Physical and Chemical Studies on Ceruloplasmin

I. THE RELATION BETWEEN BLUZ COLOR AND THE VALENCE STATES OF COPPER*

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In studies first reported almost 15 years ago, Holmberg and Laurel isolated and characterized a globulin of plasma which they named ceruloplasmin (1). In their investigations they found, among other facts, that the protein was intensely blue (1), had a molecular weight of 151,000 (1), contained 8 atoms of copper per molecule (1), and possessed a moderate amount of oxidase activity toward p-phenylenediamine and several other organic compounds (2). It has since been learned that hereditary deficiency or absence of ceruloplasmin in human beings is associated with a pathological increase in the copper content of almost all tissues, a condition known as Wilson’s disease (3, 4). The present study is part of a series of investigations of ceruloplasmin which we hope will contribute to understanding of both the pathogenesis of this illness and the physiological role which ceruloplasmin plays in the copper metabolism of normal individuals.

Two aspects of the chemistry of ceruloplasmin proved to be particularly relevant to the studies reported in this paper. First, the protein’s 8 copper atoms, which could be removed reversibly (5), did not have identical properties; second, ceruloplasmin’s intense blue color could be reversibly abolished by ascorbic acid and other reducing agents (1). The nonidentity of the 8 copper atoms has been demonstrated in four ways by experiments which showed that (a) approximately 4 of them were relatively easily exchangeable for free ionic copper (6); (b) 4 could be rendered dialyzable by chymotrypsin digestion (7); (c) only some, which were cupric, gave a signal in electron paramagnetic resonance studies (8); and (d) only some, which were cuprous, reacted with 2,2’-biquinoline (9). Taken together, these findings were compatible with two conclusions: that the blue color of the protein was dependent on at least some of its copper atoms being in a particular valence state, probably cupric, and that all of the copper atoms has been demonstrated in four ways by experiments which showed that (a) approximately 4 of them were relatively easily exchangeable for free ionic copper (6); (b) 4 could be rendered dialyzable by chymotrypsin digestion (7); (c) only some, which were cupric, gave a signal in electron paramagnetic resonance studies (8); and (d) only some, which were cuprous, reacted with 2,2’-biquinoline (9). Taken together, these findings were compatible with two conclusions: that the blue color of the protein was dependent on at least some of its copper atoms being in a particular valence state, probably cupric, and that all of the copper atoms in the protein were not in the same valence state.

The work that we are reporting here represents an effort to determine the different characteristics of each of the copper atoms in the ceruloplasmin molecule. We have done this principally by correlating changes in the optical absorption spectrum with changes in electron paramagnetic resonance and proton relaxation rates. Further, the complete optical spectrum of the protein has been obtained and analyzed for the first time, and this has allowed us to place certain restrictions on some of the binding sites of copper in ceruloplasmin.

EXPERIMENTAL PROCEDURE

Materials—Ceruloplasmin was prepared from Fraction IV-1 of Cohn as previously described (10). Ceruloplasmin-copper refers to copper which is tightly bound to ceruloplasmin and which cannot be removed by passage of the solution through a column of a chelating resin, Chelex 100. Solutions of ceruloplasmin were freed of nonceruloplasmin copper by passage through such columns (10).

The purity of a preparation of ceruloplasmin can be estimated by the ratio of its blue color, measured in 0.2 M acetate buffer at pH 5.2, to total protein. We have defined this ratio as r1 = O.D.1 cm "O.D. 1 cm. For pure ceruloplasmin r1 is about 0.048, and the preparations we have used have had values of this ratio of 0.040 to 0.043.

When a solution of ceruloplasmin has also been freed of non-ceruloplasmin copper, it has proven useful to characterize it further by the ratio of the absorption at 610 mλ to copper content. We have defined this ratio as r2 = O.D.1 cm "O.D. 1 cm. C is the concentration of ceruloplasmin-copper in micrograms per milliliter. The preparations used in our experiments have generally had values of r2 from about 0.020 to 0.024.

Apoceruloplasmin was prepared by removing copper from ceruloplasmin as previously described (5). Dialysis of the final material against 1.0% sodium chloride produced a solution of apoceruloplasmin which was stable for at least several months when frozen.2 Apoceruloplasmin was studied in its copper-free form and after recombination with copper to form reconstituted ceruloplasmin. Reconstitution was effected by addition of copper and ascorbic acid (5), and the resulting ceruloplasmin was purified by column chromatography.

2 The stabilizing effect of 1.0% sodium chloride on apoceruloplasmin was discovered by J. T. Sgouris.

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by adsorption and elution from a diethylaminoethyl cellulose column followed by fractional precipitation by ethanol-chloroform (9:1, volume for volume).

L-Ascorbic acid was obtained from J. T. Baker Chemical Company.

A stock solution containing 1000 μg of cupric ion per ml was prepared by dissolving 3.9326 g of reagent grade CuSO₄·5H₂O in water to make 1000 ml. More dilute solutions were prepared from this stock solution and from 0.1 mM acetate buffer, pH 5.4.

2,2′-Biquinoline (cuprine) obtained from K. and K. Laboratories, Inc., New York, was used for the determination of cuprous copper, according to the method of Felsenfeld (9), and for the determination of total copper in the presence of an excess of ascorbic acid. The latter method gave the same result, for purified ceruloplasmin, as the more cumbersome method utilizing wet digestion and dicyclohexanoneoxalylidihydrazone (6).

Chelex 100 (Bio-Rad) is a chelating resin consisting of iminodiacetate groups attached to a styrene-divinylbenzene lattice.

Ceruloplasmin and apoceruloplasmin solutions were concentrated by ultrafiltration through collodion bags (Schleicher and Schuell).

A modified Thunberg tube (Fig. 1) was used when several anaerobic measurements of optical absorption, EPR, and PRR were desired on the same solution before and after additions of ascorbic acid, and without transfer of the solutions to different containers. The portion of this tube which permitted the measurement of optical density had a path length of 1 mm and could be fixed in a precisely reproducible position in a Bausch and Lomb Spectronic 505 recording spectrophotometer. Other spectrophotometers used were a Beckman DU, a Zeiss PMQ II, and a Cary model 14.

EPR Methods and Apparatus—The usefulness of electron paramagnetic resonance in studying magnetic ions and atoms is by now firmly established (11, 12). By this method the presence of certain magnetic ions and free radicals can be detected quantitatively, and information can be obtained about the strength and symmetry of the ligand field in the vicinity of magnetic ions.

Experiments were all performed at room temperature with a superheterodyne microwave spectrometer (13) operating at 9500 megacycles per second. A Varian 4500 unit was used to provide a 100 kc per second modulation field and to detect the resonance signal. The magnetic field was obtained by means of a 12-Inch Varian magnet.

Even though the signal to noise ratio of the EPR line would have been greatly increased at liquid nitrogen or liquid helium temperatures, we chose to make most EPR signal intensity measurements at room temperature for two reasons. First, this allowed us to study the ascorbic acid reduction of ceruloplasmin almost continuously during the course of the reaction. Second, it permitted us to correlate measurements of EPR, PRR, and optical absorption made under the same conditions. Furthermore, we found, in pilot experiments, that EPR intensity measurements on native ceruloplasmin gave the same results whether obtained at room temperature or at the temperature of liquid nitrogen.

The sample solutions were in flat quartz cells (Varian Associates) of 0.5-mm inside thickness which could be fitted to the modified Thunberg tube (Fig. 1). The cell was inserted in the center of a rectangular cavity operating in the TE102 mode.

To be able to monitor quantitatively the paramagnetic signal obtained from the sample in the cavity during the course of an experiment, it was necessary to insure that the amount of power coupled into the cavity remained constant. This was achieved by adjusting the power incident on the cavity to a fixed arbitrary level (approximately 100 microwatts) and adjusting the loaded Q of the cavity so that a fixed fraction of this power (approximately 10%) was reflected from the resonant cavity.

PRR Methods and Apparatus—Another type of experiment capable of giving information about molecules containing magnetic ions is based on the measurement of the proton relaxation rate (14-19) in aqueous solutions of these molecules. The information obtained from PRR experiments in part duplicates and in part supplements that yielded by EPR experiments, as will be apparent in the following review of those of the physical principles which bear on the present problem.

The spin lattice relaxation time, T₁, of protons is the time required by the longitudinal proton magnetization of the sample to come into equilibrium with an external magnetic field after thermal equilibrium has been disturbed. The relaxation rate, 1/T₁, arises principally from the magnetic dipolar interaction between the protons, unless paramagnetic ions are present in the solution. Then the proton-electronic dipolar magnetic interactions dominate 1/T₁ even at millimolar concentrations of paramagnetic ions. The relative motion of these ions and the protons exposes the latter to a fluctuating magnetic field, causing Larmor frequency transitions to take place which tend to restore the thermal equilibrium. This manifests itself by a shortening of T₁.

The relaxation rate produced by a given concentration of paramagnetic ions depends greatly on whether, and how, these ions are bound to other molecules (19). Consequently, if the proton relaxation rate of water attributable to the presence of a certain concentration of paramagnetic ions is R, and if the relaxation rate is changed to R’ when the same concentration of ions is bound to other molecules in the solution, it is useful to define a proton relaxation enhancement factor, E = R'/R. The relaxation rates R and R' are determined from the experimentally observed rates, 1/T₁ and 1/T₁*, by subtracting, from each, the relaxation rate caused by proton-proton magnetic interactions in water, or common buffers, which is equal to approximately 0.34 sec⁻¹ at room temperature.

Generally, very small values of E arise when the paramagnetic ion is relatively inaccessible to the water molecules, as may occur if the ion is embedded within a macromolecule. Values of E exceeding unity, on the other hand, are associated with ions bound to the outside of large molecules, where they are almost as accessible to water molecules as the ions in pure water but where the relative motion between protons and ions has been slowed down. This slowing down provides a greater intensity of the fluctuating magnetic field at the Larmor frequency, so that the relaxation rate is increased. A more detailed discussion of these phenomena has been given elsewhere (19).

The determination of the extent to which a measured proton relaxation rate depends on enhancement is of considerable importance in interpreting results of studies on ceruloplasmin. We are, of course, interested primarily in the effect of ceruloplasmin-copper on the PRR. Yet, nonceruloplasmin copper also has an effect on the measured relaxation rate. Although most of the nonceruloplasmin copper which contaminates solutions of ceruloplasmin can be removed by an EDTA-type of chelating resin,
plasmin-copper, to the outside of the ceruloplasmin molecule, a very small amount of such copper may greatly, and deceptively, increase the PRR of a solution of ceruloplasmin.

We were able to eliminate this uncertain effect of nonceruloplasmin copper on PRR by making relaxation rate measurements in the presence of EDTA. Fig. 2 shows the results of adding increasing amounts of EDTA to a solution of free cupric ions, with a reduction of $\xi$ from unity to about 0.3. Experiments in which EDTA was added to solutions of ceruloplasmin showed that the relaxation rates of the solutions were reduced. Thus, it appeared that EDTA was able to remove loosely bound non-ceruloplasmin copper from ceruloplasmin, to reduce its $\xi$ to about 0.3, and thereby to lower the apparent $\xi$ of ceruloplasmin-copper to close to its true value (Table I).

All measurements of relaxation times were performed in a magnetic field of approximately 12,000 oersteds. The magnetization of the sample was measured by a pulsed nuclear magnetic resonance (20), and the relaxation time was obtained by the two-pulse method suggested by Carr and Purcell (21). In this technique the equilibrium proton magnetization of the sample was suddenly disturbed by the application of a pulse of RF power at the proton Larmor frequency of 50 megacycles per second. The return to thermal equilibrium was observed by sampling the magnetization of the protons by a second pulse, which followed the original pulse by a variable time interval.

Both experimental resonance methods give direct information about the presence of paramagnetic cupric ions, but each has a distinct advantage beyond this. EPR results can be used also to characterize, at least in part, the ligand field of a metal ion in a complex molecule; PRR studies may provide some insight about the site of binding of such an ion to a macromolecule.

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**Fig. 1.** A modified Thunberg tube used to obtain measurements of EPR, PRR, and optical densities of ceruloplasmin solutions during the course of anaerobic decolorization experiments. A, the RF coil in the magnetic field needed for proton relaxation measurements; B, 1-mm path length optical cuvette; C, 0.5-mm quartz cuvette (Varian Associates) for EPR measurements; D, bulb for ascorbic acid solution, which is later mixed with the ceruloplasmin solution in A and B. The stopcock at the right of the figure is closed after the assembly has been flushed with nitrogen and evacuated. Recolorization can be achieved by opening the stopcock to air or by producing oxygen by electrolyzing a small quantity of water in the bulb, E.

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**Fig. 2.** The proton relaxation enhancement factor of Cu$^{2+}$ as a function of the ratio of the concentration of EDTA to that of Cu$^{2+}$.

The Cu$^{2+}$ molarity was kept constant at $10^{-3}$ M. The enhancement factor for Cu$^{2+}$ ions in the presence of an excess of EDTA is 0.31.
RESULTS

EPR Results—The typical EPR line obtained from a concentrated solution of ceruloplasmin closely resembled the line obtained by Malmström and Vännäs, who have measured its g values and hyperfine structures (8).

On reduction by ascorbic acid, the blue color of ceruloplasmin decreased and ultimately disappeared, with complete decolorization in a vacuum requiring ascorbic acid equivalent to 50% of the copper content of the protein (10). We correlated the absorption of ceruloplasmin in the red region of the spectrum with the valence state of its copper by measuring the optical density of a solution of 610 mμ within 3 minutes of the measurement of the EPR spectrum of the protein. Measurements were made at various times during the 1 to 2 hours required for decolorization to proceed to its equilibrium value. Several experiments were performed with the addition of different amounts of ascorbic acid relative to the concentration of ceruloplasmin. Although, as shown in Fig. 5, the optical absorption spectrum of ceruloplasmin contained three other lines, these were found to have intensities proportional to the line of 610 mμ no matter what the degree of reduction.

Table I

Proton relaxation rates of several copper complexes

This table shows the results of several PRR experiments involving inorganic copper and ceruloplasmin-copper under various conditions. The first column gives R, the proton relaxation rate, in sec⁻¹, divided by C, the concentration of total copper, in micrograms per ml, which, in all experiments, was between 100 and 300 μg per ml. The value of R has been corrected for the specific relaxation rate of water protons caused by proton-proton interactions in water and in apoceruloplasmin or completely decolorized ceruloplasmin. The second column gives E, the enhancement factor, which is equal to the corrected R/C divided by the corrected R/C of cupric ions in water, and which is independent of copper concentration in the range studied. The horizontal lines give results of various experiments as follows.

1. Cu²⁺ ions, as CuCl₂, in water, which serves as the reference for the other measurements.
2. Cu²⁺ ions in water with EDTA equal to, or greater than, twice the concentration of copper, which insures that essentially all of the Cu²⁺ ions are complexed.
3. Ceruloplasmin as effluent from a Chelex column. A small, variable amount of nonceruloplasmin copper, up to 1% of total copper, having an E of approximately 8, as shown in Table II, causes the variation in R/C.
4. The same samples as in 3, with sufficient EDTA to complex all of the nonceruloplasmin copper ions. The E of these ions is reduced from as much as 8 to 0.3, as shown in Fig. 2, so that these ions do not appreciably influence the PRR results.
5 and 6. Decolorized ceruloplasmin and apoceruloplasmin in the presence of EDTA as in 4. No relaxation attributable to paramagnetic ions is observed.

| Experiment | R/C | s |
|------------|-----|---|
| 1. Cu²⁺ in H₂O | 0.0096 | 1.00 |
| 2. Cu²⁺ in H₂O + excess EDTA | 0.0029 | 0.30 |
| 3. Ceruloplasmin after passage through Chelex resin | 0.0024-0.0030 | 0.25-0.31 |
| 4. Ceruloplasmin (of 3) + EDTA | 0.0017 | 0.18 |
| 5. Ceruloplasmin (decolorized) + EDTA | 0.0000 | 0.00 |
| 6. Apoceruloplasmin + EDTA | 0.0000 | 0.00 |

Fig. 3. The concentration of Cu²⁺ ions in ceruloplasmin, determined from the integrated intensity of the EPR signal, as a function of the optical density at 610 mμ. Both variables have been normalized to a concentration of 1 μg of ceruloplasmin-copper per ml. The symbols correspond to the following number of ascorbic acid equivalents per gram atom of ceruloplasmin-copper: circle, 0.0; square, 0.5; triangle, 0.3; triangle, 0.2; diamond, 0.1. The solid circle, ●, refers to reconstituted ceruloplasmin. The highest value for any symbol, except for reconstituted ceruloplasmin, represents the value obtained before addition of ascorbic acid. Lower points represent measurements during the decolorization produced by ascorbic acid. The final values obtained after recolorization by the admission of air into the tube are indicated by an f next to the symbol. The apoceruloplasmin, before reconstitution, was colorless and gave no EPR signal.

Fig. 3 shows the results of these experiments normalized for the copper contents of the various solutions of ceruloplasmin used.

A comparison of the integrated intensity of the EPR spectrum of ceruloplasmin with that of a standard solution of CuCl₂ permitted us to calculate the fraction of ceruloplasmin-copper, ρ, which gave the EPR signal in each measurement with the assumption that all the cupric ions contribute to the EPR signal (22). The average of ρ for the six samples of native ceruloplasmin shown in Fig. 3, without ascorbic acid, was 0.29.

When ascorbic acid in varying amounts in relation to ceruloplasmin-copper was added to these samples of ceruloplasmin, there were relatively slow decreases in both the intensity of blue color (O.D.₆₁₀) and the concentration of cupric ions (integrated EPR signal). Within the precision of our measurements (approximately 3% for spectrophotometry and 10% for EPR), the points from the various samples measured at different times all fell on a straight line which passed through the origin, as shown in Fig. 3.

When oxygen was admitted to the modified Thunberg tube after such a decolorization experiment, most of the blue color originally present in the solution was recovered. The points marked f in Fig. 3 represent the final measurements made on such "recolorized" solutions of ceruloplasmin, and they fall on the same straight line that fitted the points obtained during decolorization.

The point at the extreme upper right of Fig. 3 shows the meas-
measurements made on a sample of reconstituted apoceruloplasmin. This ceruloplasmin possessed a greater fraction of its copper as cupric ion, as measured by EPR, than any native ceruloplasmin that we studied. Yet it also possessed a proportionately greater optical absorption at 610 nm, so that the point corresponding to these measurements fell on the straight line drawn through points derived from measurements on native, and partially or completely decolorized, ceruloplasmin.

The reciprocal slope of the straight line of Fig. 3 is equal to 0.070 per mg of ceruloplasmin cupric ion per ml. This value leads to a gram atomic extinction coefficient at 610 nm of 4400 for ceruloplasmin cupric ion. The average gram atomic extinction coefficient for all the ceruloplasmin-copper, which is the parameter found in the literature, is about 1200 to 1300 (1).

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**PRR Results**—In experiments analogous to the ones just described, we again correlated the optical absorption of ceruloplasmin at 610 nm with the valence state of its copper—now determined, however, by measurements of the proton relaxation rate. These experiments were carried out with solutions which had been passed through Chelex columns, with and without the addition of EDTA, in efforts to minimize and estimate the relaxation-enhancing effect of nonceruloplasmin copper which we have already discussed.

Fig. 4 shows typical results of several such experiments in a vacuum. The solid curve connects points obtained in the absence of EDTA, and the dashed curve is drawn through points obtained in the presence of EDTA equivalent to 2% of the total copper present. The solid curve lies above the dashed curve by an amount which reflects the enhancement caused by the free copper impurity in the solutions.

Since the two curves differ from each other by a constant amount throughout their extent, it appears that the loosely bound nonceruloplasmin copper is not reduced by ascorbic acid as readily as ceruloplasmin-copper is. This is in accord with the fact that nonceruloplasmin copper is much less active as a catalyst in the oxidation of ascorbic acid, in the presence of ceruloplasmin, than ionic copper in the absence of this protein (10).

When oxygen was admitted to the Thunberg tubes after decolorization in the presence of EDTA, approximately 60 to 70% of the blue color returned, as in the EPR experiments, and the PRR increased toward its initial values. In the absence of EDTA, however, the final value of the PRR was above the original value, perhaps because a small amount of ceruloplasmin-copper had been converted into free copper because of denaturation of approximately 1% of the protein during the experiment.

Completely decolorized ceruloplasmin and apoceruloplasmin possess residual relaxation rates. Both of these are equal to about 0.0012 sec⁻¹ per total copper per ml, or per an equivalent amount of protein per ml in the case of apoceruloplasmin. EDTA does not abolish either of these residual relaxation rates, which probably result from proton-proton magnetic interactions in the protein molecules and their effects, rather than those of paramagnetic ions, on the protons of the solvent, water.

If we accept the result of our EPR experiments that the average fraction of paramagnetic copper ions in our ceruloplasmin is 0.29, we can calculate the average enhancement factor for the cupric ions in ceruloplasmin, from Table I, as 0.0017 / (0.29)

### Table II

| Ceruloplasmin-copper | Nonceruloplasmin cupric ion | R | ΔR | ΔR/(Cu²⁺)non-CP | ε<sub>non-CP</sub>Cu²⁺ |
|----------------------|-----------------------------|---|----|----------------|-------------------|
| µl/ml                | sec⁻¹                       |   |    |                |                   |
| 291                  | 0                           | 0.83 | 0  | 0.077 | 8.0 |
| 226                  | 20                          | 2.35 | 1.54 | 0.075 | 7.8 |
| 216                  | 56                          | 5.00 | 4.22 | 0.081 | 8.1 |
| 208                  | 90                          | 7.80 | 7.06 | 0.072 | 7.5 |
| 185                  | 160                         | 12.2 | 11.5 | 0.070 | 7.5 |

This table shows the results of PRR experiments involving ceruloplasmin and inorganic copper. The first column gives the concentration of ceruloplasmin-copper, which decreases in successive lines because of dilution by the added solution of non-ceruloplasmin cupric ions (indicated in the subscripts by non-CP Cu²⁺). The concentration of the latter is given in the second column. R is the measured relaxation rate; ΔR is R minus the R for ceruloplasmin-copper corrected for dilution. ΔR/(Cu²⁺)non-CP is the specific relaxation rate for the added cupric ions, and ε<sub>non-CP</sub>Cu²⁺ is the enhancement factor for these ions.
Cupric ions added to ceruloplasmin, in contrast, have an enhancement factor greater than unity and are, consequently, probably bound to the outside of the ceruloplasmin molecule. The results of an experiment demonstrating this are shown in Table II, where from 1 to about 6 added cupric ions, per molecule of ceruloplasmin, have an $E$ which is close to 8.

Optical Measurements—The absorption spectrum of ceruloplasmin was investigated by Holmberg and Laurell (1), and its most prominent feature was found to be a strong absorption peak at a wave length of 610 $\mu$m. In the present investigation we re-examined the optical absorption spectrum by comparing native ceruloplasmin with ceruloplasmin decolorized by the addition of $L$-cysteine in a double beam spectrophotometer. Essentially similar spectra were obtained when buffer, or ceruloplasmin decolorized by ascorbic acid, was used as a reference except that in these cases the 332-$\mu$m line was not as well resolved since it appeared, respectively, as a shoulder either on the intense 280 $\mu$m absorption line due to protein or on the intense 260-$\mu$m absorption line due to the excess ascorbic acid required for decolorization in the presence of air.

The results are shown in Fig. 5, in which we have plotted as solid lines the optical density of a ceruloplasmin solution in phosphate buffer, $\Gamma/2 = 0.15$, pH 7.4, as a function of the energy of the incident light in wave numbers. It is apparent that the spectrum, although not completely resolved, consists of at least four lines. Consequently, we have attempted to resolve the observed spectrum into four lines with gaussian shapes. These lines are shown by dashed curves in Fig. 5, and their physical characteristics are listed in Table III. The extinction coefficients and oscillator strengths appearing in this table are calculated on the assumptions that only one optical transition is involved in each line and that the only chromophoric centers are, as is implicit in our EPR and PRR results, cupric ions.

The temperature dependence of the 610 $\mu$m absorption line of ceruloplasmin was determined by measuring its magnitude and wave length at 5° and at 55°. Over this temperature range the protein was not denatured. The absorption did not change, to a precision of 1%, and the wave length of the absorption maximum remained constant within 10 $\mu$m.

**DISCUSSION**

We shall consider, in turn, the conclusions to be drawn from the EPR, PRR, and optical results.

The EPR results suggest, first, that all of the chromophoric copper atoms in ceruloplasmin are cupric. The optical absorption of ceruloplasmin, in various degrees of decolorization, is always proportional to the paramagnetic resonance signal (Fig. 3). The shape of the EPR signal is characteristic of a cupric ion in an approximately axial field (8), and the hyperfine structure of this signal shows that the cupric ions are well separated from other cupric or cuprous ions since this structure is characteristic of magnetically isolated nuclei with spin equal to 3/2. We shall treat this in greater detail under discussion of the optical results. As expected, apoceruloplasmin has no EPR signal.

The fraction of the total ceruloplasmin-Cu which is cupric, $\rho$, has an average value of 0.29 for these samples, which indicates that between 2 and 3 of ceruloplasmin’s 8 copper atoms are cupric.

Although $r_3$, the optical density at 610 $\mu$m, per total copper content, of ceruloplasmin reconstituted from apoceruloplasmin, is generally similar to that of native ceruloplasmin, one sample of reconstituted ceruloplasmin had a considerably greater $r_3$ and a correspondingly greater EPR signal (Fig. 3). For it $\rho$ is 0.42, indicating that more than 3 of its copper ions are cupric. This suggests that $\rho$ is a variable parameter of ceruloplasmin dependent on the previous history of the sample of protein being studied.

Vångård and Malmström have obtained values of $\rho$ near 0.5 with preparations of ceruloplasmin supplied by the Kabi Company. No values of $\rho$ greater than this have been reported, suggesting that no more than half of the copper atoms in a ceruloplasmin molecule can exist in the cupric state.

We conclude from the linear relationship between the fraction

t $T$. Vångård, personal communication.
of ceruloplasmin copper which is cupric and optical density throughout the range covered by our EPR experiments that there is a 1:1 correspondence between the paramagnetic and the chromophoric centers of ceruloplasmin; i.e. all cupric ions, and only cupric ions, are chromophoric. Any hypothetical chromophoric non-paramagnetic or paramagnetic non-chromophoric ions would preserve the linear relation of Fig. 3 only if they had the same optical or EPR spectrum, respectively, and the same reaction rate for ascorbic acid reduction as the chromophoric-paramagnetic ions. Since these spectra and the ascorbic acid reaction rate are sensitive to the nature of the chemical binding of the ions, we consider the existence of more than one type of paramagnetic or chromophoric ion very unlikely.

The PRR results confirm the conclusion that all of the chromophoric coppers are cupric, since decolorized ceruloplasmin solutions have the same residual proton relaxation rate as apoceruloplasmin. As we have already indicated, this residual rate arises most probably from magnetic proton-proton interactions in and between the protein and solvent. This confirms that decolorized ceruloplasmin has no paramagnetic centers. Although such centers might, in principle, have a line width too great to make them observable by EPR, this would not prevent them from contributing to the proton relaxation rate.

The PRR results further suggest that the decolorization proceeds in two separate stages. When ascorbic acid equivalent to approximately 30% of the ceruloplasmin-copper, or less, is added, both the blue color and the relaxation rate decrease to a value apparently determined by the amount of ascorbic acid. When ascorbic acid equivalent to 50% of the protein copper content is added, a sudden further drop in the relaxation rate is observed during the course of the reduction.

In order to consider the different relaxation rates produced by the various cupric ions of ceruloplasmin, we note that the slope of the curves in Fig. 4 at any point is proportional to the E of the cupric ions undergoing reduction at that point. There is a marked increase in E when p has been reduced to approximately half of its maximal value. It is therefore useful to consider the decolorization as proceeding in two well defined stages, in the course of each of which the value of E drops monotonically by more than an order of magnitude.

The shape of the curve shown in Fig. 4 could be due to the existence of four distinct cupric ion sites which are reduced in a definite order and whose E's extend over an order of magnitude. Since the EPR and optical experiments show only a single type of paramagnetic and chromophoric center, this seems unlikely to us.

It is also possible that the cupric centers with high E's (those most accessible to water) are the first to be decolorized, and that those with low E's (relatively less accessible) are decolorized on the average somewhat later. When about half the cupric ions are decolorized, the protein configuration may undergo a change which makes the remaining cupric centers considerably more accessible. Such a configurational change may possibly result from the alteration in the electrostatic properties of the protein as the reduction proceeds.

Finally, the optical results allow us to consider what is probably the most interesting question concerning the physical properties of ceruloplasmin: "Why is ceruloplasmin so blue?" Although we cannot answer this question unambiguously, we shall try to give a brief discussion of possible origins for the intense absorption of ceruloplasmin in the red, and eliminate those models for the chromophoric centers which are at variance with one or another of the experimental results.

For cupric ions, lines in the visible region can arise only from transitions between d-orbitals whose degeneracy has been removed by the ligand electric field (22). Since such transitions rarely have oscillator strengths, f, greater than $10^{-4}$, even ligand fields of low symmetry, which can admit an appreciable amount of p-wave functions to the cupric d-orbitals, can hardly account for the observed oscillator strengths.

Another source for strong absorption that has been discussed (23, 24) is an optical transition occurring when the Cu nucleus is displaced from its equilibrium position as a result of thermal motion. In such a case the parity considerations which forbid d to d transitions are no longer applicable. A mechanism of this type is, however, expected to have an appreciable temperature dependence for the optical density, and this is not found in ceruloplasmin, as indicated above.

Chromophoric centers which permit charge transfer transitions can easily attain very high values of f. For such transitions to occur there must exist in the vicinity of the cupric ion a suitable electron donor. Organic donors having pi-orbitals could fill this function, provided that the excited pi-levels are low enough in energy. Since apoceruloplasmin and reduced ceruloplasmin show no absorption bands at wave lengths greater than 280 mp, we conclude that no pi-levels of sufficiently low energy exist. Consequently, organic donors can be excluded.

The only remaining potential electron donors in ceruloplasmin are the cuprous ions. In considering cuprous-cupric pairs, one must immediately exclude equivalent cupric ions with a shared electron, since such pairs would exhibit an EPR line whose hyperfine structure would consist of seven lines, characteristic of two Cu nuclei, each with a spin of 3/2 in all possible relative orientations. It seems likely, therefore, that the pairs of copper ions in their ground state consist of a cupric ion connected through a ligand bridge to a cuprous ion in a different ligand field. The d^2-electron wave function of the cupric ion is sufficiently localized to lead to a one-nucleus hyperfine structure (four lines) for the EPR spectrum but nevertheless has enough density at the cuprous ion to lead to a large electric dipole optical absorption. The various lines of the observed ceruloplasmin absorption spectrum correspond to levels of the cuprous (d^9) electronic configuration. This structure is considered to retain its stability when the cupric ion is reduced to cuprous, but the configuration cupric-cupric is unstable, possibly owing to the increased electrostatic repulsion.

A chromophoric center like the one postulated above would be very similar to the ferrous-ferric center in Prussian blue, which has been discussed by Robin (25), or the aurous-auric center in cesium aurous auric chloride discussed by Elliott (26). A more detailed discussion of the optical properties of this center will be published elsewhere.

**SUMMARY**

1. The valence states of ceruloplasmin-copper and the intense blue color of the protein have been investigated and related to one another by measurements of electron paramagnetic resonance, proton relaxation rates, and the optical spectrum of native and decolorized ceruloplasmin and of apoceruloplasmin.

2. The fraction of the total ceruloplasmin-copper which is cupric, ρ, is variable and probably is a function of the history of
the particular preparation. In six samples of ceruloplasmin, \( p \)
had an average value of 0.29.

3. All cupric ions, and only cupric ions, of ceruloplasmin-
copper are chromophoric.

4. The extinction coefficient of ceruloplasmin, \( \text{O.D.}^{1	ext{em}}_{\lambda_{\text{max}}} \),
is best expressed per gram atom of ceruloplasmin-cupric ion per
liter, and is 4400.

5. The unusually large absorption coefficient of ceruloplasmin
is due to a chromophoric center which allows charge transfer
transition probably in cuprous-cupric pairs.

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