved from this useful observation.



It is at the terminal oxidase stage, presumably cytochrome oxidase, that copper enzymes may be intimately involved in the susceptibility of tissues to radiation damage. According to a proposal of J. Schubert (3), radiation produces organic peroxides which oxidize Cu(I) proteins to Cu(II) proteins. Somehow the Cu(II) proteins become "fixed" in this oxidation state and thus can no longer react with molecular oxygen, thus impairing respiratory metabolism. The evidence for these ideas is only indirect at present. Schubert reports that protection can be afforded against all types of ionizing radiations, even after exposure, by stabilization of Cu(I), destruction of the peroxides, or reduction of tissue oxygen. Conversely, Cu(II) stabilizers produce increased sensitivity to radiation. Schubert also notes a positive correlation of the comparative radiation sensitivity of animals and their copper contents. However, this correlation does not extend to individual tissues. The most radiosensitive tissues in man are the spleen, pancreas, and the cells producing leucocytes which have the least copper and the least cytochrome oxidase.

In a recent discussion of the importance of the copper in cytochrome oxidase for the oxygen utilization of animals and plants (4), Chance (5) noted that the reaction of oxygen with the copper ion of cytochrome oxidase is only one-tenth as fast as the reaction of oxygen with the ferrous hematin of cytochrome oxidase. His doubts about the significance of copper in the intracellular function of cytochrome oxidase are at variance with the views of Griffith (6), who summarized the studies showing "a specific association of copper with cytochrome oxidase which also suggests a role for copper as an active oxidation-reduction catalyst in the reaction catalyzed by cytochrome oxidase."

Oxygen Interaction with Copper Proteins and Enzymes

Our present knowledge of how oxygen reacts with the copper proteins can only be described as primitive and incomplete. This is due to two main reasons: (*a*) the lack of appropriate application of a variety of sensitive physical methods for measuring these interactions using fast reaction techniques and (*b*) the difficulty in obtaining sufficient quantities of most of the Cu enzymes for adequate study.

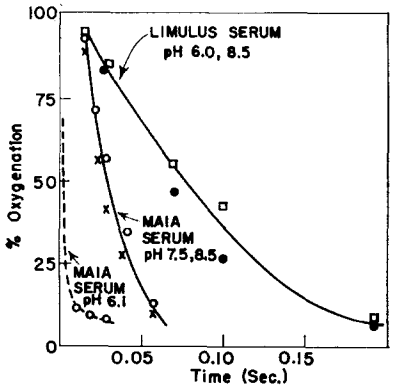


FIGURE 1. Relative rates of oxygenation of two hemocyanins at 20° under approximately "physiological" conditions. The oxygenation was measured by blue color formation using Hartridge-Roughton rapid flow techniques. Data adapted from Millikan (7).

Unique among the Cu proteins is the interaction of oxygen with hemocyanins which may be written as follows:—

$$Cu_2 \text{ protein} + O_2 \rightleftharpoons O_2\text{-}Cu_2 \text{ protein}$$

$$\text{Hemocyanin} + O_2 \rightleftharpoons \text{Oxyhemocyanin}$$

(colorless) (blue)

The rates of these reactions were estimated under physiological conditions in a classical paper by G. A. Millikan in 1933 (7) using rapid flow methods developed earlier by Hartridge and Roughton (8). The dissociation of oxygen from hemocyanin takes 0.1 second to reach completion in the horseshoe crab (*Limulus*) serum and 0.04 second in spider crab (*Maia*) serum, as shown in Fig. 1. The dissociation was also accelerated at pH 6.0. While the association process was too rapid to be estimated with any appreciable accuracy, Millikan made an educated guess of a half-reaction time of 0.003 second. These reaction times are reminiscent of the times for the corresponding reactions of hemoglobin. Also, as with hemoglobin, the equilibrium of the hemocyanin-oxygen interaction has been extensively studied (7, 9) and will be mentioned later. *Met*hemocyanin is reported not to be able to reversibly bind oxygen (10).

The rate of oxygen interaction with the oxidative copper enzymes can only be inferred since oxygen binding is usually a prelude to a rapid oxidation of a reduced form of the enzyme as shown in the following reaction sequence:

$$Cu(I)_4 \text{ protein} + O_2 \rightleftharpoons O_2\text{—}Cu(I)_4 \text{ protein}$$

$$O_2\text{—}Cu(I)_4 \text{ protein} + 4H^+ \rightleftharpoons Cu(II)_4 \text{ protein} + 2H_2O$$

The formation of the Cu(II) protein can usually be followed without disturbing its state in the protein by determining its electron spin resonance signal. But to our knowledge, rapid flow methods have not been systematically

TABLE II
HALF-SATURATION OXYGEN CONCENTRATIONS OF COPPER PROTEINS

Cu Protein	Estimated half-saturation Molar concentration $[O_2]$ at 20° (±2)	Reference
Hemocyanin	4×10^{-6} M* to 6.5×10^{-5}	Millikan (7) Prosser (9)
Cytochrome oxidase	4×10^{-6}	Chance (12)
Ascorbate oxidase	2.2×10^{-4}	Thimann *et al.* (13)
Ceruloplasmin	3.9×10^{-6}	Osaki (15)
Cu(II) catalysis of ascorbate	2×10^{-4}	Frieden (60)
Uricase	2×10^{-4}	Mahler (43)
Tyrosinase	5.5×10^{-5}	Osaki (15)
Baker's yeast	1.7×10^{-6}	Winzler (55)
Rat liver cells	5×10^{-7}	Longmuir (61)
Numerous microorganisms	1×10^{-8} to 3×10^{-6}	Longmuir (61)
Rat liver mitochondria	8×10^{-9}	Baender and Kiese (62)

* These correspond to 2 and 36 mm Hg respectively.

applied to these oxidative reactions. Over 95 per cent of the reduced Cu of ascorbate-reduced laccase and ceruloplasmin was reoxidized in less than 0.1 second (11). Modification of the oxygen electrode and/or more rapidly responding ESR systems may be needed to study these reactions in more detail. Recently the rates of some of the reactions in which ceruloplasmin is oxidized and reduced have been studied by Broman *et al.* (11).

Quantitative Studies of the Interaction of Oxygen with Copper Proteins

That oxygen binding or oxygen concentration can play a significant role in the rate-determining step or steps during the over-all reaction involving a Cu enzyme is suggested by the data in Table II. Second order rate constants for the reaction between oxygen and the Cu protein might be more meaningful

but these data are not available except as noted later for ceruloplasmin (11). In Table II the equilibrium constants for the dissociation, K_m, or half-saturation oxygen pressures, have been estimated from scattered data in the literature, usually involving manometric or polarographic methods. For cytochrome oxidase and hemocyanin the oxygen interactions are very intense, indeed. The equilibrium constants for the hemocyanins all fall conveniently under the partial pressure of oxygen in air (158 mm Hg) (7, 9). From a variety of data, the half-saturation pressure for cytochrome oxidase appears to be within the range of 10^{-6} M (1 to 2 mm Hg). It must be near this if cytochrome oxidase is to function properly in the respiratory chain since it has been estimated that the pO_2 of the mitochondrion is no more than 1 mm

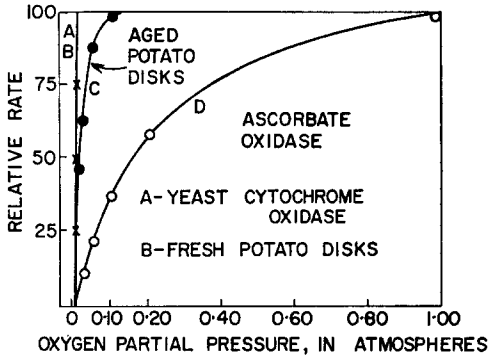


FIGURE 2. The dependence of oxygen uptake on the partial pressure of oxygen. The relative rate on the vertical axis is the per cent of the maximum oxygen consumption. Identifications are as follows: *A*, yeast cytochrome oxidase data of Winzler (55); *B*, freshly cut 0.5 mm potato disks at 15° (indistinguishable from *A*); *C*, disks kept 1 day at 25°, measured at 15°; *D*, plant ascorbate oxidase measured at 25°. Data adapted from Thimann *et al.* (13).

Hg. In 1957, Chance (12) concluded that the oxygen affinity of the respiratory system is so high that no changes of rate will occur until the oxygen concentration has fallen to about 4 μM (2 mm Hg) at 25°. The concentrations of oxygen necessary for half the maximum respiration in many intact cellular systems show a similar high oxygen affinity.

In contrast are the data, also included in Table II, for several isolated systems in which Cu ions play an essential catalytic role. One of the most complete studies has been reported for ascorbate oxidase by Thimann *et al.* (13) and is shown in Fig. 2. The K_m is about 16 per cent O_2 or 2.2×10^{-4} M. From this and related data it was concluded that the oxygen uptake of potato disks could not be due to a terminal oxidase of relatively low oxygen affinity such as ascorbate oxidase. Cytochrome oxidase is, therefore, a more logical candidate. Since ascorbate oxidase is very sensitive to "protection or

stabilization" by a variety of substances, it was later observed that the lack of saturation by air could still be observed on a protected enzyme (Fig. 3) (14). Estimations of the half-saturation oxygen concentrations for several other copper ion–sensitive systems, listed in Table II, give values in the 10^{-4} range, for uricase, and free Cu(II) catalysis. These data suggest that the protein did not contribute significantly to oxygen binding.

Since we could find no precise data related to tyrosinase and ceruloplasmin, the effect of oxygen on the activity of these enzymes has been studied (15) with the data shown in Figs. 4 *a* and 4 *b* and also in Table II. The K_m for tyrosinase, 5.5×10^{-5} M, is somewhat under that for the typical copper

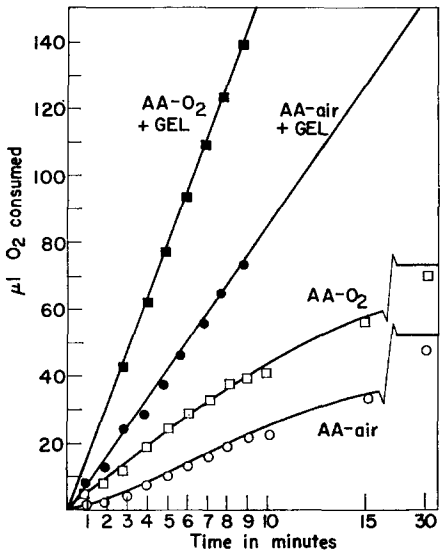


FIGURE 3. Comparative effects of air and 100 per cent oxygen on gelatin-protected and unprotected plant ascorbate oxidase. Each experiment included 0.010 M phosphate buffer, pH 7.2, 0.020 M ascorbate, 1.0 mg per ml gelatin, and 5.0 units ascorbate oxidase at 30°. Data from Frieden and Maggiolo (14).

enzyme listed in Table II. The K_m for the ceruloplasmin-catalyzed oxidation of ascorbate was unexpectedly low, about 4×10^{-6} M. Thus in its apparent affinity for oxygen, ceruloplasmin is comparable to hemocyanin and cytochrome oxidase. Except for the tendency of hemocyanin to aggregate, ceruloplasmin resembles hemocyanin much more closely than cytochrome oxidase in numerous chemical properties. It is even possible that ceruloplasmin represents a vestigial but highly evolved form of hemocyanin. It is of interest that Broman (16) has suggested that the Cu of ceruloplasmin is the direct precursor of the Cu of tissue cytochrome oxidase. Regardless of these speculations, it is clear that in what is now known about their relative interactions with oxygen, hemocyanin, ceruloplasmin, and cytochrome oxidase are substantially different from the other copper proteins or catalytic systems listed in Table II.

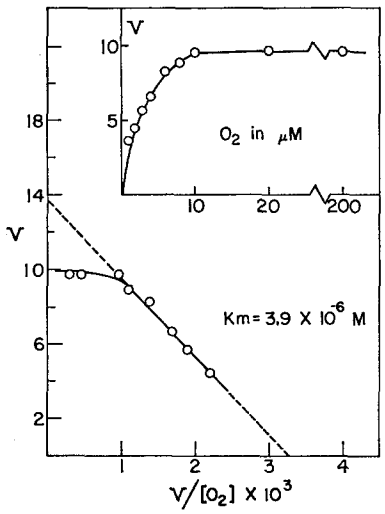


FIGURE 4*a*

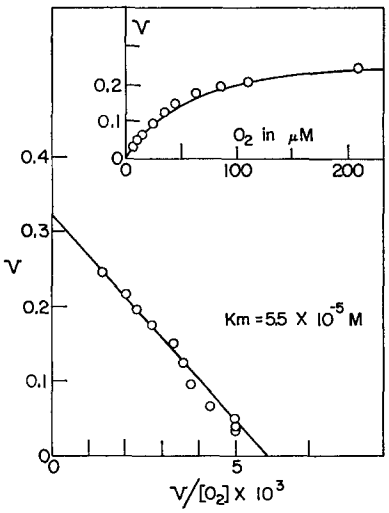


FIGURE 4*b*

FIGURE 4 *a*. The determination of the K_m of ceruloplasmin with respect to oxygen. The velocity of oxygen uptake in the oxidation of ascorbate in 0.2 M acetate buffer at pH 5.2 by 0.3 μM ceruloplasmin was measured polarographically at various concentrations of oxygen at 30°C. Ascorbate concentration was kept high enough (170 μM) to get zero order rate with respect to the substrate. Neocuproine was added up to 15 μM to prevent effect by any unforeseen inorganic copper ion contamination.

FIGURE 4 *b*. The determination of the K_m of tyrosinase with respect to oxygen. The velocity of tyrosine oxidation measured by absorbance change at 475 mμ, v, is plotted against v/S or S at various oxygen concentrations between 2 and 200 μM. The reaction mixture containing 3×10^{-4} M tyrosine in 0.022 M phosphate buffer was equilibrated to desired oxygen concentration by bubbling the gas mixture through the solution in a cuvette at a rate of 20 ml/min. at 30°C. After 20 minutes of equilibration time, 20 μl of tyrosinase was added to the reaction mixture.

Electronic Transitions and the Origin of the Blue Color of Copper Complexes and Proteins

With several exceptions (non-oxygenated) all Cu proteins have strong visible absorptions, resulting in blue colors (see Table III), except for monoamine and diamine oxidases which are pink (17, 32), probably due to the presence of a Cu(II) pyridoxal–phosphate complex. This lack of absorption in the visible for tyrosinase and hemocyanin (non-oxygenated) is usually associated with the exclusive cuprous state of the metal ion. We now consider the structural basis for the intense blue color of copper proteins and chelates.

Copper complexes with planar and distorted or regular octahedral structures have parity forbidden *d-d* transitions in the visible region. These molecules have a center of symmetry and cannot obtain the intensity for *d-d* transition (even-even) by the electric dipole transition selection rule. The

observed intensities of octahedral ($\epsilon \cong 1$–150) and planar ($\epsilon \cong 5$–250) (18) copper complexes are borrowed from the intensities of either their allowed *d-p* transition or allowed charge transfer transitions (charge transfer from ligand to metal or the reverse) by a coupling mechanism between molecular vibrations and electronic motions (so-called vibronic process).

Recent efforts to find copper compounds which might serve as models for blue compound formation have been summarized by Dawson (19). Of a

TABLE III
OXIDATION STATE OF Cu AND THE COLOR OF SOME Cu PROTEINS

Cu protein	Cu/mole	ESR method	Chemical method	Visible light Absorption properties	
				Maximum mμ	Molar extinction coefficient
Hemocyanin	20–200	Cu^{+1}	Cu^{+1}	No color	
Oxyhemocyanin	20–200	Cu^{+1}	$Cu^{+1} = Cu^{+2}$	347	6,100
				585	750
Azurin	1	Cu^{+2}		625	3,500
Galactose oxidase	1	Cu^{+2}		(Pink-yellow)	
Monoamine oxidase (plant, plasma)	4	Cu^{+2}		380	2,500
				480 (Pink)	1,100
Diamine oxidase	1		Cu^{+2}	500 (Pink)	
Tyrosinase, mushroom	4	4 Cu^{+1}	4 Cu^{+1}	No color	
Neurospora	1	Cu^{+1}	Cu^{+1}	No color	
Laccase lacquer tree	4, 6	Cu^{+2}		610	1,300
Fungal	4	2 Cu^{+1}, 2 Cu^{+2}			
Dopamine hydroxylase	4–7	Cu^{+2}		No color	
Ascorbate oxidase	8		1 Cu^{+1}, 3 Cu^{+2}	606	770
Ceruloplasmin	8	4 Cu^{+1}, 4 Cu^{+2}	Cu^{+1}, Cu^{+2}	605	1,200
R. vernicifera blue protein	1		Cu^{+2}	608	4,000
Plastocyanin	2	2 Cu^{+2}	Cu^{+2}	597	4,900
Transsulfurase	1			420	3,230
				570	300

See references 47, 50 *a*, and 57 for appropriate sources.

series of Cu(II) complexes with proteins and certain protein components including imidazole, glycine peptides, lactoglobulin, and bovine serum albumin, the highest molar extinction coefficient reported was 95 at 580 mμ for Cu(II)-*bis*-tetraglycine. This value is an order of magnitude below the molar extinction coefficient (per Cu) of the Cu proteins listed in Table III. Thus, Dawson concluded that the type of bonding between copper and the ligands in naturally occurring copper proteins, characterized by a high extinction around 600 mμ over a relatively wide pH range, is very different

than that responsible for the lower absorption, the pH-dependent wave lengths of Cu(II) complexes with non-specific proteins or peptides.

Copper complexes with bulky ligands have tetrahedral or some similar structure: like tetrahedral $CuBr_4^{-2}$ or $CuCl_4^{-2}$. These structures have no center of symmetry; thus, the *d-d* transition is not strictly forbidden by the selection rule. Nevertheless, the intensity ($\epsilon \leqq 50$–750) is still lower than $\epsilon = 800$ which is observed in a very long wave length region; *e.g.*, $\epsilon = 600$ for $CuCl_4^{-2}$ at 1050 mμ. However, very strong charge transfer (CT) transitions appear in the visible for these complexes.

TABLE IV

CHARGE TRANSFER BANDS OF TETRAHEDRAL COPPER COMPLEXES (20)

	mμ	ε	*Assignment*
$CuCl_4^{-2}$	377	1,700	$\pi \rightarrow 3d$
	274	4,000	$\sigma \rightarrow 3d$
$CuBr_4^{-2}$	585	1,000	$\pi \rightarrow 3d$
	512	2,100	$\pi \rightarrow 3d$
	338	4,200	$\sigma \rightarrow 3d$
	266	2,300	$\sigma \rightarrow 3d$

TABLE V

CHARGE TRANSFER BANDS OF $[Co(NH_3)_5X]^{+2}$ (20)

Complex	CT ($\pi \rightarrow d$)	ε	CT ($\sigma \rightarrow d$)	ε
	mμ		*mμ*	
$Co(NH_3)_5Cl$	270	600	278	20,000
$Co(NH_3)_5Br$	313	850	253	18,500
$Co(NH_3)_5I$	380	2,700	287	16,500

Tetrahedral $CuCl_4^{-2}$ and $CuBr_4^{-2}$ have CT bands shown in Table IV (20). No stable CuI_4^{-2} exists because an electron is transferred very easily from the iodine to the Cu(II) by a small thermal excitation. If a stable CuI_4^{-2} existed one would expect the CT bands to appear in a much redder region than that of $CuBr_4^{-2}$. The position of the CT bands of the imaginary CuI_4^{-2} can be deduced from the data on the CT bands of $[Co(NH_3)_5X]^{+2}$ shown in Table V (20). $Co(NH_3)_5$ I has a CT band at a considerably higher wavelength than the corresponding bromide or chloride. Thus we might therefore expect the longest corresponding band for CuI_4^{-2} to be in the 600 to 700 mμ range. The CT bands of tetrahedral Cu(II) complexes have intensities comparable to those of the absorption bands for the blue color of Cu proteins (Table III), whereas the CT bands of the usual planar or octahedral Cu(II) complexes always appear in the ultraviolet region.

The stability of the Cu(II) state in Cu proteins might be less than that of $CuBr_4^{-2}$ or slightly more than that of CuI_4^{-2}. Actually the bands responsible for the blue colors of Cu proteins are less energetic than that of $CuBr_4^{-2}$. Therefore, we could expect the Cu sites responsible for the blue color of Cu proteins to have tetrahedral structures. This intense color is due to CT bands, $\pi \rightarrow 3d$ and/or $\sigma \rightarrow 3d$ transitions, which appear at a longer wave length region than that of $CuBr_4^{-2}$. The electron affinity of the site estimated by optical spectra (Franck-Condon principle) is more than that of $CuBr_4^{-2}$ and possibly comparable or slightly less than that of CuI_4^{-2}.

A convenient model system for further exploration of the copper locus in copper proteins is now being provided by the copper chelate shown in Fig. 5. This is the most intensely blue copper complex known $\epsilon = 14{,}000$ at 610 mμ and was first noted, but not characterized, by Nilsson (21). It is one of the

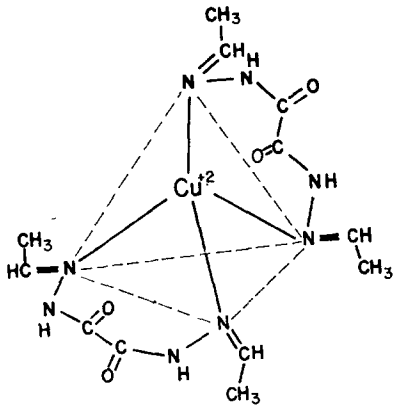


FIGURE 5. Postulated structure of the blue copper chelate formed by one copper ion, two molecules of oxalyldihydrazide, four molecules of acetaldehyde.

cuprizones, a series of colored copper chelates of oxalyldihydrazide and various aldehydes or ketones such as acetaldehyde or cyclohexanone. The stoichiometry of this chelate is oxalyldihydrazide to acetaldehyde to Cu ion of 2:4:1. It also has two additional absorption maxima at 380 and 260 mμ, similar to several copper proteins; e.g., hemocyanin, 350 mμ; laccase and ceruloplasmin, 404 and 340 mμ (22).

A tetrahedral structure (Fig. 5) is proposed for this chelate because of the high intensity of the blue color. Planar or octahedral structures of Cu(II) chelates also have blue colors but not as intense. The deep blue color of the chelate in Fig. 5 is believed to be due to charge transfer transitions from the π-orbitals of the ligand to the central d orbitals or from the sigma orbitals of the ligand to the d orbitals. These electronic transitions produce deep visible absorption only in the visible wave length range for the tetrahedral or similar structures. We hope to report in detail on some of the other interesting properties of this series of compounds at a later date.

Oxidation State of Copper Ion in Copper Proteins

In attempting to describe the sequence of events leading to the reactions of oxygen with the Cu proteins, it is of prime importance to know the oxidation state of the Cu protein in its resting or native state. Oxidized hemocyanin, *met*hemocyanin, like (oxidized) *met*hemoglobin, and (oxidized) *met*hemerythin, are reported to be unable to bind molecular oxygen (10). In addition to spectral data, Table III presents a summary of the latest available data on the Cu(I) or Cu(II) state of copper proteins as determined by electron spin resonance (ESR) signal or magnetic susceptibility measurements, and by

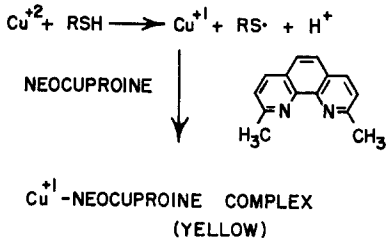


FIGURE 6 *a*. Reduction of Cu(II) to Cu(I) by sulfhydryl compounds in the presence of neocuproine.

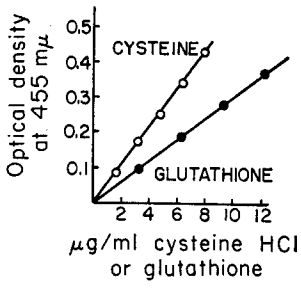
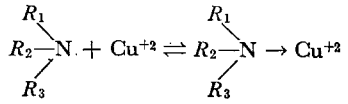


FIGURE 6 *b*. Quantitative reaction of cysteine and glutathione by the reduction of Cu(II) in the presence of neocuproine. Each 4.0 ml of reaction mixture contained 5.0×10^{-5} M cupric chloride, 2.5×10^{-5} M neocuproine, 0.01 M phosphate buffer, pH 7.0, and the indicated amount of cysteine or glutathione. The optical density at 455 mμ was measured at 25°C. Data from Frieden (24).

chemical measurements, principally the method of Felsenfeld (23). Each method suffers from certain limitations. The ESR test is based on the typical paramagnetic nature of Cu(II) and the diamagnetic nature of Cu(I). But false negatives for Cu(II) can be obtained when the Cu(II) is involved in spin coupling with oxygen or another ligand or in the formation of a diamagnetic dimer or a covalent Cu Cu bond. The chemical method involves the determination of Cu(I) with a Cu(I)-preferring reagent, biquinoline, after removal of the copper from the protein with acid. EDTA is also included to chelate Cu(II) and thus prevent Cu(II) reduction by various protein functional groups, particularly SH groups. The possibility of reducing the released Cu(II) has been clearly pointed out earlier (24) (Fig. 6 *a*). This reaction is quantitative for reducing agents such as glutathione and cysteine (Fig. 6 *b*) (24). Though the reduction of Cu(II) proceeds most rapidly with cysteine

residues, it has been found that *N*-substituted tyrosines can also reduce Cu(II) in the presence of a "Cu(I)"-preferring agent, 2,9-dimethyl-1, 10-phenanthroline (neocuproine), at respectable rates (25).

With these basic facts in mind we now proceed to examine the problem from a more theoretical viewpoint. Coordination is a binding between acid and base by partial donation of the electron pair of the base to the vacant orbital of acid. This type of charge transfer takes place in the coordination of metal complexes:



For copper ion complexes such as $Cu(NH_3)_4^{+2}$, the lone pair electrons of ammonia (ligand) are transferred to the central Cu(II) ion. Therefore, the net charge of the Cu ion becomes less than +2 (probably less than +1). The charge donation due to a coordination bond formation gives an electroneutralized Cu ion. This does not signify the actual reduction of the metal ion. The formal oxidation state of the central Cu ion is still divalent or Cu(II).

In the case of blue copper proteins which have cupric ion as a functional group, a similar electron donation takes place from the coordinating groups of proteins and probably hydration water. Sometimes, electron donation of this type may occur from an instantaneously coordinating substrate molecule. Even in this case the oxidation state of Cu may be still maintained from this electron donation. However, all known substrate molecules have high energy labile π-electrons. If a tetrahedral local structure can be assumed for the Cu site of blue-colored copper proteins, the π-electron of the highest occupied orbital of the substrate will merge with the central *d* orbital of copper, and accordingly, the positive hole of the *d* orbital (due to an unpaired electron of the d^9 shell of Cu(II) will transfer to the ligands including the instantaneously coordinated substrate molecule. Molecular orbital theory predicts that the mixing between the *d* orbitals of copper and σ- and π-orbitals of ligands is much higher than that of strong field complexes of planar or octahedral structures. This delocalization still does not signify a reduction of Cu ion. However, when the coordination bond is broken, the substrate leaves one of the delocalized π-electrons at the Cu site resulting in the reduction of Cu(II) to Cu(I).

The cation radical formed from the substrate will transfer its proton to some nearby available proton acceptor and then, disproportionation or any other secondary chemical reaction will take place. The proposed cation radical formation has been experimentally observed by Broman *et al.* (11) by ESR studies of the catalytic action of laccase and ceruloplasmin. The reduced coppers can retain their accepted electrons because of the *higher* stability of

tetrahedral Cu(I) configuration, and will furnish their electrons to oxygenated site(s) along the hydrated protein helical structure or by direct coupling between the two copper ions.

HEMOCYANIN

Deoxygenated hemocyanin contains strictly cuprous ions which do not give an ESR signal or absorption band in the visible (11*a*). The molecular orbital theory of a tetrahedral coordination complex shows that top filled orbitals are an admixture of *d* orbital of copper and π- and σ-orbitals of the ligands and that they are mainly localized at the central metal orbitals. The *t* orbitals

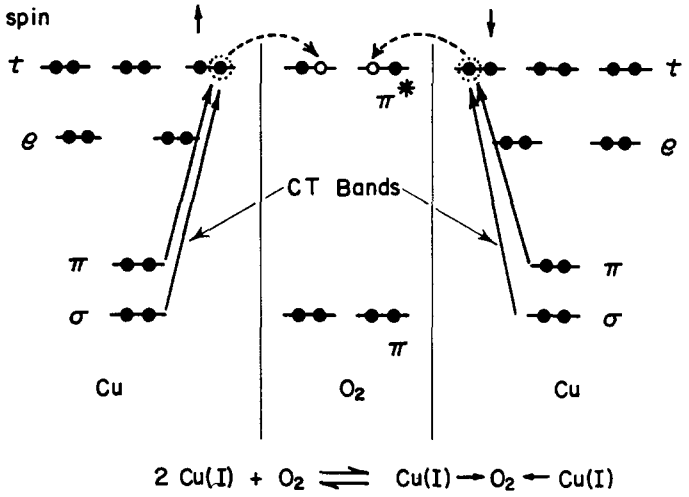


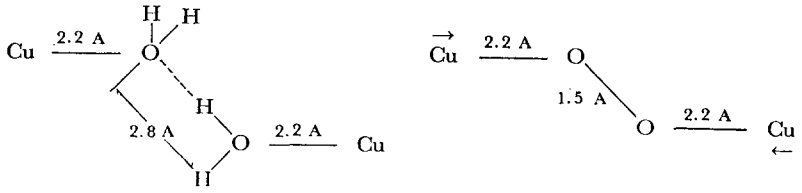
FIGURE 7. Electron configuration of oxyhemocyanin based on molecular orbital theory Two arrows at spin indicate spin coupling of the unpaired electron of each copper atom. The filled and open circles indicate electrons and holes (vacancies), respectively.

are an admixture of $d\pi$-, π-, and σ-orbitals, while the *e* orbitals consist of $d\sigma$- and π-orbitals. When oxygenated, one of the top filled orbital electrons of each cuprous ion goes into the half-filled π-orbital of O_2 (Fig. 7).

This results in a vacancy of the top filled *t* orbital. Consequently, charge transfer transitions take place from the π- and σ-molecular orbitals, which are mainly composed of the π- and σ-orbitals of coordinating ligands, to this vacancy in the top *t* orbitals. The blue color of hemocyanin arises from the CT induced by the bridged O_2, and not as the result of CT between the metal and O_2 which has been assumed to cause the blue color. The actual electronic structure of oxygenated hemocyanin may closely resemble Cu(II)–$(O_2)^{-2}$–Cu(II). However, the environment (probably due to hydrophobic groups) may be analogous to the case of hemoglobin, which requires that the oxygen molecule return its borrowed electron when deoxygenated. Since the

charge separation is not preferred in the hydrophobic environment, this tendency prevents a permanent oxidation of Cu(I) to Cu(II). The diamagnetism of oxygenated hemocyanin can be explained by a coupling of unpaired electrons of each metal ion, even though the oxidation state of the copper of oxygenated hemocyanin is very close to Cu(II). The hemocyanin structure proposed here can also explain the results of numerous chemical methods to determine the valence or oxidation state of copper in oxyhemocyanin which involves the separation of the metal ion from the protein prior to the determination and always shows some mixture of Cu(I) and Cu(II).

It has been proposed, partly by analogy with hemoglobin, that the combination of oxygen with hemocyanin is accompanied by changes in the conformation of the protein. The distance between the two coppers in hemocyanin can be estimated from the closest packing radius of the water molecule, *ca.* 1.4 A obtained by x-ray studies of ice, by assuming that the deoxygenated hemocyanin has two coordinated water molecules.



While the O-O distances of peroxide, superoxide, and oxygen show systematic contraction from 1.5 A (for O_2^{-2}) to 1.2 A (for O_2), in each case they are less than 1.5 A.

Assuming the same bond length for Cu—O, 2.2 A, for the oxygenated and the deoxygenated, we find a remarkable shift of two coppers due to the oxygenation. Therefore we would expect some conformational change in the protein part due to the oxygenation, very similarly to the conformational change observed for hemoglobin. However, the evidence for these changes in hemocyanin, recently summarized by Manwell (26), is admittedly equivocal, particularly because of the difficulty in distinguishing changes in the associated solvent from actual changes in the protein. Closely involved is the question of whether hemocyanin has functional subunits, since subunits have been reported which bind oxygen less avidly than the aggregate molecules. The disaggregation of hemocyanin effected by lowering divalent ion concentration, particularly calcium ion, has also been suggested as a mechanism for modifying the oxygen transport function of hemocyanin (27).

THE COPPER OXIDASES

The top filled orbitals of Cu(II) site have one vacancy which behaves as an electron acceptor for the substrate. The intense blue color or CT bands of

Cu proteins are due to the electron transition from the bonding π- and σ-orbitals to this vacancy in the t orbitals.

The Cu(I) site which is found originally in the enzyme or converted from Cu(II) by reduction has a closed shell for the top filled orbitals. When oxygenated, one of the top filled orbital electrons will transfer to the oxygen molecule and create a vacancy in the t orbitals. However, it depends upon the environment of the site of the oxygenation whether the electron transfer is tentative and will be restored upon deoxygenation, or whether the electron transfer results in the reduction of oxygen. Probably the Cu proteins which behave as oxidases have active sites surrounded by hydrophilic groups and water, which facilitate the oxidation of Cu(I) by O_2. Because of unstable t electrons, there is a possibility of a two electron transfer from the t orbitals of each closely located copper to the oxygen, resulting in the direct reduction of O_2 to H_2O. However, it is not reasonable to assume this direct reduction, unless those two copper sites are joined to neighboring reduced Cu sites (reduced by substrate) through some electron-conducting mechanism such as a hydrated helical protein structure.

Water or Hydrogen Peroxide As the Product of Cu Ion- or Cu Enzyme-Catalyzed Oxidations

One of the reaction characteristics which has been used to distinguish between copper ion–catalyzed oxidations and certain closely related enzymic oxidations has been the production of a stoichiometric amount of hydrogen peroxide by the free metal ion catalysis (28). None of the more familiar copper oxidases such as tyrosinase, ascorbate oxidase, laccase, or ceruloplasmin produces hydrogen peroxide (9, 28, 52). Yet it has been long known or accepted that the copper ion–catalyzed oxidations of ascorbate, *p*-phenylenediamine (*p*PD), and uric acid produce hydrogen peroxide (28, 29, 52). In recent years several new copper oxidases have been discovered which, in contrast to the previously mentioned enzymes, do produce stoichiometric amounts of hydrogen peroxide. These include galactose oxidase (30), monoamine oxidase (17), diamine oxidase (32), and uricase (29). The enzyme, uricase, of course, had been known for a long time but the evidence for its being a copper enzyme has been reported only recently (29). The reactions known to involve hydrogen peroxide production are as follows:—

$$\text{Ascorbate} + O_2 \xrightarrow{Cu^{+2}} H_2O_2 + \text{dehydroascorbate}$$

$$p\text{-Phenylenediamine} + O_2 \xrightarrow{Cu^{+2}} H_2O_2 + \text{oxidation products of } p\text{PD}$$

$$\text{Uricate} + O_2 \xrightarrow[\text{pH 11}]{Cu^{+2}} H_2O_2 + \text{oxidation products of uricate}$$

$$\text{Uric acid} + O_2 \xrightarrow{\text{uricase}} H_2O_2 + \text{allantoin} + CO_2$$

$$\text{D-Galactose} + O_2 \xrightarrow{\text{galactose oxidase}} H_2O_2 + \text{D-galactohexodialdose}$$

$$\text{Benzylamine} + O_2 \xrightarrow[\text{oxidase}]{\text{plasma monoamine}} H_2O_2 + \text{benzaldehyde} + NH_3$$

$$NH_2CH_2CH_2CH_2CH_2NH_2 \xrightarrow[\text{oxidase}]{\text{diamine}} H_2O_2 + \text{oxidation products of } n\text{-butyl diamine}$$

We now raise the question as to why some Cu oxidases produce hydrogen peroxide while others produce water. We can evaluate the thermodynamic possibilities from the data presented in Table VI. The E'_0 values of neutral pH's virtually exclude hydrogen peroxide as a product of certain blue Cu

TABLE VI
STANDARD OXIDATION-REDUCTION POTENTIALS*

	E_0 (pH = 0)	E'_0 (pH, 7.0)
K/K^+	+2.93‡	
HO_2/O_2	+0.13	+0.55
Hemoglobin Fe^{+2}/Fe^{+3}		+0.14
Ascorbate/dehydroascorbate	−0.39	−0.06
Cu^{+1}/Cu^{+2}	−0.15	
H_2O_2/O_2	−0.68	−0.26
Cytochrome *c*; reduction-oxidation	−0.46	−0.26
Hydroquinone/quinone	−0.70	−0.28
$Fe(CN)_6^{-4}/Fe(CN)_6^{-3}$	−0.36	
Pseudomonas aeruginosa blue Cu protein‡		−0.33
Plastocyanin§		−0.37
R. vernicifera blue Cu protein‖		−0.42
Ceruloplasmin¶; Cu^{+1}/Cu^{+2}		−0.39
Laccase**; Cu^{+1}/Cu^{+2}		−0.42
$CuCl/Cu^{+2}$	−0.54	
$CuBr/Cu^{+2}$	−0.64	
H_2O/O_2	−1.23	−0.82
CuI/Cu^{+2}	−0.86	
$Cu(CN)_2^-/Cu^{+2}$	−1.12	
H_2O/H_2O_2	−1.77	−1.35

*Data at 20–30° from Latimer (63) or Lardy (64).
‡ Horio *et al.* (67).
§ Katoh *et al.* (68).
‖ Omura (69).
¶ Blumberg *et al.* (65).
** Nakamura (66).

enzymes, *e.g.* laccase, ceruloplasm–catalyzed oxidations since they have potentials which are lower than the H_2O_2/O_2 couple of −0.26 v. The H_2O/O_2 potential establishes water as a possible product of the reaction between the Cu(I) form of the enzyme and oxygen. Either reaction is possible for free copper ion catalysis with a potential of −0.15 v. It is of interest that perhydroxy, HO_2, is excluded as a product of any cuprous or ferrous ion oxidation if its potential is as high as has been reported (63) —+0.13 v (pH = 0) or +0.55 v when corrected to pH 7.0.

It is apparent that the Cu enzymes which produce hydrogen peroxide

must have potentials around −0.26 v or less negative if the Cu(I)/Cu(II) reaction is involved. These enzymes would then have a significantly different structure from the Cu enzymes listed in Table VI, since the latter have E'_0 less than −0.33 v. The low potential for laccase, ceruloplasmin, and other Cu proteins is probably due to the stabilization of the tetrahedrally coordinated Cu(I) form of the enzyme. Clearly this kind of stabilization cannot exist for the Cu(I) form of hydrogen peroxide–producing Cu enzymes. We would expect the coordination of copper ion in these enzymes to be either planar or octahedral. It is of interest to note that the effect of ligands is to uniformly decrease the Cu(I)/Cu(II) potential but to consistently increase the Fe(II)/Fe(III) couple. The simplest explanation is to assume that these effects are due to preferential stabilization of Cu(I) and Fe(III) by the respective ligands.

Correlation of the Number of Enzyme Copper Atoms and Water or Hydrogen Peroxide Formation

The discussions on the relative potentials merely delineate the possible reactions. Which reaction actually occurs is determined by separate kinetic factors. A major problem posed by the exclusion of perhydroxy and hydrogen peroxide as the product of certain Cu enzyme-catalyzed oxidations is that there must eventually be a four electron transfer. As shown in Table VII, those enzymes which produce water have four or more copper atoms per molecule. Favorable positioning of at least two of these copper atoms would facilitate a four electron reduction of oxygen to water. Except for monoamine oxidase the Cu enzymes which produce hydrogen peroxide and thus serve as two electron transfer oxidases have only one copper atom per molecule of enzyme. Monoamine oxidase is reported (17, 33, 34) to have four copper atoms per enzyme molecule but still produces hydrogen peroxide, the only copper enzyme with more than one copper atom per molecule known to produce hydrogen peroxide. It is also unusual in that it has two molecules of pyridoxal phosphate per 255,000 gm, the molecular weight of monoamine oxidase. Thus there is a structural basis for the unique behavior of this enzyme. The presence of pyridoxal phosphate suggests that we might expect a different reaction mechanism for monoamine oxidase, involving an oxidative removal of an amino group through transfer to pyridoxal phosphate. It is also conceivable that the detailed mechanism of monoamine oxidase deamination could involve direct participation of only one or two of the total of four coppers found in the molecule. This effects only a one or two electron or hydride ion transfer. Another possible exception to this correlation is *Neurospora* tyrosinase, which has only one cuprous ion per 33,000 gm (35) for which no hydrogen peroxide has been reported as a product, although its

absence has not been verified. No data are available on the oxidation-reduction potential of the Cu(I)/Cu(II) of this enzyme which might reflect on the likelihood of hydrogen peroxide as a possible product.

The foregoing considerations may also apply to copper enzymes which have two copper atoms per molecule but no example of this has yet been found. Of interest and value in properly interpreting mechanisms of these enzymes will be isotopic studies such as those of Bentley and Neuberger (36), who proved that the oxygen in the hydrogen peroxide produced by uricase came directly from molecular oxygen. Additional oxygen isotope studies would be helpful in elucidating these mechanisms further.

TABLE VII
CORRELATION OF NUMBER OF ENZYME COPPER ATOMS AND H_2O_2 FORMATION

Cu enzyme or catalytic system	Cu/mole	Water or H_2O_2 formation	Reference
Uricase	1	H_2O_2	(29)
Galactose oxidase	1	H_2O_2	(30)
Diamine oxidase	1	H_2O_2	(31)
Cu(II) ascorbate	(1)	H_2O_2	(28)
Cu(II)-*p*-phenylenediamine	(1)	H_2O_2	(28)
Cu(II) uricate	(1)	H_2O_2	(30)
Monoamine oxidase	4*	H_2O_2	(17)
Tyrosinase (mushroom)	4	H_2O	(59)
Laccase	4, 6	H_2O	(57)
Dopamine-β-hydroxylase	4–7	H_2O	(38)
Ascorbate oxidase	8	H_2O	(58)
Ceruloplasmin	8	H_2O	(28)

* Enzyme also contains pyridoxal phosphate.

In general these two electron transfer oxidases do not serve as important terminal oxidases. Their major role appears to be to effect certain specific syntheses and metabolic changes, *e.g.*, galactohexodialdose formation; uric acid and aromatic monoamine disappearance. As Mason (37) has emphasized, the disadvantages of reduction mechanisms which result in the formation of such reactive intermediates as superoxide or hydrogen peroxide are twofold, (*a*) a random attack upon functioning components of the cell, and (*b*) the inefficiency of such oxidations compared to oxidation to water because half or more of the electron affinity of the oxygen molecule is thereby lost to metabolism. Thus the enzymes which produce hydrogen peroxide have probably been relegated to a highly specific or minor role in metabolism.

All mechanisms proposed for the two or four electron transfer oxidases assume a cyclic valence change involving reduction of Cu(II), probably to Cu(I) protein by substrate, usually forming free radical intermediates and

then the subsequent oxidation of Cu(I) protein by molecular oxygen. This has been confirmed for several copper enzymes; *e.g.*, ascorbate oxidase (38), ceruloplasmin (11), laccase (11), dopamine hydroxylase (31), and also copper ion catalysis (1, 3). It remains to be confirmed for the enzymes which produce H_2O_2. It was mentioned earlier that the ESR signal of plasma amine oxidase does not change from the Cu(II) state during enzymic activity (34).

While the ESR method is virtually the only one now available for the study of cyclic changes in copper, the possibility of false negatives makes conclusions based on it tentative. For example, if during the cyclical exchange, the various reaction rates are such that the steady state level of Cu(II) during enzymic activity remains at more than 95 per cent of the total copper, the

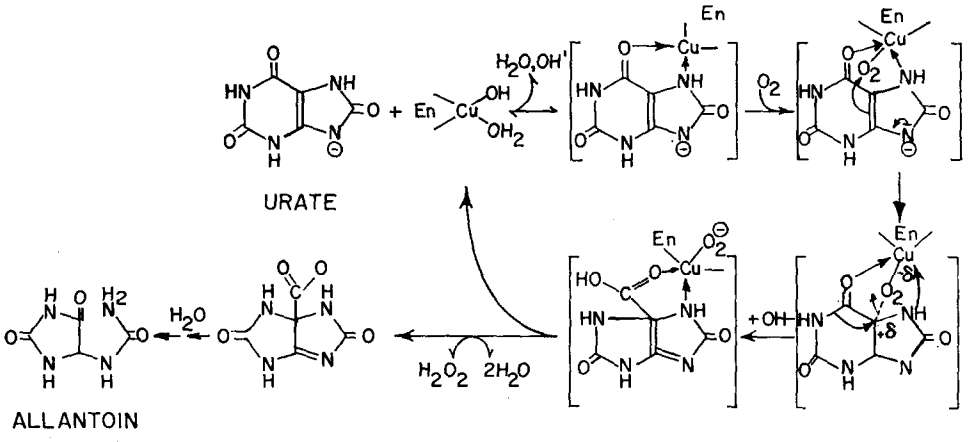


FIGURE 8. Postulated mechanism of action of uricase as proposed by Mahler (29).

presence of the remaining 5 per cent of Cu(I) might not be detectable. An oxidative deamination taking place in the presence of pyridoxal phosphate or some other prosthetic group is therefore possible, but it would be difficult to deduce a consistent mechanism for the oxidation of the primary alcohol of galactose and the production of hydrogen peroxide without an intermediate change in Cu(II) state of the enzyme, particularly when no other prosthetic group or coenzyme is involved.

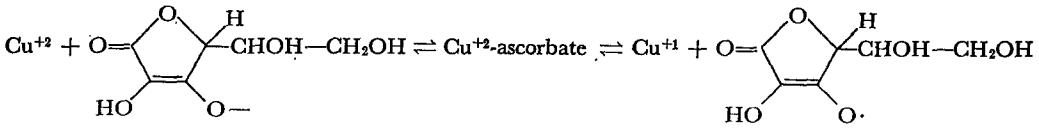
For the enzyme uricase a mechanism that does not involve any valence change for the copper ion has been proposed by Mahler and his associates (29). Uricase is a difficult enzyme to prepare and characterize and, in the light of more current methods, the evidence for its copper content must be regarded as mostly indirect. As shown in Fig. 8, Cu(II) ion is pictured as providing (*a*) a locus of simultaneous attachment for both the electron donor, uricase, and the electron acceptor, oxygen, (*b*) a strong polarizing force for drawing electrons away from uric acid, and (*c*) a means of shuttling electrons

to oxygen from orbitals shared with uric acid. The ultimate products of uricase action probably arise from non-enzymatic side reactions which occur subsequently. The reaction with oxygen is depicted as a displacement by oxygen of one of the protein Cu bonds. Another possibility is a transition from the strong planar copper complex of a coordination number of 4 to the much weaker octahedral copper complex of a coordination number of 6.

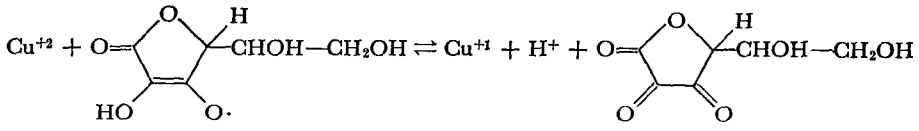
THE CU(II)–CATALYZED OXIDATION OF ASCORBIC ACID

Probably the most widely studied of the free copper ion catalyses is the Cu(II)–catalyzed oxidation of ascorbate (39–42). Although this reaction has found wide use, particularly in the study of Cu(II)–complexing reactions (39), the exact mechanism has still not been unequivocally elucidated, despite many intense efforts (39–42). The basic features of the reaction which must be accounted for include:

1. The catalytic role of Cu(II) and the changing order of the reaction with respect to Cu(II) as shown in Fig. 9.
2. The oxidation of ascorbate to one mole of dehydroascorbate consuming one mole of O_2 and producing one mole of H_2O_2.
3. The cyclic interconversion of Cu(I) and Cu(II).
4. In the neutral pH range (5–9) the reactive species is the ascorbate monoanion which is converted to the relatively stable ascorbate semiquinone, recently isolated and characterized by Levandoski *et al.* (42 *a*). The sequence of reactions may be pictured as follows:—



This free radical could react with O_2 to produce dehydroascorbate, but it is also possible for it to react with another Cu(II), regenerated, if necessary, by reaction with oxygen as shown in the following reactions:



In order to account for a stoichiometric amount of hydrogen peroxide by the most simple kinetic mechanism possible it would be convenient first to postulate the formation of the perhydroxy radical $HO_2^{\cdot}$, as the result of a one electron transfer from Cu^{+1} to O_2. But a consideration of the potentials involved (see Table VI) makes the formation of perhydroxy extremely unlikely, although it has not been excluded experimentally. If $HO_2^{\cdot}$ were formed, it would not survive for any extended period because of its high

reactivity with Cu(I) or other intermediates and its strong tendency to dismutate in aqueous solutions to hydrogen peroxide and oxygen. For Cu(I) oxidation, the reduction of oxygen to H_2O_2 or water is both thermodynamically feasible but the formation of water could involve a four electron transfer which is virtually impossible when the Cu ions are not assembled in a favorable structure such as in a Cu protein. We propose a two electron transfer by

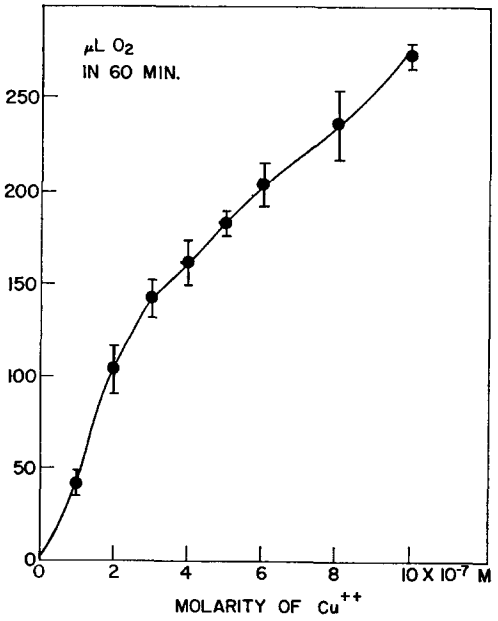


FIGURE 9. The effect of varying Cu(II) on the rate of oxidation of 0.020 M ascorbate in 0.010 M phosphate buffer, pH 7.1 at 30°. The Warburg method was used to measure oxygen uptake with air as the gas phase (Wahlborg and Frieden, 56).

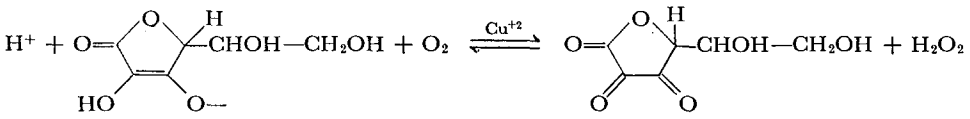
either of the following two mechanisms:

$$Cu^{+1} + O_2 \rightleftharpoons Cu^{+1}\text{—}O_2 \xrightleftharpoons{Cu^{+1}} Cu_2^{+1}\text{—}O_2 \rightleftharpoons Cu_2^{+2}\text{—}(O_2)^{-2} \xrightleftharpoons{2H^+} 2Cu^{+2} + H_2O_2$$

or

$$Cu^{+1} + O_2 \rightleftharpoons Cu^{+1}\text{—}O_2 \rightleftharpoons Cu^{+2}\text{—}(O_2)^{-1} \xrightleftharpoons{Cu^{+1}} Cu^{+1}\text{—}(O_2)^{-1}\text{—}Cu^{+2} \rightleftharpoons$$
$$Cu^{+2}\text{—}(O_2)^{-2}\text{—}Cu^{+2} \xrightleftharpoons{2H^+} H_2O_2 + 2Cu^{+2}$$

These reactions while not being the only ones possible could account for the over-all stoichiometry and other features cited.



This brief discussion of these mechanisms has not included possible chain mechanisms or reactions which are bimolecular with respect to Cu(II). It is also of interest to note that the Cu(II)–catalyzed oxidations of *p*-phenylene-

diamine and uric acid also result in the production of stoichiometric amounts of H_2O_2.

MECHANISMS OF CU ENZYME-CATALYZED OXIDATIONS

Except for tyrosinase (and possibly galactose oxidase) which qualifies as a mixed function oxidase (monoxygenase), most of the Cu oxidases are thought to be electron transfer oxidases. Their uniqueness, probably shared with many Fe enzymes, may arise from the fact that their action is characterized by a one electron transfer which is ultimately multiplied to a two or four electron transfer to oxygen.

With the above correlations and information on the state of oxidation and with the helpful discussions and suggestions of Mason (37) and Mahler (43), we have attempted to formulate general oxidative mechanisms for the Cu enzymes which should take into account the basic features of these reactions summarized below.

1. Two electron transfer oxidases which could result in formation of hydrogen peroxide as a primary product.
2. Four electron transfer oxidases which produce water and usually occur in two steps of two electrons at a time.
3. The number of copper atoms per molecule of enzyme or active enzyme subunit.
4. The probable oxidation state of copper in the enzyme molecule during rest and activity.
5. The necessity of treating oxygen in its diatomic state until it undergoes at least partial oxidation to $(O_2)^{-2}$ prior to any cleavage of the oxygen molecule.
6. The sequence of reactions of the catalytic copper with oxygen and oxidizable substrates, *e.g.* when the enzyme exists as Cu^{+2} in the resting or prereaction state, the first reaction is a one electron reduction of each Cu^{+2} to Cu^{+1}. The probable single exception is tyrosinase where all the copper atoms appear to exist as Cu^{+1} (59). There also may be enzymes in which no cyclic change in the valence of Cu ion occurs which will have to be described by different mechanisms.
7. Finally, these mechanisms are consistent with numerous accessory facts including the production of free radical intermediates, exchangeability of the copper ions (44 *b*, 44 *c*), induction periods, copper ion in representative model systems, etc.

It has been realized that any mode of action involving the cyclical valence change of $Cu^{+2} \rightleftharpoons Cu^{+1}$ raised important problems arising from the fact that the metal undergoes a one electron change, the oxidizable substrate a one or two electron change, and the oxygen molecule eventually, either a two or four electron change. It seems likely that most substrates can reduce Cu^{+2} to

Cu^{+1} by a one electron transfer. The most pressing dilemma has been one of electron inventory and relates to the reduction of the oxygen molecule to either peroxide or water. The following oxygen reduction reactions are possible:

Consecutive one electron reduction:

$$Cu^{+1} + O_2 \rightleftharpoons Cu^{+1}—O_2 \rightleftharpoons Cu^{+2}—(O_2)^{-1} + Cu^{+1} \overset{2\,H^+}{\rightleftharpoons} 2Cu^{+2} + H_2O_2$$

Two electron reduction:

$$Cu_2^{+1} + O_2 \rightleftharpoons Cu_2^{+1}—O_2 \rightleftharpoons Cu_2^{+2}—(O_2)^{-2} \overset{2\,H^+}{\rightleftharpoons} Cu^{+2} + H_2O_2$$

Four electron reduction:

$$Cu_4^{+1} + O_2 \rightleftharpoons Cu_4^{+1}—O_2 \rightleftharpoons Cu_4^{+2}—(O_2)^{-4} \overset{4\,H^+}{\rightleftharpoons} Cu_4^{+2} + 2H_2O$$

As explained earlier, we must exclude perhydroxy (HO_2) formation which forbids a single one electron reduction. The expected product for these and analogous reactions is hydrogen peroxide or further oxidation states of oxygen. The relatively high stability of hydrogen peroxide may explain how two electron transfer reactions can lead exclusively to hydrogen peroxide, but unless the Cu enzyme itself possesses catalase activity, a four electron transfer is necessary for water formation from oxygen. None of the Cu enzymes has significant catalase activity. In this connection, it is of interest that while there are several Cu oxidases with one copper per molecule, none has been found to have two coppers per molecule.

If the enzyme has a sufficient number of adjacent and accessible metal ions as Cu(I), the requisite number of electrons can be transferred. A final alternative is suggested by the formation of highly active free radical substrate intermediates such as the ascorbate-free radical, which could reduce a Cu(II) immediately so that the resulting Cu(I) can transfer another electron to an attached partially reduced oxygen. A number of these possibilities are depicted in Tables VIII and IX for two and four electron transfer oxidases with a different number of electron acceptances.

In Table VIII, the first two reactions provide for a one electron donation followed by two consecutive one electron acceptances by oxygen. A simultaneous two electron acceptance by O_2 would simplify the problem of hydrogen peroxide formation by a series of analogous reactions but no two electron copper oxidases are known. The possibility that the enzyme molecule acts in pairs still remains. Certain Cu oxidases with more than two Cu atoms per molecule might react with oxygen two Cu units at a time. Numerous reasonable paths for an ultimate four electron transfer are available. For example, the $En Cu_2^{+2}(O_2)^{-2}$ complex could react with an intermediate sub-

strate species to convert a two electron transfer to an over-all four electron transfer as shown:

$$En\text{-}Cu_2^{+2}\text{—}(O_2)^{-2} + 2\ AH\cdot \rightleftharpoons En\text{-}Cu_2^{+1}\text{—}(O_2)^{-2} + 2\ A + 2\ H^+$$
$$En\text{-}Cu_2^{+1}\text{—}(O_2)^{-2} \rightleftharpoons En\text{-}Cu_2^{+2}\text{—}(O_2)^{-4} \xrightleftharpoons{4\ H^+} 2\ H_2O + En\text{-}Cu_2^{+2}$$

For those copper enzymes which have at least four accessible Cu(I) (*e.g.*, ceruloplasmin, ascorbate oxidase, tyrosinase), we can postulate a simultaneous or four electron transfer on an intramolecular basis as depicted in Table IX. For the other copper proteins, we must assume intermolecular reactions or some mechanism other than a four electron transfer, but it is not likely to involve the O—O bond or the production of peroxide intermediates

TABLE VIII

MECHANISM OF THE TWO ELECTRON TRANSFER Cu OXIDASES

Two consecutive electron transfer with one Cu atom per catalyst molecule and H_2O_2 formation.

1. Removal of an electron from substrate:
   $En\text{-}Cu^{+2} + AH_2 \rightleftharpoons En\text{-}Cu^{+2}\text{—}AH_2 \rightleftharpoons En\text{-}Cu^{+1} + AH\cdot + H^+$
2. First electron transfers to oxygen:
   $En\text{-}Cu^{+1} + O_2 \rightleftharpoons En\text{-}Cu^{+1}\text{—}O_2 \rightleftharpoons En\text{-}Cu^{+1} \rightarrow O_2\ [En\text{-}Cu^{+2}\text{—}(O_2)^{-1}]$
3. Second electron transfer from substrate:
   $En\text{-}Cu^{+1} \rightarrow O_2 + AH\cdot(AH_2) \rightleftharpoons En\text{-}Cu^{+1} \rightarrow O_2^- \xrightleftharpoons{2\ H^+} En\text{-}Cu^{+2} + H_2O_2 + A(AH\cdot) + H^+$
4. Second electron transfer from second catalyst molecule:
   $En\text{-}Cu^{+1} \rightarrow O_2 + En\text{-}Cu^{+1} \rightleftharpoons En\text{-}Cu^{+1} \rightarrow O_2 \leftarrow Cu^{+1}\text{-}En \xrightleftharpoons{2\ H^+} 2\ En\text{-}Cu^{+2} + H_2O_2$

of other oxygen species for which there is no good evidence to date. The implications of a concerted reaction with one oxygen molecule, as Broman (16) has emphasized for ceruloplasmin, suggest that optimally four reactable copper ions must be adjacent to one another, although two copper atoms may be sufficient if a two electron transfer takes place for each copper as discussed previously. Another possible consequence is that the activation of the oxygen molecule may be at least partially due to energy accruing from a distorted tertiary structure arising from conformational changes during enzyme oxidation. Certain subtle structural changes have been associated with the function of ceruloplasmin. The role of the four additional Cu(I) atoms of ceruloplasmin may be to serve in a special way as shown in Table IX. The association of the $Cu_2^{+1} \ldots Cu_2^{+2}$ is shown only to indicate some mechanism for electron transfer but not necessarily a direct charge transfer, nor any Cu^{+1}–Cu^{+2} interaction in the resting enzyme.

MECHANISM OF TYROSINASE CATALYSIS

We now come to tyrosinase, heretofore regarded as a unique Cu enzyme because it is a mixed function oxidase and includes among its functions the hydroxylation of monophenols to diphenols and the oxidation of diphenols to quinones, although these activities may be due to different subunits (37, 44, 44 *a*). Chemically, tyrosinase is the only Cu oxidase in which all the copper ion appears to remain in the reduced form even in the resting state (47, 59).

TABLE IX
MECHANISM OF THE FOUR ELECTRON TRANSFER Cu OXIDASES

1. Removal of four electrons from four (or less) substrate molecules (not simultaneous):
$En\text{-}Cu_4^{+2} + 4\ AH_2 \rightleftharpoons En\text{-}Cu_4^{+1} + 4\ AH\cdot + 4\ H^+$

2. Transfer of four electrons to oxygen:

$$Cu_2^{+1}\ Cu_2^{+1} + O_2 \rightleftharpoons Cu_2^{+1}\text{—}O_2\text{—}Cu_2^{+1} \rightleftharpoons \begin{matrix} Cu^{+2}\text{—}O^{-1}\ Cu^{+1} \\ | \\ Cu^{+2}\text{—}O_{-1}\ Cu^{+1} \end{matrix} \rightleftharpoons$$

(Enzyme)

$$\begin{matrix} Cu^{+2}\text{—}O^{-2}\text{—}Cu^{+2} \\ Cu^{+2}\text{—}O_{-2}\text{—}Cu^{+2} \end{matrix} \xrightleftharpoons{4\ H^+} En\text{-}Cu_4^{+2} + 2\ H_2O$$

(Enzyme)

Possible special mechanism for ceruloplasmin (4 Cu^{+1}, 4 Cu^{+2})

$$Cu_2^{+1} \cdots Cu_2^{+2}\ Cu_2^{+2} \cdots Cu_2^{+1} + 2\ AH_2 \rightleftharpoons 4\ H^+ + 2\ A +$$

(Enzyme)

$$Cu_2^{+1} \cdots Cu_2^{+1}\ Cu_2^{+1} \cdots Cu_2^{+1} \xrightleftharpoons{O_2} Cu_2^{+1} \cdots Cu_2^{+1}\text{—}O_2\text{—}Cu_2^{+1} \cdots Cu_2^{+1} \rightleftharpoons$$

(Enzyme)

$$Cu_2^{+1} \cdots Cu_2^{+2}\text{—}O_2^{-4}\text{—}Cu_2^{+2} \cdots Cu_2^{+1} \rightleftharpoons Cu_2^{+2} \cdots Cu_2^{+1}\text{—}O_2^{-4}\text{—}Cu_2^{+1} \cdots Cu_2^{+2} \rightleftharpoons$$

(Enzyme)

$$Cu_2^{+2} \cdots Cu_2^{+1}\ Cu_2^{+1} \cdots Cu_2^{+2} + O_2^{-4} \xrightleftharpoons{4\ H^+} 2\ H_2O$$

(Enzyme)

Many other Cu enzymes, where the resting state of the copper is Cu(II), involve reaction with oxygen after reaction with the substrate since it is presumed that only the Cu(I) states interact with oxygen and the substrate converts the Cu(II) to Cu(I). This order is reversed in tyrosinase and $EnCu^{+1}\text{–}O_2$ forms must occur before the substrate is oxidized. As with ascorbate oxidase, exchange with free Cu^{64} ion is observed only during catalytic activity of tyrosinase (44 *b*, 44 *c*).

Fig. 10 depicts a widely proposed mechanism of tyrosinase action. A possible early form of the enzyme-oxygen complex is $EnCu_2^{+2}(OOH)^{-1}$, which has been proposed as the hydroxylating form of tyrosinase (43 *a*). This hypothesis

receives support by analogy from detailed studies of certain *ortho*hydroxylating model systems by Konecny (45) and Havinga and his associates (46). Konecny (45) found that the system H_2O_2–Cu(II) will hydroxylate benzene and benzoate to phenol and salicylate. Havinga *et al.* (46) extended this to systems more closely related to tyrosinase in the Cu(II)–catalyzed *ortho*hydroxylation of phenols and aniline in the presence of amines. The hydroxylating intermediate is proposed as $-Cu^{+2}(OOH)^{-1}$. Hydrogen peroxide can be formed by subsequent oxidation of some of the oxidation products. In the intermediate drawn in Fig. 10, Cu(II) acts as an acid catalyst which polarizes the O—O bond so that it will cleave ionically with the electron pair remaining on the oxygen near the copper. The resulting (OH^+) fragment hydroxylates

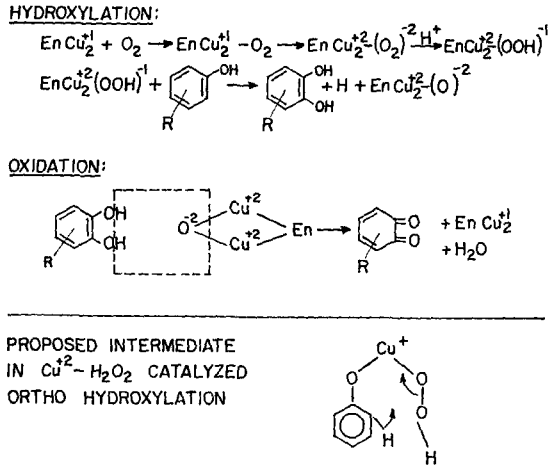


FIGURE 10. Upper figure, proposed mechanism of hydroxylation and oxidation by tyrosinase. Lower figure, proposed intermediate in the Cu(II)–H_2O_2–catalyzed *ortho*-hydroxylation of phenols and aromatic amines.

the benzene ring in a typical electrophilic displacement reaction. As emphasized by Ingraham (46 *a*), in this reaction Cu(II) acts in the same way as two protons in other acid-catalyzed hydrogen peroxide oxidations.

An alternative mechanism based on a hydrogen transfer mechanism from substrate to oxygen without a change in the apparent formal charge of the cuprous ion can also be envisaged.

In its other major type of reaction involving direct oxidation of diphenols to quinones and beyond, tyrosinase is not atypical of other Cu enzymes. The ability of the protein to keep all its copper in the Cu(I) state even in the presence of oxygen until substrate interacts, is a striking feature but is also not unique. From ESR data Malmström and Neilands (47) believe that four of the eight (8) coppers of ceruloplasmin are maintained permanently in the Cu(I) state. There are also several Cu(I) reagents, such as biquinoline and

neocuproine, which form extremely stable chelates with Cu(I), thus insulating Cu(I) against the oxidizing effects of dissolved oxygen.

CERULOPLASMIN AS AN ASCORBATE OXIDASE

Copper ion catalysis has been compared most extensively with enzymic catalysis using ascorbate as substrate. Pertinent data on the catalysis of ascorbate oxidation by Cu(II), squash ascorbate oxidase, and ceruloplasmin are shown in Table X.

The fact that the Cu ion catalysis of ascorbate oxidation is unique in producing hydrogen peroxide has been noted earlier. The pH optima of these reactions are clearly different. The K_m with respect to O_2 for ceruloplasmin is surprisingly low, 4×10^{-6} M, whereas the protein of ascorbate oxidase does not contribute significantly to oxygen binding. Both enzymes have much smaller K_m's with respect to ascorbate. Obviously plant ascorbate oxidase is a much more specific and effective catalyst in terms of catalyzing

TABLE X

COMPARISON OF THE CATALYSIS OF ASCORBATE OXIDATION BY Cu(I), SQUASH ASCORBATE OXIDASE, AND CERULOPLASMIN

Property	Cu(II)	Plant ascorbate oxidase	Ceruloplasmin
pH optimum	≥ 7.5	5.6	6.0
Moles H_2O_2 produced	1	0	0
K_m for ascorbate, M	7×10^{-3}	2.4×10^{-4}	1.3×10^{-5}
K_m for O_2, M	2×10^{-4}	2.2×10^{-4}	3.9×10^{-6}
Activity/Cu(Vm)	150	10^5	12
Reference	(28)	(58)	(28)

the oxidation of a limited group of substrates and in greatly increasing the catalytic activity per atom of Cu.

While some doubts had been raised about the ability of ceruloplasmin to catalyze ascorbate oxidation (48) we believe that this has now been firmly established (28). Crucial data comparing the properties of Cu(II) and ceruloplasmin catalysis are summarized in Table XI. Except for the fact that these catalytic systems have oxygen, ascorbate, and copper in common, they are different in every other respect. Numerous kinetic differences such as dependence on pH, the effect of ascorbate concentration, and relative activation energy, have been found. As discussed earlier, Cu(II) reactions produce stoichiometric amounts of H_2O_2 where the enzyme produces none. Perhaps the most important difference is the specific sensitivity of ceruloplasmin to inhibition by citrate and the specific inhibition of Cu(II) reactions by neocuproine, serum albumin, and certain other copper ion chelators.

TABLE XI

SUMMARY OF DIFFERENCES BETWEEN CERULOPLASMIN- AND Cu(II)-CATALYZED OXIDATION OF ASCORBATE

Parameter or property	Ascorbate oxidation catalyzed by	
	Ceruloplasmin	Cu(II)
K_m (μM)	13	7,000
Zero order constant, $\mu M\ min.^{-1}\ \mu M^{-1}$	14.4	150
As measured at 265 mμ	11.9	
As measured by O_2 uptake	11.4	
First order rate constant, *min./μM copper*	0.053	0.023, 0.033
Reaction order at 100 μM ascorbate	Zero order	First order
Activation energy, *kcal/mole*	12.5	16.5
Optimum pH	6.0	>7.5
H_2O_2 formation	None	Stoichiometric
Inhibition (per cent) by		
10 μM citrate	94	3
16 μM neocuproine	0	100
0.2 per cent albumin	0	99

Kinetic parameters were obtained at pH 5.2 and 30° unless otherwise stated in the original paper (28).

We emphasize that establishing the catalytic activity of ceruloplasmin independent of copper ion catalysis is a matter of some subtlety as illustrated in Fig. 11. It can be seen that at over 10^{-4} M ascorbate concentrations, the activity of this copper protein is less than that for a corresponding amount of Cu(II). However, the serum ascorbate concentrations, which are generally in the 10^{-5} M range, fall well within the range in which ceruloplasmin activity

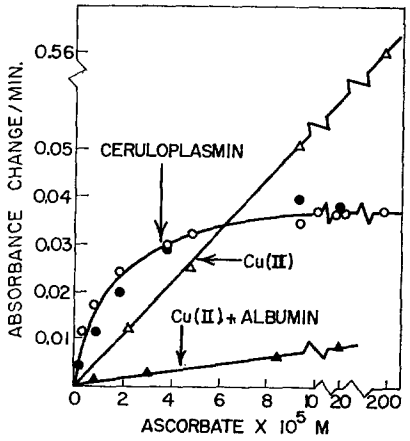


FIGURE 11. Kinetic difference between catalysis of ascorbate oxidation by ceruloplasmin and Cu(II) in 0.02 M acetate buffer pH 5.2 at 30°. The rate of ascorbate oxidation as the absorbance change at 265 mμ per minute is plotted against substrate concentration. Ceruloplasmin (1.95×10^{-7} M) or Cu(II) (1.6×10^{-6} M) was used with or without 0.15 per cent bovine serum albumin as indicated (from Osaki *et al.*, 28).

is quantitatively greater, particularly in the presence of serum proteins such as albumin.

From these relationships we concluded that there must be some control of the ascorbate oxidase activity of this serum enzyme. This regulation appears to be effected through the inhibition of this copper oxidase by prevailing serum levels of citrate, data for which are shown in Table XII (49). Also as shown in Table XII, neither neocuproine nor serum albumin affected the

TABLE XII

EFFECTS OF CITRATE, NEOCUPROINE, AND SERUM ALBUMIN ON ASCORBATE OXIDATION BY CERULOPLASMIN AND Cu(II)

Inhibitor	Concentration	Inhibition of ascorbate oxidation by		
		Ceruloplasmin pH 5.2	Ceruloplasmin pH 7.0	Cu(II) pH 5.2
	μM	*per cent*	*per cent*	*per cent*
Citrate	0.2	42		0
	0.33		40	
	0.4	62		0
	1.67		53	
	2.0	81		0
	5.0	88		0
	10.0	94		0
	16.7		87	
	84.0		97	
	100.0*	95		18
	μM			
Neocuproine	1.6	0		37
	2.3	0		60
	3.3	0		100
	16.0	0		100
	per cent			
Bovine serum albumin	0.01	0		12
	0.03	0		40
	0.05	0		60
	0.10	0		80
	0.20	0		99

All experiments were carried out at pH 5.2 and 30° unless otherwise stated. In the citrate inhibition experiments 38 μM ascorbate, and in the neocuproine and albumin inhibition experiments 36 μM ascorbate, were used. Citrate and neocuproine concentrations are micromolar; bovine serum albumin concentration is given as per cent. Ceruloplasmin concentration was $1.5 \times 10^{-7} M$ except in citrate inhibition, in which 1.6×10^{-7} and $2.7 \times 10^{-7} M$ ceruloplasmin were used at pH 5.2 and 7.0, respectively. Cu(II) concentration was $1.6 \times 10^{-6} M$.

* Corresponds to the normal range of citrate concentration in human plasma. Data from Osaki *et al.* (28).

ascorbate oxidase of ceruloplasmin in contrast to the situation with respect to Cu(II) ion. Though the molecular activity of ceruloplasmin is relatively low, it must be remembered that its concentration in normal sera is relatively high, 2×10^{-6} M, comprising 1 out of every 300 of serum protein molecules.

The enzymic activity of ceruloplasmin could be sheer coincidence or accidental. Oxidation of ascorbate to dehydroascorbate which is not coupled to phosphorylation or terminal oxidation might be regarded as biochemically wasteful. Under certain conditions, however, it may be desirable to have a convenient serum mechanism for this reaction. Martin and Mecca (50) have shown that dehydroascorbic acid penetrates the brain, eye, and erythrocytes more rapidly and to a greater extent than ascorbic acid. Except for this we must conclude tentatively that the biological function of ceruloplasmin is still related to copper transport or transfer.

CATALYTIC ACTION OF CERULOPLASMIN

As the final part of this brief survey of copper proteins and oxygen we consider the catalytic activity of ceruloplasmin further because recent studies with a variety of current methods have made this particular oxidase one of the Cu enzymes most studied in detail (11). While typical kinetic studies of ceruloplasmin are quite feasible, the slowness of its reactions has permitted simultaneous electron spin resonance and spectrophotometric measurements in a stopped-flow apparatus to provide more specific kinetic data than with any other Cu oxidase.

Many aspects of the catalytic activity of ceruloplasmin and its inhibition have been recently reviewed (50 *a*). In addition to ascorbate, two other classes of compounds serve as substrates for ceruloplasmin. One group have in common the fact that they are all reducing agents including hydrosulfite, hydroxylamine, thioglycolate, and hexacyanoferrate. The third group—aromatic polyamines and polyphenols—are by far the most numerous and well studied substrates including *p*-phenylenediamine (*p*PD) and serve as the basis for the close comparison of ceruloplasmin with laccase.

A most complete study of a large number of arylamine and polyphenol substrates of ceruloplasmin has been made by Peisach and Levine (51). They showed that the rates of oxidation of a large number of this kind of substrate are directly related to Hammett sigma values. Compounds having substituents with high positive sigma values (electron withdrawing) show little or no activity, whereas compounds with negative sigma values (electron releasing) were reactive. Thus they concluded that the ease of oxidation of the substrate increased with increasing electron density in the ring. The compounds tested can be placed in the following order with respect to ease of oxidation:

*p*PD > *N*,*N*-dimethyl *p*PD > *N*-methyl *p*PD > *p*-aminophenol > hydroquinone > *N*,*N*-dimethyl *m*-PD > *m*-PD

For the oxidation of *p*PD, Peisach and Levine (51) proposed the following reaction sequence which proceeds through four steps:

1. The formation of a charge-transfer complex between substrate and ceruloplasmin Cu(II).

$$AH_2 + \text{ceruloplasmin Cu(II)} \rightleftharpoons AH_2\text{-ceruloplasmin-Cu(II)}$$

2. Transfer of a single electron from substrate to ceruloplasmin Cu(II) to form a free radical.

$$AH_2\text{-ceruloplasmin Cu(II)} \rightleftharpoons AH\cdot + H^+ + \text{ceruloplasmin Cu(I)}$$

3. The loss of another electron from the free radical either through disproportionation or by reaction with the enzyme.

$$2\ AH\cdot \rightleftharpoons A: + AH_2$$
$$AH\cdot + \text{ceruloplasmin Cu(II)} \rightleftharpoons A: + H^+ + \text{ceruloplasmin Cu(I)}$$

4. The diradical species then reacts with more *p*PD to form a product usually measured in colorometric ceruloplasmin tests and the ceruloplasmin Cu(I) is oxidized by molecular oxygen.

$$A: + AH_2 \rightleftharpoons \text{product}$$

A similar mechanism was proposed for durenediamine oxidation in which the diradical product rearranges and is hydrolyzed. Broman *et al.* (11) suggested that the radical intermediate formed in the oxidation of *p*PD is the AH_2^+ and not the AH·. However, the principle involved is the same.

Peisach and Levine (51) confirmed the earlier work of Holmberg and Laurell (52) that monoamines (aniline) and monophenols (pentamethylphenol) are not oxidized by ceruloplasmin. The factors common to all aryl substrates tested are that each possesses a minimum of two electron-supplying groups, and no strong electron-withdrawing groups, suggesting definite electronic requirements in addition to the steric requirements.

Walaas *et al.* (53) have studied the oxidation of catecholamines by ceruloplasmin. The mechanism of the reaction is as follows:—

$$\text{Ceruloplasmin-Cu(II)} + \text{catecholamine} \overset{k_1}{\rightleftharpoons} \text{ceruloplasmin Cu(I)} + \text{catecholamine (oxid.)}$$

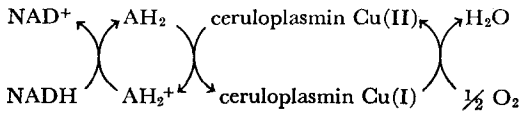
$$\text{Ceruloplasmin Cu(I)}_n + \frac{n}{4}\,O_2 + nH^+ \rightleftharpoons \text{ceruloplasmin Cu(II)}_n + \frac{n}{2}\,H_2O$$

The catecholamine oxidation product may be a free radical which reacts further as in the mechanism outlined previously.

The relative rates of oxidation of typical catecholamine substrates expressed as k_1 in $m^{-1}\text{sec.}^{-1}$ are: dopamine, 143; noradrenaline, 120; adrenaline, 99;

isopropylnoradrenaline, 85; dopa, 7. The larger rate constants for compounds with unsubstituted amine side chains suggest that this group is involved in the interaction with ceruloplasmin. If the π-electrons are primarily involved in the binding of the *p*PD series referred to earlier, the interaction of the catecholamines may occur at a different site on ceruloplasmin.

The oxidation of NADH and NADPH has been observed in a system containing ceruloplasmin and appropriate substrates. Walaas and Walaas (54) have shown that the reaction proceeds as:



Thus a free radical is probably an intermediate of the ceruloplasmin-catalyzed reaction. These radicals in turn oxidize the NADH or NADPH by acting as one electron acceptors. There is no evidence of a direct reaction between NADH and ceruloplasmin. By measuring the moles of NAD^+ or $NADP^+$ formed in 5 minutes the relative rates were determined as follows:—

	Dimethyl *p*PD	Noradrenaline	*p*PD	Adrenaline	Serotonin
With NADH	0.195	0.185	0.14	0.04	0.03
With NADPH	0.20	0.20	0.17	0.06	0.08

An intensive study of the role of copper in the catalytic action of both laccase and ceruloplasmin using combined fast reaction techniques and ESR measurements was recently reported by Broman *et al.* (11). The kinetics of the reduction of the Cu(II) of ceruloplasmin by various substrates were studied using ESR changes. The rate of formation and decay of a positively charged free radical formed from *p*PD was estimated as shown in Fig. 12 which summarizes data for two different concentrations of O_2. With only 0.15 mM O_2, the oxygen was rapidly depleted and the *p*PD oxidation product reached a maximum and quickly decayed. In pure O_2, the Cu(II) was quickly converted to Cu(I), but the radical persisted for several minutes until the *p*PD (or O_2) was exhausted. The mechanism postulated by these workers which accounts for the data is as follows:—

$$\textit{En}\text{-}Cu^{+2} + AH_2 \rightleftharpoons \textit{En}\text{-}Cu^{+2}AH_2 \rightleftharpoons \textit{En}\text{-}Cu^{+1}(AH_2)^+ \rightleftharpoons \textit{En}\text{-}Cu^{+1} + (AH_2)^+$$
$$\textit{En}\text{-}Cu_4^{+1} + O_2 + 4\ H^+ \rightleftharpoons \textit{En}\text{-}Cu_4^{+2} + 2\ H_2O$$

From data similar to those of Fig. 12, Broman *et al.* (11) were able to estimate many of the parameters involved in ceruloplasmin-catalyzed oxidation of various substrates. These data shown in Table XIII clearly indicate the

superiority of *p*PD as a substrate, include values for $t_{1/2}$, the approximate half-time for reduction to the steady state level, c_s/c_o, the bivalent fraction of the valence-changing Cu during the steady state, k_1, the first order rate

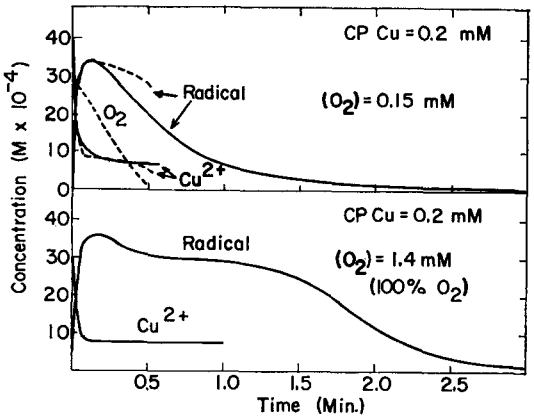


FIGURE 12. Experimental (solid lines) and calculated (dashed lines) ESR absorption of Cu^{+2} and of free radical in experiments with ceruloplasmin and *p*PD. The calculated curves are obtained with $k_1' = 7.4 \times 10^3\ M^{-1} \times min.^{-1}$, $k_2'h = 71\ M^{-1/4} \times min.^{-1}$, and $k_d = 1.25 \times 10^4\ M^{-1} \times min.^{-1}$. The upper part of the figure shows results obtained with solutions equilibrated with air; the lower part shows results when pure O_2 had been bubbled through the substrate solution (from Broman *et al.*, 11).

TABLE XIII

KINETIC PARAMETERS FOR CERULOPLASMIN IN 0.1 *M* TRIETHYLAMINE—ACETIC ACID BUFFER (pH 5.5) AT 17° C, DETERMINED BY ESR MEASUREMENTS

Substrate	$t_{1/2}$	c_s/c_0	k_1	$q'O_2$	t_s	$q''O_2$	k_2
	min.		$min.^{-1}$	$min.^{-1}$	*min.*	$min.^{-1}$	$min.^{-1}$
PPD	0.015	0.20	37	0.92	0.4	0.9	9.2
Pyrogallol	0.065	0.47	5.6	0.34	1.4	0.27	5.0
Catechol	0.08	~0.48	4.5	~0.27	3	0.12	4.2
Quinol	0.4	0.80	0.35	0.035	10	0.036	1.4
Hydroxylamine	—	>0.7	—	—	>20	—	—

Initial concentrations: total copper (c_{tot}), $8 \cdot 10^{-4}$ M; Cu^{+2} (c_0), $4 \cdot 10^{-4}$ M; O_2 (u_0), $2.9 \cdot 10^{-4}$ M; and substrates, $5 \cdot 10^{-3}$ M.
Data from Broman *et al.* (11).

constant for the reduction, $q'O_2$ and $q''O_2$, the rate of oxygen consumption calculated by two different methods, t_s, the duration of the steady state, and finally, k_2, the first order rate constant for the reoxidation reaction. The data are also consistent with the postulated mechanism which involves the reduction of *E*n-Cu(II) by the substrate followed by reoxidation by O_2.

Note Added in Proof After this paper was presented and prepared for publication, an excellent article by A. S. Brill, R. B. Martin, and R. J. P. Williams dealing with many similar problems entitled Copper in biological systems appeared in Electronic Aspects of Biochemistry, (B. Pullman, editor), New York, Academic Press, Inc., 1964, 519.

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