EXCHANGE OF CERULOPLASMIN COPPER WITH IONIC Cu^{64} WITH REFERENCE TO WILSON'S DISEASE [1]

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Ceruloplasmin is a blue alpha-globulin which is normally present in human plasma at a concentration of 15 to 30 mg. per ml. (1–4). This protein contains 8 atoms of copper per molecule, and has a molecular weight of 150,000 (1). Ceruloplasmin's physiological function is unknown although its plasma level rises in various diseases and in pregnancy (3, 5–7), and is lower than normal in patients with hepatolenticular degeneration (Wilson's disease) (2, 3, 8), the nephrotic syndrome (3), and in the newborn infant (6). In the nephrotic syndrome, the low level of ceruloplasmin is apparently due to urinary loss of ceruloplasmin (3). In Wilson's disease, however, the deficiency or absence of the protein is probably the consequence of a specific, inherited inability to synthesize ceruloplasmin at a normal rate (9). Several groups of investigators believe that the primary abnormality of Wilson's disease is a deficiency of ceruloplasmin which leads, by an unknown mechanism, to excessive net absorption of dietary copper (2, 10–13).

In studies on the chemistry of ceruloplasmin, attempts have been made to remove reversibly its tightly bound copper, but these have been unsuccessful (14, 15). Nor have experiments demonstrating exchange of radioactive ionic copper with the copper in ceruloplasmin been reported. Radioactive ionic copper, however, has been exchanged with the tightly bound copper of another protein, ascorbic acid oxidase, which shows some resemblances to ceruloplasmin (16–18). The aim of the experiments reported in this paper was to determine if exchange could be effected between ceruloplasmin copper and ionic copper. The results obtained indicate that such an exchange can occur under certain conditions.

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METHOD

Experimental Procedure

The design of the experiment was as follows. A stock solution (Solution 1) was made which contained 47.0 mcg. per ml. of ionic copper, labelled with $copper^{64}$, and 47.0 mcg. per ml. of ceruloplasmin copper. A 2.0-ml. aliquot of this solution was diluted to 25.0 ml., and served as a control (Solution 2) which contained all the ceruloplasmin, radioactive copper, and free ionic copper used in preparing Solutions 3 and 4. To a second 2.0-ml. aliquot, 20 mg. of ascorbic acid was added, after which all the ionic copper, but not the ceruloplasmin copper, was removed by ion exchange resins, and dilution to 25.0 ml. was made (Solution 3). Another solution was prepared exactly as Solution 3, except that no ascorbic acid was used (Solution 4). Solution 5 was prepared in identical fashion from a solution which contained the same amount of labelled ionic copper and ascorbic acid as 2.0 ml. of Solution 1, but no ceruloplasmin. The entire experiment was done in duplicate, providing an A and B set of solutions. Solutions 2, 3, 4 and 5 were analyzed for radioactive copper, free ionic copper, total copper, and ceruloplasmin, except that Solution 5 obviously contained no ceruloplasmin. The presence in a solution of radioactivity and ceruloplasmin in the absence of appreciable free ionic copper indicated that exchange had occurred.

Apparatus

Beckman DU Spectrophotometer modified for use with a) cuvettes of 5.0-cm. path-length and 2.5-ml. volume, and b) thermospacers for maintaining solutions at constant temperature.

Gas-flow radiation detector and scaler (Nuclear Instrument and Chemical Corp., Model Nos. D-47 and 161-A).

Materials

Ceruloplasmin. Ceruloplasmin was prepared from Fraction IV of Cohn (19) by fractionation using methanol and chloroform.[2] The preparation used, AK-38S,

[2] Fraction IV was obtained through the kindness of Dr. James A. McComb, Director, Dr. John M. Newell, and Mr. Lewis H. Larsen of the Massachusetts Public Health Biologic Laboratories, and of Dr. Sam T. Gibson, Director, of the American National Red Cross Blood Program. The preparation of the ceruloplasmin was carried out by Dr. H. O. Singher and Dr. Alan Keltz of the Ortho Research Foundation, Raritan, New Jersey.

TABLE I

Ceruloplasmin, Preparation AK-38S

1 Total protein*	2 Ceruloplasmin†	3 Ceruloplasmin as % of total protein	4 Total copper‡	5 Free copper§	6 Ceruloplasmin copper‖	7 Calculated ceruloplasmin copper¶	8 Ratio of ΔO.D. at λ to ΔO.D. at 610 mμ
mg./ml.	*mg./ml.*		*mcg./ml.*	*mcg./ml.*	*mcg./ml.*	*mcg./ml.*	
17.1	14.3	83.6	49.6±0.9	2.6±.14	47.1	48.6	

λ mμ:	*540*	*580*	*610*	*660*
AK-38S:	0.36	0.82	1.00	0.75
H. and L.:**	0.37	0.85	1.00	0.68

* Nitrogen content × 6.67.
† $\Delta O.D._{610}^{5\,cm.} \div 0.34$.
‡ Wet digestion. See methods. Average of five determinations.
§ See methods.
‖ Ceruloplasmin copper = (Column 4) − (Column 5).
¶ (Column 2) × copper content of ceruloplasmin = (14.3) (0.0034).
** H. and L. values read from Figure 1 of Reference 1.

was a clear-blue solution which had been sterilized by Seitz filtration. Table I gives some characteristics of this preparation together with a comparison of its absorption spectrum with that given by ceruloplasmin prepared by Holmberg and Laurell (1). AK-38S had oxidase activity toward paraphenylenediamine, as shown below. If allowed to stand at about 2° C. at pH 4.8, platelike crystals of ceruloplasmin precipitated out of solution, although the yield was so small that crystallization was not used preparatively.

Copper^{64}. Copper^{64} acetate was obtained from Abbott Laboratories, Oak Ridge, Tennessee. The preparation used for the experiments reported in this paper contained 3.2 mc. per mg. at the time of shipment from Oak Ridge. Its concentration, determined in triplicate, was 2.17 mg. of copper per ml. Dilutions were made quantitatively with sufficient stable copper sulfate to produce both the desired number of counts per minute (c.p.m.) and final copper concentration.

For determination of copper^{64}, duplicate 1-ml. aliquots of the solutions to be analyzed were dried on aluminum planchettes and counted. All solutions, except Solution 5, had almost the same buffer and protein content so that absorption of β-particles by salt and protein was roughly equal in each sample which was radioactive. All counts were corrected for decay of radioactivity, using a half-life of 12.8 hours, and for background, which never exceeded 16 c.p.m. Proper operation of the counting equipment was controlled daily by counting a carbon^{14} standard, and determining the background radioactivity.

Resins. The cation exchange resin, Amberlite IR-120(H), Analytical Grade (Rohm and Haas Co., Philadelphia, Pa.), was washed in a column with water and then, successively, with twice its volume of 5 per cent HCl, four volumes of water, eight volumes of 5 per cent NaCl and eight volumes of water. This entire cycle was repeated a second time, using eight volumes of 5 per cent HCl instead of two. The resin was finally washed with water overnight so that the effluent pH was 6.4 (16). This resin, now on the sodium cycle, was analyzed for total copper by the method described below and found to contain 0.26 mcg. of copper per ml. of resin.

The chloride cycle of the anion exchange resin, Amberlite IRA-400 (Rohm and Haas), was converted quantitatively to the OH form by passing over it, in a column, approximately 60 volumes of 6 per cent sodium hydroxide followed by 2 volumes of 6 per cent carbonate-free potassium hydroxide, after which the effluent was free of chloride. The resin was then washed with about 15 volumes of water so that the effluent was no longer alkaline to litmus and put on the acetate cycle by adding 5 per cent acetic acid to it in a beaker until the supernatant solution stayed acid, after stirring, to litmus. The resin was washed with distilled water to neutrality. Analysis, after wet digestion, showed it to contain 1.0 mcg. of copper per ml.

Resin columns were made in 90 × 1 cm. (external diameter) lengths of glass tubing. As used, the resin columns consisted, successively, from top to bottom, of 10 ml. of anion exchange (acetate) resin, 20 ml. of cation exchange resin (sodium), 10 ml. of acetate resin, 10 ml. of sodium resin, and a loose plug of acid-washed glass wool. Each column was washed with 200 ml. of pH 5.8, $\Gamma/2 = 0.1$ acetate buffer before use. Solutions were passed through the columns at room temperature at 2 to 4 drops per minute. Each column was used only once. In each column the ratio of sodium-cycle-cation-exchange-resin to ionic copper in Solutions 3, 4 and 5, before "columnization," was always 2 ml. resin per 3 mcg. of copper. Such a column would be expected to effect complete removal of ionic copper since in the experiments of Joselow and Dawson half as much of a very similar cation-exchange resin (Amberlite IR-100), treated and used in almost the same manner, removed all traces of ionic copper (16). The acetate-cycle-anion-exchange-resin was added to the columns in order to remove ascorbic acid which changes the blue color, enzymatic activity and light absorption at 280 mμ of solutions of ceruloplasmin.

Quantitative determination of ceruloplasmin—Spectrophotometric

This method, slightly modified from that of Holmberg and Laurell (1), was applied, in the studies to be reported below, only to purified ceruloplasmin fractions of plasma. With slight changes, to be reported in detail in a subsequent paper, the method is applicable to citrated plasma itself, although for unknown reasons it will not work with serum. This analysis depends on the blue color of ceruloplasmin, since measurement of the optical density (O.D.) of the solution at 610 mμ, before and after destruction of the blue color, allows the concentration of ceruloplasmin to be calculated from its extinction coefficient.

Procedure. Using 5.0-cm. path-length cuvettes, the optical density of the solution to be analyzed is measured in the Beckman Spectrophotometer at about six wavelengths between 540 and 660 mμ including 610 mμ. After adding 30 mg. of ascorbic acid crystals to the contents of the cuvette, and mixing, optical densities are measured again at the same wavelengths when the optical density at 610 mμ has fallen to a minimum. The differences in optical density (ΔO.D.) before and after addition of the ascorbic acid, plotted against wavelength, should give a curve with a maxium at about 605 to 620 mμ similar to that found by Holmberg and Laurell (Table I). The concentration of ceruloplasmin in mg. per ml. is found by dividing $\Delta O.D._{610}$ by the extinction coefficient, which is 0.34, for a 1 mg. per ml. solution in a cell of 5-cm. path-length.[3]

The standard deviation of results obtained by this method, calculated from seven sets of duplicate determinations, was 0.0072 mg. ceruloplasmin per ml. The accuracy can be estimated from the results in Table I where ceruloplasmin copper, calculated in Column 7 as the product of the ceruloplasmin concentration, measured by this method, and the content of copper in ceruloplasmin (1) agreed with the independently determined ceruloplasmin copper given in Column 6 within 3 per cent.

Quantitative determination of ceruloplasmin—Enzymatic

A method of quantitatively measuring the oxidase activity of ceruloplasmin is of value in determining whether the protein has been enzymatically inactivated by the experimental procedures. This measurement was of importance since ascorbic acid oxidase appears to be inactivated when it functions enzymatically (16). The following method was developed to measure this activity spectrophotometrically by allowing ceruloplasmin to catalyze the oxidation of paraphenylenediamine (ppd) to colored compounds at 30.0° C. in a Beckman Spectrophotometer.

Use of this method depends on the assumptions that 1) ceruloplasmin catalyzes the oxidation of ppd, 2) there is sufficient dissolved oxygen in the solution not to limit the rate of reaction, and 3) the ionic composition of the various solutions is not different enough to change the rate of reaction. That the first assumption is true was shown by Holmberg and Laurell (5) and also has been demonstrated in our laboratory by the fact that the serum from patients with Wilson's disease who lack ceruloplasmin, ppd is oxidized at essentially the same rate as in saline controls. The second assumption seems validated by the findings of Holmberg and Laurell (14) and by the fact that the measured rate of oxidation almost always remains constant except in mixtures containing more than about 1 mg. of ceruloplasmin. With respect to the third assumption (20), the ionic composition of the various solutions of these experiments undoubtedly differs. Thus, Solution 2 contains about 2 mcg. of ionic copper per ml. which are not present in Solutions 3 and 4. Further, Solutions 3 and 4 have had practically all of their cations and anions exchanged, respectively, for sodium and acetate because of the columnizations to which they are subjected. However, the amount of ionic copper present in Solution 2 can be neglected since there is no significant difference between its enzymatic activity and that of solutions which lack free copper but are otherwise identical in composition to Solution 2. Furthermore, Solution 2 contains about as much acetate buffer as the columns contribute to Solutions 3 and 4, and differences in the content of other anions can probably be neglected (20). Enzymatic analyses of these solutions, carried out in this manner, appear to be useful as quantitative indicators of whether the protein has lost enzymatic activity.

Procedure. Pipette 1.0 ml. of the ceruloplasmin solution into a clean, dry 1.0-cm. path-length cuvette and add, successively, 1.5 ml. of 0.15 M sodium chloride, and 0.5 ml. of a 2.0 per cent solution of recrystallized paraphenylenediamine dihydrochloride (Fisher No. P 98) in an acetate buffer of pH 5.20 ± 0.02, and ionic strength 1.2. Make 8 to 10 timed readings at about one-minute intervals of the optical density of the solution against saline at 530 mμ. The spectrophotometer must be equipped with thermospacers to maintain the cuvette chamber at 30.0 ± 0.1° C. The rate of autoxidation of ppd, which is significant only when the unknown contains less than 0.1 mg. of ceruloplasmin per ml., can be measured using a control solution containing no ceruloplasmin. The pH of the solutions should be 5.00 ± 0.04 at the end of the determination.

Plot the optical densities obtained against time. The slope of the straight line on which the last 6 to 8 points should fall represents the rate of oxidation of ppd, and is proportional to the concentration of ceruloplasmin in the solution.

From eight sets of duplicate determinations the standard deviation of the slopes obtained was 9×10^{-4} ΔO.D. per minute.

It is possible to calibrate this procedure with known amounts of ceruloplasmin and relate the measured slope to ceruloplasmin concentration. This may be done in

[3] For convenience we have calculated ceruloplasmin concentration from ΔO.D. at 610 mμ, using the equivalent extinction coefficient to that used by Holmberg and Laurell at 605 mμ, *i.e.*, $\epsilon^{1\%}_{1\text{ cm.}} = 0.68$, since no appreciable difference in the ΔO.D. of ceruloplasmin solutions can be detected at these two wavelengths.

TABLE II

Determination of free copper in solutions of ceruloplasmin

	Tubes		
	1	2	3
Unknown solution or blank	3.0 ml.	3.0 ml.	3.0 ml.
50% ethanol	0.4 ml.		
50% ethanol saturated with DCO		0.4 ml.	0.4 ml.
0.15 M NaCl	0.4 ml.	0.4 ml.	
Standard copper:			
1 mcg. per ml.			0.4 ml.
O.D.$^{5\text{ cm.}*}_{600\ m\mu}$ A-3	0.039	0.084	0.187
O.D.$^{5\text{ cm.}*}_{600\ m\mu}$ Blank	−0.020	−0.004	0.103

* Read one hour after addition of all reagents.

saline or in serum. The details and results of such a calibration, which are not relevant to the present experiments, will be reported subsequently.

Quantitative determination of copper—Total copper

The procedure developed in this laboratory for the quantitative determination of the total copper content of biological materials using wet digestion and dicyclohexanoneoxalyldihydrazone (DCO) (Eastman Organic Chemicals, No. 7175) is a combination and modification of two procedures described in the literature (21, 22). The method, details of which will be published in a separate paper, has been applied to ceruloplasmin solutions, serum and urine. In analyses of ceruloplasmin solutions containing from 1.4 to 3.7 mcg. of copper, the standard deviation of the results was 0.039 mcg. of copper, with recoveries of added copper of 96 to 102 per cent.

Quantitative determination of copper—Free copper

In a solution containing ceruloplasmin and free ionic copper, DCO will react only with the ionic copper to produce a deep blue color at a pH of from 7 to 9.

Procedure. Each unknown sample is diluted with water to contain no more than 0.5 mcg. of free copper per ml., and then further diluted with an equal volume of 25 per cent sodium acetate solution. Reagents are added to three 3.0-ml. aliquots of each unknown and blank solution, in the manner indicated in Table II. Optical densities are read one hour later. The optical densities given in Table II are the actual analyses of one determination on Solution A-3 (see Table IV), and the corresponding blank.

Calculations. The calculation of the free copper content of the unknown solution requires that the rise in optical density in unknown Tube 2 be corrected for the optical density of unknown Tube 1 and for the optical density due to the free copper in the reagents as measured in the blank. Tube 3 is the standard in both unknowns and blank sets. For example, the calculation of the results given in Table II is as follows:

$$\text{Free copper in A-3 (mcg. per ml.)} = \frac{0.084 - 0.039 - \left(0.016 \times \frac{0.103}{0.107}\right)}{0.103} \times 0.4 \times \tfrac{3}{3} = 0.078$$

In the numerator of this equation the first term is the optical density produced in the unknown solution by DCO, and the second term is the optical density of the same solution without DCO. The third term is the optical density due to the combination of DCO with the copper in the blank, corrected by the ratio of the optical densities produced by DCO and 0.4 mcg. of copper in

TABLE III

Components and preparative treatment of solutions 1 to 5

Solution	AK-38S*	Std. A†	Std. B‡	Solution A-1	Solution B-1	Acetate buffer pH 5.8, Γ/2 =0.1	Ascorbic acid (10 mg./ml.)	Water	Treatment
	ml.	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	
A-1	4.0	4.0							None
A-2				2.0		10.0			Diluted with water to 25.0 ml.
A-3				2.0		4.0	2.0		Columnized§
A-4				2.0		4.0		2.0	Columnized§
A-5		1.0				4.0	2.0	1.0	Columnized§
B-1	4.0		4.0						None
B-2					2.0	10.0			Diluted with water to 25.0 ml.
B-3					2.0	4.0	2.0		Columnized§
B-4					2.0	4.0		2.0	Columnized§
B-5			1.0			4.0	2.0	1.0	Columnized§

* See Table I for free and ceruloplasmin copper content of AK-38S.

† Std. A made to contain 44.5 micrograms of copper per ml. and 240,000 counts per minute per ml. at arbitrary zero time.

‡ Std. B made to contain 44.5 micrograms of copper per ml. and 385,000 counts per minute per ml. at arbitrary zero time. Both standards were made from quantitative mixtures of standard cupric sulfate solutions and radioactive cupric acetate.

§ Passed through resin column, of composition given in text, at 2 to 4 drops per minute. The first 21.0 ml. of effluent were rejected and the next 20.0 ml., representing all of the material introduced into the column which could be recovered, were collected, diluted to 25.0 ml. with water, and labelled A-3, A-4, A-5, *etc.*

TABLE IV

Results of experiments to demonstrate exchange of ceruloplasmin copper and free ionic copper *

Solution	Free copper	Total copper	Ceruloplasmin copper	% of Solution 2	Ceruloplasmin $\Delta O.D._{410}^{1 cm.}$ +0.34	% of Solution 2	Enzymatic activity	% of Solution 2	Net corrected counts/min. per ml.†
	mcg./ml.	*mcg./ml.*	*mcg./ml.*		*mg./ml.*		*ΔO.D./min.*		
A-2	1.98	3.74	1.76	100	0.274	100	0.0182	100	10,280
A-3	0.078	1.46	1.38	78	0.190	69	0.0153	84	2,585
A-4	0.005	1.36	1.35	77	0.247	90	0.0133	73	27
A-5									2
B-2	2.00	3.69	1.69	100	0.288	100	0.0170	100	15,017
B-3	0.045	1.41	1.36	80	0.212	74	0.0136	80	3,708
B-4	0.018	1.36	1.34	79	0.224	78	0.0131	77	31
B-5									0
Std. A	44.0§	44.3§							240,000‡

* All values given are averages of duplicate determinations except total coppers, which are averages of triplicates; ceruloplasmin coppers, which are calculated as (total-free) copper; and the free copper of B-3, which is a single determination.

† Counts are corrected for background and 12.8 hour half-life of copper[64]. Self-absorption assumed equal in all planchettes.

‡ Value given is 25 times net corrected c.p.m. measured in a 1:25 dilution of Std. A.

§ Theoretical value is 44.5 mcg. per ml.

the unknown and in the blank solutions. The factor $\frac{2}{3}$ corrects the final result for the two-fold dilution of the unknown by the 25 per cent sodium acetate and for the 3-ml. aliquot taken.

The standard deviation of the method, in 12 sets of duplicate determinations on solutions with an ionic copper concentration of 0.005 to 0.2 mcg. per ml., was 0.0115 mcg. per ml.

The accuracy of the method and the fact that it actually measures only non-ceruloplasmin copper are shown by the results in Table IV where analyses of Solutions A-2 and B-2 which, undiluted, contained 1.88 mcg. per ml. of free copper and the same amount of ceruloplasmin copper gave 1.98 and 2.00 mcg. per ml. for free copper, respectively. Table IV also shows that very low levels of free copper were found in the 3 and 4 solutions, which had been passed over ion-exchange columns to remove ionic copper, but which still contained about 1.4 mcg. per ml. of ceruloplasmin copper.

RESULTS

The composition and method of preparation of Solutions 1 to 5, for the A and B experiments, are shown in Table III. When the ascorbic acid was added in the preparation of Solution 3, the ceruloplasmin instantly turned colorless. The effluent from the column, however, was blue, indicating that the decolorization is reversible. The quantitative results of the experiments are given in Table IV.

DISCUSSION

The results will be discussed with reference to 1) whether they demonstrate that exchange of ceruloplasmin copper with ionic copper occurred, 2) the extent of such exchange, 3) the mechanism of the exchange, and 4) a hypothesis relating ceruloplasmin to absorption of dietary copper and to Wilson's disease.

1) To show that exchange of ceruloplasmin copper with free ionic copper occurred, it is necessary to find (a) a significant number of counts in the effluent solution above those accounted for by any free ionic copper, together with (b) ceruloplasmin which has not increased its copper content. It will be seen from the results given in Table IV and the following discussion that significant exchange occurred when ceruloplasmin was mixed with copper[64] in the presence of ascorbic acid.

From the results given in Table IV on Solutions A-4 and B-4, it is apparent that no exchange of ceruloplasmin copper with radioactive copper took place when the two were mixed in buffer without ascorbic acid. The 27 and 31 c.p.m. found in A-4 and B-4, respectively, are accounted for by the small amount of free copper found in these effluents since, for A-4, $\frac{0.005}{1.98} \times 10{,}280 = 26$ c.p.m. and, for B-4, $\frac{0.018}{2.00} \times 15{,}017 = 135$ c.p.m. The other control solutions demonstrated that ascorbic acid does not prevent removal of free ionic copper by the columns since Solutions A-5 and B-5 show neither free copper nor counts. Both of these sets of results also show that the columns used effec-

tively remove over 99 per cent of the free ionic copper present in A-2 and B-2. It is also clear that no appreciable amount of the copper content of the resins is released into these solutions.

In Solutions A-3 and B-3, significant exchange occurred since a) the free copper found in the effluent solutions cannot account for the counts found, and b) there is no evidence that the effluent ceruloplasmin contained a greater amount of copper than before treatment.

a) The free copper present in these solutions, though higher than that in the A-4 and B-4 solutions, accounts for only $\frac{0.078}{1.98} \times 10{,}280 = 405$ c.p.m. in A-3, and $\frac{0.045}{2.00} \times 15{,}017 = 338$ c.p.m. in B-3. Furthermore, there is only about a 2 per cent chance that either 0.078 or 0.045 mcg. of copper per ml. could be larger than 0.102 or 0.080, respectively, since 0.078 ± 0.024 and 0.045 ± 0.035 are the 99 per cent confidence ranges of each of these values based on the precision of the method and the number of samples analyzed.[4] Even the upper limits of these values for free copper could not account for as many as 20 per cent of the counts found in A-3 and B-3.

b) It is unlikely that in Solutions A-3 and B-3 radioactive copper was simply bound tightly to ceruloplasmin in addition to the copper already there. This is primarily because the amount of ceruloplasmin copper found in A-3 and B-3, 1.38 and 1.36 mcg. per ml., respectively, is almost exactly the same as that found in A-4 and B-4, 1.35 and 1.34 mcg. per ml., respectively, where no exchange occurred. These ceruloplasmin copper values indicate that about 80 per cent of the ceruloplasmin in A-2 and B-2 was recovered in the columnized 3 and 4 solutions, the rest presumably remaining in the column. Therefore, the conclusion that radioactive copper was not simply added to the ceruloplasmin in A-3 and B-3 is strengthened by the fact that the recovery of ceruloplasmin in the 3 and 4 solutions is also roughly 80 per cent when calculated on the basis of the ratio of the intensity of blue color ($\Delta O.D._{610}$) or enzymatic activity in A-3, A-4, B-3, and B-4 to these values in A-2 and B-2.

2) The results given in Table IV can be treated quantitatively to calculate the fraction of ceruloplasmin copper which was exchanged under the experimental conditions. In these calculations we shall take into account the loss of ceruloplasmin in the columns, using ceruloplasmin copper as a measure of the amount of ceruloplasmin recovered. The quantitative conclusions reached would be only slightly different if the intensity of blue color or enzymatic activity were used for estimating the recovery of ceruloplasmin.

Let m = Ceruloplasmin copper in Solution 2 in mcg. per ml.
m′ = Ceruloplasmin copper in Solution 3 in mcg. per ml.
n = Ionic copper in Solution 2 in mcg. per ml.
n′ = Ionic copper in Solution 3 in mcg. per ml.
α = Net corrected counts per minute per ml. in Solution 2.
β = Net corrected counts per minute per ml. in Solution 3.
x = Fraction of ceruloplasmin copper exchanged.

Since the counts found in Solution 3, corrected for the amount of ceruloplasmin recovered, must equal the counts incorporated into the ceruloplasmin plus the counts due to the free copper which leaked through the column,

$$\frac{m}{m'}\beta = \frac{mx\,\alpha}{n + mx} + \frac{(n')\,\frac{(m)}{(m')}\,\alpha}{n + mx}$$

and

$$x = \frac{n\beta - n'\alpha}{m'\alpha - m\beta}$$

In Solution A-3, $x = 0.448$; and in Solution B-3, $x = 0.479$. Thus, about 45 to 48 per cent of ceruloplasmin copper appears to have exchanged with the free radioactive copper in the solution under the conditions of preparation of Solution 3.

No attempt has been made, in the work described in this paper, to do more than to effect the exchange of ceruloplasmin copper with ionic copper under the one set of conditions described. In these circumstances, apparently half of the copper

[4] We are indebted to Professor John W. Fertig, of the Columbia University School of Public Health and Administrative Medicine, for his kind assistance in the statistical analysis of our results.

of ceruloplasmin, or an average of 4 atoms of copper per molecule, seems to have been removed and replaced by ionic copper from the milieu. It is obvious that the extent of the exchange reaction may be very susceptible to changes in (a) the duration of time over which the ceruloplasmin, ascorbic acid, and ionic copper are in contact, (b) the temperature at which this contact occurs, (c) the concentration of substrate or reductant added, whether ascorbic acid, as here, or some other substance, and (d) the ionic composition of the solution including, of course, the pH and the concentration of ionic copper. No attempt was made to control the first two variables and the solutions were mixed and columnized at room temperature as rapidly as was convenient. In our experiments, ascorbic acid was used instead of paraphenylenediamine, the substrate toward which ceruloplasmin is most active (14), because considerable difficulty was experienced in freeing solutions of the colored products of ppd oxidation by means of ion exchange columns. The ionic composition selected was close to that under which ceruloplasmin most rapidly catalyzes the oxidation of ppd, that is, "in acetate buffers between pH 5 and 6" (20). The ionic copper concentration was chosen to be equal to the ceruloplasmin copper concentration for simplicity.

3) Some insight into the mechanism of the exchange of ceruloplasmin copper and ionic copper may be gained by comparing our results with those obtained by Joselow and Dawson in studying the plant copper-protein, ascorbic acid oxidase (16–18). This blue-green enzyme has the same molecular weight as ceruloplasmin, 150,000, and contains 0.26 per cent of tightly bound copper which, like ceruloplasmin copper, is not removed by a sulfonic cation exchange resin. In experiments which were similar to those reported in this paper, and which were, in fact, the models for our work, these authors found that (a) there was no exchange of enzyme copper and ionic copper when the two were mixed without oxygen or substrate, (b) only when the enzyme was actively catalyzing the oxidation of ascorbic acid did exchange, to the extent of 30 per cent, occur, and (c) irreversible inactivation of the enzyme occurred apparently simultaneously with its enzymatic activity. The authors speculated that the exchange might be either the consequence of enzyme copper becoming more "accessible to . . . displacement by radiocopper" while structural rearrangements involved in the enzymatic inactivation were occurring, or that enzymatic activity was accompanied by the copper of ascorbic acid oxidase shuttling between the divalent and monovalent states, and that exchange took place when the enzyme copper was monovalent. As support for this second hypothesis, Joselow and Dawson pointed out that a shift from divalent to univalent copper may be accompanied by a change from a strong square coplanar configuration for the directed valences of copper to a weaker tetrahedral configuration. In the case of ceruloplasmin, no loss of enzymatic activity followed exchange, as is apparent from Table IV (except for the ceruloplasmin trapped in the column), so that the exchange cannot be ascribed to loosening of the copper-protein bond due to permanent structural rearrangement. Therefore, the possibility is even greater in the case of ceruloplasmin than in that of ascorbic acid oxidase that exchange is consequent to reduction of the ceruloplasmin copper to the monovalent state.

4) Since about half of the copper in ceruloplasmin can be exchanged for ionic copper, the conclusion seems inescapable that ceruloplasmin can release at least about half of its tightly bound copper into the solution reversibly. It may be of interest to speculate as to the possible relevance of such release to the relation of ceruloplasmin to absorption of dietary copper, and to Wilson's disease.

The mechanism whereby copper is absorbed is unknown but the simplest assumption concerning it would be that there is diffusion of ionic copper, or copper bound to small molecules like amino acids, from intestinal lumen into blood. Such diffusion would presumably occur only if, and when, the concentration of diffusible copper in the intestinal lumen were higher than the concentration of diffusible copper in the plasma circulating through the intestine. Since the average adult ingests 2.5 to 5 mg. of copper per day (23), and the total daily volume of gastrointestinal secretions in an adult is about 8,200 ml. per day (24), the average concentration of copper in the intestinal lumen is 0.3 to 0.6 mcg. of copper per ml. In normal subjects the plasma concentration of non-ceruloplasmin copper is less than 0.1 mcg. per ml. (25), and the concentration of cerulo-

plasmin copper is about 1.1 mcg. per ml. (3). Since only non-ceruloplasmin copper appears to be diffusible across semi-permeable membranes like the placenta, and blood-brain barrier (6, 12), probably only such copper is capable of diffusing from plasma across the membranes of the intestine into its lumen. Thus, the gradient of diffusible copper, about 0.4 mcg. per ml. in intestinal lumen to 0.1 mcg. per ml. in intestinal plasma, would favor diffusion from lumen to plasma. If, however, all or part of the ceruloplasmin copper in intestinal blood were released from the protein, thereby becoming diffusible, the concentration gradient would be reversed since there would be up to 1.1 mcg. of diffusible copper per ml. of plasma. Thus, under such conditions, the concentration of ceruloplasmin present in normal subjects would give rise to sufficient diffusible copper in intestinal plasma to prevent absorption of much of the usual dietary copper. Furthermore, if such were the normal situation, a consequence of a deficiency or absence of ceruloplasmin, which is characteristic of patients with Wilson's disease (2, 3, 8), would be increased absorption of dietary copper. In fact, just such an excessive absorption of copper, in comparison with the copper absorbed by normal subjects, seems to occur in patients with Wilson's disease (12, 26–28).

Perhaps the almost completely speculative nature of this mechanism needs further emphasis. Its relation to the present work lies in the fact that our results have demonstrated that ceruloplasmin can release at least half of its copper reversibly, *in vitro*. But we have not determined that such release can occur *in vivo*, or, if it does occur, that the protein and copper can exist dissociated for an appreciable length of time. Nor have we determined if conditions exist in the intestinal circulation that would make such a release more likely to occur there than in blood in other parts of the vascular system. For example, the ratio of the concentration of ascorbic acid to that of ceruloplasmin was about 25 times as great in Solution 3 before columnization as it is in normal plasma (1–4, 29). Although the amount of ascorbic acid used in preparing Solution 3 was about 10 times greater than that necessary to reduce the ceruloplasmin, we do not know if the concentration of ascorbic acid in intestinal plasma is sufficient to reduce and bring about exchange of ceruloplasmin copper, or whether there are other reductants or substrates present that can produce these effects.

The foregoing provides a theoretical role for ceruloplasmin in regulating the absorption of copper, and in its relation to a hypothesis of the natural history of Wilson's disease which several groups of authors have proposed (2, 10–13). The subject destined to develop this illness is presumably born with a deficiency or absence of ceruloplasmin. Absorption of a greater portion of the dietary copper than occurs in normal subjects would result from this deficiency, perhaps in the manner proposed above. After a sufficient period of time, which probably varies both with the concentration of ceruloplasmin and the amount of copper ingested, symptoms and signs of the disease appear. These are apparently due to the tissue damage which the deposits of the excess copper produce (30, 31), although the mechanism of copper toxicity is unknown. The clinical features of the disease apparently reflect injury to those organs which seem most susceptible to damage by copper, *i.e.*, the liver (32), brain and kidney (33), since the copper seems to be deposited rather ubiquitously in the body (12).

SUMMARY

About half of the 8 atoms of copper present in each molecule of the plasma protein, ceruloplasmin, have been shown to be capable of exchanging with radioactive copper^{64}. The exchange has been produced only when the protein and copper^{64} are mixed in the presence of sufficient ascorbic acid to reduce reversibly the blue ceruloplasmin to its colorless form. The existence of such an exchange implies that ceruloplasmin copper can be reversibly released from the protein, at least in the presence of ascorbic acid. It is probable that reduction of ceruloplasmin copper to the monovalent form is essential for exchange to occur.

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