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Title: EFFECT OF CADMIUM ON CERULOPLASMIN LEVELS IN THE RAT

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Injection of a 2.0 mg Cd^{2+}/kg B.W. (I.P. as $CdCl_2$ in .05 M NaAc, pH = 6.9-7.2, .15 M or μ = .15 with NaCl) resulted in a decrease in ceruloplasmin (EC 1.12.3) p-phenylenediamine oxidase activity within one hour of injection. Maximal decrease (40%) occurred within six to eight hours, with levels rising slowly over the duration of the experiment (28.5 hours postinjection).

Cadmium-109 (36 μCi carrier free $^{109}CdCl_2$ in .05 M HCl, .15 M NaCl) was injected (I.P.) into rats and plasma obtained five hours later. Purification of the ceruloplasmin using DEAE and Sephadex G-200 chromatography revealed significant cadmium-109 in the ceruloplasmin fraction. *In vitro* binding was not observed.

Attempts were made to purify ceruloplasmin utilizing, DEAE, hydroxylapatite and Sephadex G-200 chromatography coupled with ethanol-chloroform precipitation. The product

obtained was spectrophotometrically pure and deemed electrophoretically homogeneous, however antibody produced in rabbits to this antigen was not homogeneous as revealed by immunoelectrophoresis and Ouchterlony immunodiffusion.

The precipitin response of the antiserum obtained was investigated with respect to the effect of dilution, pH and NaCl concentration.

The equivalence point was shown to remain essentially independent of dilution (over a range of 1.3 to 10), pH (from 5.8 to 8.0) or NaCl concentration (.01 M to .25 M). The point of maximal protein precipitation was affected by pH and the amount of protein precipitated was decreased by dilution and unaffected by changing salt concentration.

The experiments involving cadmium suggest the mechanism of cadmium induced inhibition of ceruloplasmin oxidase activity does not involve decreased synthesis. The in vivo binding of cadmium to ceruloplasmin may be the cause of decreased oxidase activity.

Effect of Cadmium on Ceruloplasmin Levels in the Rat

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EFFECT OF CADMIUM ON CERULOPLASMIN LEVELS IN THE RAT

I. INTRODUCTION

As stated by Nordberg (1) the toxicity of cadmium has been recognized since 1858. In the last four decades it has come under increasing scrutiny, first as an industrial hazard and later as a ubiquitous metal present in the environment with possible chronic toxicity (2,3,4). Schroeder (2) has reported a possible correlation between cadmium and hypertension in humans; Carrol (3) has presented evidence for a relationship of cadmium in air to cardiovascular death rates, and cadmium has been recognized as a causative factor in the Japanese outbreak of Itai-Itai (ouch-ouch) disease (5). Consequently, in the last few years, concern about the level of cadmium in the environment and its possible effects on humans has increased dramatically. Several review articles and books have recently summarized existing knowledge concerning cadmium (2, 5-11).

On a biochemical scale, one of the well documented facets of cadmium toxicity is its interaction with enzyme systems (9). Over 40 enzymes have been reported to be inhibited by cadmium and another 25-30 are activated by it *in vivo* and/or *in vitro* (9, 11-25).

Whanger and Weswig (24) first reported that the oxidase activity of ceruloplasmin (EC 1.12.3), a copper containing

plasma protein, was inhibited, *in vivo*, by cadmium. The mechanism of this inhibition is not known and the present investigation was initiated to test several hypotheses.

The mechanics of the proposed investigation required the preparation of antiserum to rat ceruloplasmin and also the preparation of ceruloplasmin-^{14}C. A homogeneous antiserum to rat ceruloplasmin and the preparation of ceruloplasmin-^{14}C have been reported (26), however, the company responsible for immunization required mg quantities of antigen (ceruloplasmin) and the reported work resulted in µg quantities of ceruloplasmin antigen.

After a discussion of the significance of ceruloplasmin and some of the proposed mechanisms of cadmium-enzyme interactions, a series of studies to investigate the mechanisms of cadmium-enzyme interactions, a series of studies to investigate the mechanism of cadmium induced inhibition of ceruloplasmin oxidase properties are presented. Also presented is the preparation of ceruloplasmin-^{14}C and the production and characterization of antiserum to rat ceruloplasmin.

Ceruloplasmin and Cadmium-Enzyme Interactions

Ceruloplasmin

Human ceruloplasmin was first isolated by Holmberg and Laurell in 1948 (27). Ceruloplasmin is an α_2-globulin found in the plasma of many vertebrates (28). This plasma

glycoprotein, containing eight gram-atoms of copper per mole of protein, accounts for up to 98% of the plasma copper found in man and rats (29).

Ceruloplasmin is known to oxidize, *in vitro*, the following types of compounds: aromatic polyamines and polyphenols, e.g., *p*-phenylenediamine, epinephrine, dopamine, serotonin; enediols (only if iron coupled), e.g., ascorbate, hydroquinone, catechol, hydroxylamine, thioglycolate, cysteine, DOPA; and several inorganics, e.g., Fe^{+2}, $Na_2S_2O_4$, NH_2OH, $K_4Fe(CN)_6$ (30,31).

The biological significance of these *in vitro* oxidations has not been established with certainty despite extensive investigation (29,30). Osaki *et al.* (32) first suggested that ceruloplasmin fulfilled a physiological role in oxidizing ferrous to ferric iron for subsequent incorporation in transferrin. A considerable body of evidence supporting this role has been amassed since his proposal which has been summarized by Frieden (31). A major drawback in this theory has been the fact that patients with Wilson's disease (a hereditary copper metabolism disorder) have generally low to non-existent ceruloplasmin levels, yet generally show no disturbances of iron metabolism (30, 31, 33). Recently, however, Topham and Frieden (33) have reported a non-ceruloplasmin ferroxidase in the plasma of Wilson's disease patients that was not present in normal sera. While doubts still exist (34), the evidence points

very strongly to a role for ceruloplasmin in iron metabolism (35). Hampton et al. (36) reported that ceruloplasmin oxidatively deaminates histamine and suggested it may be the primary histaminase of monkey and human plasma. These results may provide new directions in the search for a physiological role for ceruloplasmin.

Cadmium-Enzyme Interactions

Potential and observed interactions of cadmium with enzymes and proteins in general, are many and varied (9). An often cited cause of enzymatic inhibition is the reaction of cadmium with sulfhydryl groups necessary to the activity of many enzymes (37). The affinity of cadmium (as well as Pb, Hg, Cu, etc.) for other ligands present in biological systems such as phosphate, cysteinyl, carboxyl and histidyl residues is well established (9,37) and could lead to inhibition or enhancement of enzymatic activity. Due to chemical similarities of cadmium (38) with metals in certain metalloenzymes, it can presumably replace the physiological metal causing concommitant inhibition or activation of enzyme activity. This is particularly feasible for zinc metalloenzymes due to the similarity of the Cd^{2+} and Zn^{2+} ions and has been shown to occur in vitro for the zinc enzyme carboxypeptidase B (20) among others (37-40). Both Cd^{2+} and Cu^{+1} are isoelectronic (24) and may form similar complexes with ligands.

A more indirect action of cadmium may occur by virtue of its ability to interfere with such vital processes as respiration and ATP formation (9, 41-45), in concentrations as low as 10^{-6} M. The consequences of such interference could be the disruption of high energy requiring processes such as protein synthesis.

The binding of cadmium (and other metals) to DNA has been noted and found to affect the helical structure (9,46, 47) of DNA. Very little evidence is available to link this interaction with in vivo biological disruption but recently it has been shown that cadmium induces piastin synthesis in pea tissue at the transcriptional level (48). The synthesis of metallothionein, the only naturally occurring cadmium containing protein, is apparently stimulated by cadmium (49, 50). An abnormal serum component was observed in the plasma of rats given intraperitoneal injections of cadmium (51). The behavior of plasma proteins following in vivo treatment with cadmium seems to be somewhat dependent upon the method of treatment (i.e., injection or feeding), the dose level, and duration of exposure (51-53). In any event, changes in plasma proteins are observed. Whether the changes in distribution in plasma protein fractions are the result of disruption of synthetic pathways or a result of other biological action is a matter of conjecture.

Cadmium has been shown (54) to interact at a fundamental level with lipid layers and is effective in expanding

them at concentrations as low as 10^{-8} M (in vitro). It has been speculated that cadmium-lipid interactions may be involved in the toxic effects of cadmium on kidney tubules, testes, nerve membranes, and mitochondria (9,54,55). Cadmium has recently been shown to cause a marked increase in the number and size of lysosomes (55), and affect lysosomal enzyme activity (56). The precise nature of a cause-effect relation between these observations is not clear.

The uptake and distribution of copper was shown to be affected by cadmium (57). Cadmium severely depressed copper uptake from ligated segments of intestine and stomach and also affected organ distribution of copper, significantly decreasing the percent of applied dose which reached the liver, the organ of ceruloplasmin synthesis.

Though not conclusively proven, it has been suggested that ceruloplasmin functions as a homeostatic control mechanism for copper (30). Others (29) have suggested a role in the transport of copper. Cadmium is known to affect the metabolism of both copper (57) and iron (58) and it has been shown that specific components of cadmium toxicity are responsive to copper, others to iron, and still others to zinc (59). In view of these facts and the proposed roles for ceruloplasmin, it was felt that investigation into the mechanism of cadmium induced inhibition of ceruloplasmin was warranted.

Hypotheses Regarding Cadmium Induced Ceruloplasmin Inhibition

Based upon the above considerations one may postulate the following hypotheses as to a mechanism of cadmium induced inhibition of ceruloplasmin activity:

1. Inhibition of ceruloplasmin synthesis as a result of cadmium interactions with DNA and/or energy metabolism.
2. Prevention or decrease of copper incorporation into ceruloplasmin during synthesis by virtue of an induced copper deficiency.
3. Incorporation of cadmium in the place of copper in the ceruloplasmin molecule during synthesis by virtue of cadmium possessing some chemical properties similar to copper.
4. Cadmium binding to ceruloplasmin after synthesis via ligands present on the protein.
5. Increased catabolism of ceruloplasmin by means of stimulated catabolic enzyme activity or through lysosomal modification.

The following experiments were designed to distinguish which of the above mechanisms was active.

II. DECAY OF CERULOPLASMIN ACTIVITY FOLLOWING CADMIUM INJECTION

Methodology

Male O.S.U. Wistar rats and male O.S.U. Brown rats (weighing approximately 280 grams, raised on Purina chow and tap water) were injected intraperitoneally with cadmium. At various times from one to 28.5 hours after cadmium injection, blood (.2-1.0 ml) was obtained from the tail (by clipping off the end of the tail) or by heart puncture. Plasma was obtained by centrifugation at 2000 X g for 15-30 minutes. The plasma was frozen until later analysis.

Dose and Form of Injected Cadmium

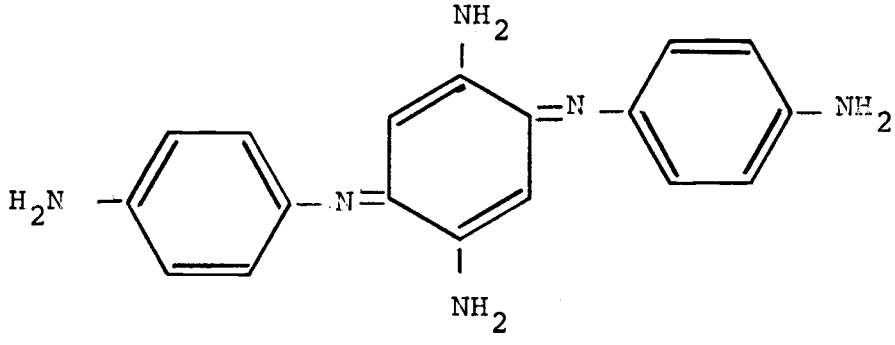
Cadmium was injected in a dose of 2.0 mg Cd^{2+}/kg B.W. as $CdCl_2$ in .05 M sodium acetate (NaAc), .15 M NaCl or μ = .15[1] with NaCl, pH = 6.9-7.2. Cadmium concentration in the solution injected was 2.0 mg Cd^{2+}/ml. A similar dose of 1.0 mg Cd^{2+}/kg B.W. was also injected in one group of rats. Control rats received 1.0 ml/kg B.W. of .05 M sodium acetate, .15 M NaCl, pH = 7.0-7.3.

Ceruloplasmin (p-phenylenediamine oxidase) Assay

The p-phenylenediamine (PPD) oxidase activity of ceruloplasmin was assayed generally as described by Houchin

[1] Ionic strength $\mu = \Sigma \frac{1}{2} MZ^2$. Where M is the molarity of a charged species and Z is the charge on the ion. Contribution from acetate was ignored.

(60). One-tenth of a ml of plasma was added to one ml of freshly prepared .1% PPD in acetate buffer (μ = 1.2, pH = 5.2) at 37°C. After 15 minutes incubation, the reaction was stopped by adding five ml of a .02% solution of sodium azide in water. The absorbance at 525 mμ was measured on a Gilford spectrophotometer against a reagent blank. The absorbance A was converted to International Units (61) by the following equation: International Units (I.U.) = A (349), where 349 is a factor converting the absorbance of the oxidation product to micromoles of Bandrowski's base formed per minute per liter of plasma under the above conditions.



Bandrowski's Base

Bandrowski's base is an oxidation product of p-phenylenediamine with the same absorption spectrum as the ceruloplasmin oxidation product (61).

The preparation of PPD is not well detailed in the literature and the only commercially available source is of a technical grade[2] which was deemed unsuitable for the assay

[2]Grade II p-phenylenediamine obtained from Sigma Chemical Company, P. O. Box 14508, St. Louis, Missouri.

procedure. A method for recrystallization of PPD to form PPD•2HCl is detailed by Henry et al. (62) but the crystals produced when this procedure was tried appeared amorphous and were deemed unsuitable. Since the label on the bottle of crystals prepared by a previous investigator indicated recrystallization from benzene, the following procedure was devised. All procedures were carried out with as little light present as possible to decrease photolytic reactions.

1. One gram of crude PPD was dissolved in 250 ml distilled benzene by stirring in a 50°-60°C water bath for 10-15 minutes. There will always be some small dark chunks left undissolved but it is thought that they are polymerization products of PPD and undesirable.
2. The hot solution was filtered through a fine filter paper, the filtrate placed in a beaker and set in a refrigerator for several hours or overnight. Crystals form readily and appear as a pink fluffy mass.
3. Crystals are filtered cold and washed once with cold benzene. A sintered glass filter works well and allows the crystals to be dried in the filter. These can be easily scraped out later.
4. The crystals are dried in the dark under vacuum overnight. The resultant crystals will be an off-white to pinkish purple and will attain a progressively more purple color with age and exposure to light.

5. The crystals should be stored in brown or opaque bottles to keep photolytic reactions at a minimum.

Results and Discussion

The decay of ceruloplasmin activity begins to drop off almost immediately following injection (Figure 1) and is seen to be virtually complete by seven hours (Figure 2). The behavior of ceruloplasmin in the controls is somewhat puzzling as there is an obvious drop in level as time progresses. It would be expected that if any change were observed it would be a rise in ceruloplasmin levels as it has been reported by Whanger and Weswig (63) that stress and/or injection increases ceruloplasmin levels. It was also the experience of this investigator that stress results in a rise in ceruloplasmin levels and not a decrease. The trend is consistently found in both control groups, however, and cannot be denied. The only explanation that may fit this data is that the loss of blood (approximately .8 ml per drawing) results in a dilution of ceruloplasmin as body fluids attempt to maintain normal blood volume.

The 24 percent decrease in control ceruloplasmin activity at the 28.5 hour sample point is also unexplainable except as a result of dilution. As it has been shown (64) that stress as minimal as 15 minutes of swimming will raise ceruloplasmin levels, it is almost inconceivable that ceruloplasmin levels in controls and/or the cadmium injected

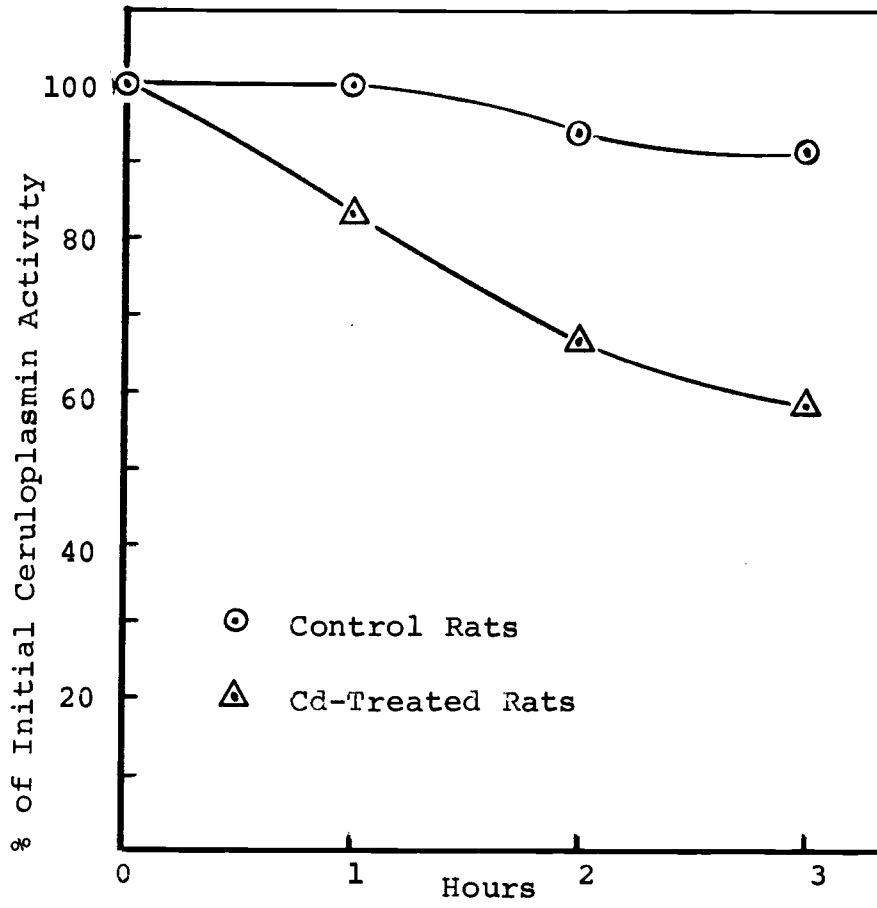


Figure 1. Effect of I.P. cadmium injection on ceruloplasmin PPD oxidase activity. Rats treated with 2.0 mg Cd^{2+}/kg B.W. as $CdCl_2$ in .05 M NaAc buffer, .15 M NaCl, pH = 6.9-7.2, or with buffer minus cadmium.

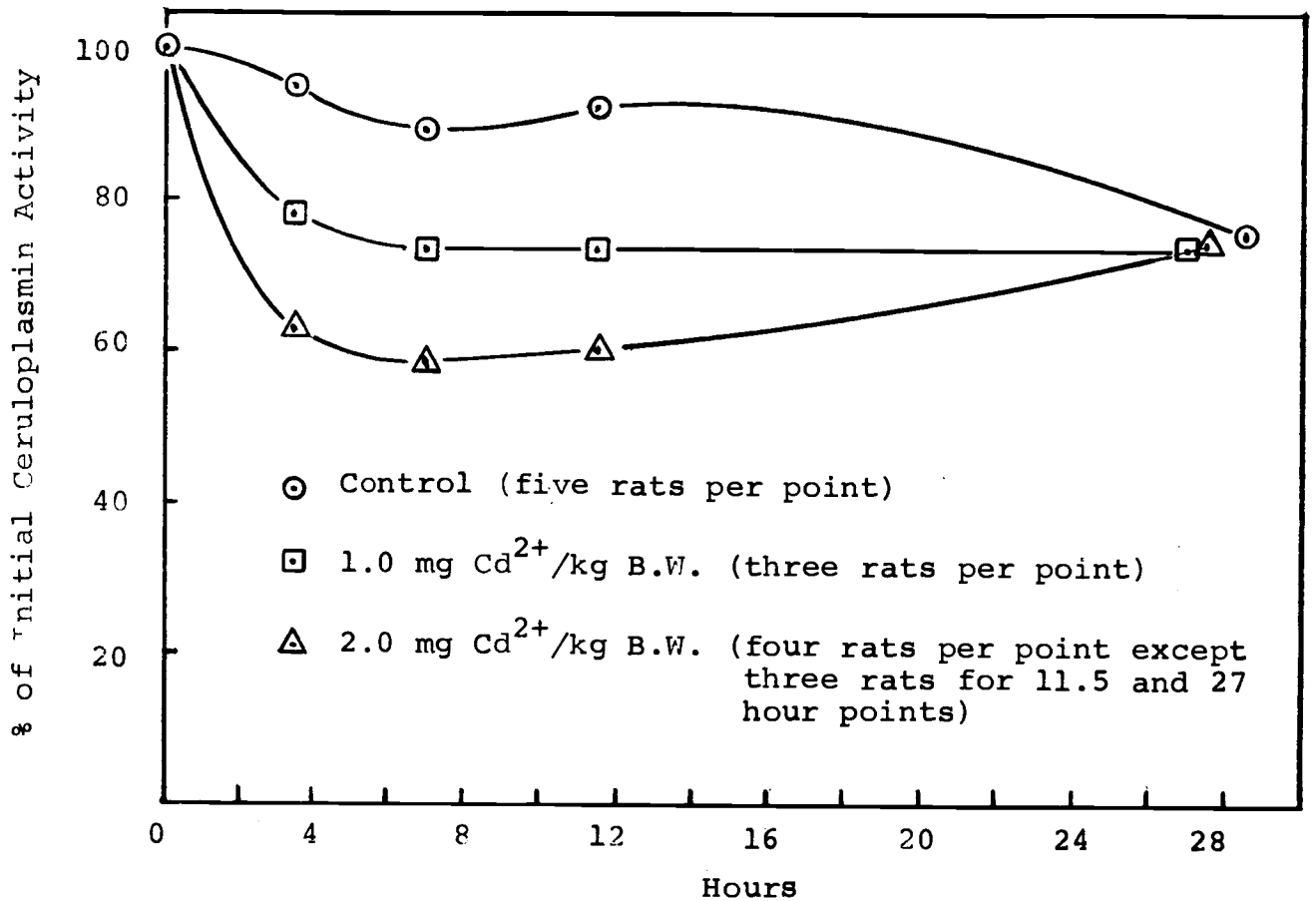


Figure 2. Effect of I.P. cadmium injection on ceruloplasmin PPD oxidase activity. Cadmium injected as 2.0 mg Cd^{2+}/kg B.W. in .05 M NaAc buffer, μ = .15, pH = 6.9-7.2, or 1.0 mg Cd^{2+}/kg B.W. as $CdCl_2$ in saline, pH approximately 5.5. Control rats received NaAc buffer minus cadmium.

rats would not rise. Therefore, the observed rise in ceruloplasmin activity in the cadmium rats is not unexpected.

Ceruloplasmin has been reported to possess a biological half-life in the plasma of 12 hours (26). Total cessation of ceruloplasmin synthesis at the time of injection would result in a decrease of 15% in three hours, if the 12-hour half-life is assumed valid. The observed 40% decrease at three hours following injection (Figure 1) strongly suggests a slowdown in synthesis is not the cause of ceruloplasmin activity decrease. Even if one corrects for the drop seen in the ceruloplasmin activity of the control rats, a 30% decrease still remains.

By a similar argument, the hypotheses that cadmium prevents or decreases copper incorporation in the apoenzyme, or that cadmium is substituted for copper in the protein during synthesis are mitigated against by these time considerations.

Although no particular conclusions may be drawn, it is interesting to note that there would appear to be a certain amount of dose dependence to the response of ceruloplasmin activity as shown in Figure 2.

Doses higher than 2.0 mg Cd^{2+}/kg B.W. were not investigated as even this dose at a pH of 5.5 resulted in 100% mortality within a few hours. Discomfort was noted in animals injected with 2.0 mg Cd^{2+}/kg B.W. at pH's around 7, however they did not die.

It should also be noted that the times of sampling for Figure 2 are only accurate to approximately plus or minus 20 minutes. This arises from mechanical difficulties in predicting the length of time to anesthetize a rat and draw blood.

III. CADMIUM-109 BINDING TO CERULOPLASMIN

Methodology

Two male O.S.U. Wistar rats of approximately 250 grams each were injected (I.P.) with 36 μCi ^{109}Cd.[3] Five hours after injection blood was obtained from the abdominal aorta of each rat, and the plasma obtained by centrifugation of the blood. Plasma samples were counted in a Packard Auto-Gamma counting system with a 3"x3" well type NaI crystal detector. A blank was prepared by adding .1 ml of a .8 μCi/ml solution of ^{109}Cd in .1 N HCl to three ml of serum. This amount of cadmium-109 corresponds roughly to two times the amount of activity found in three ml of plasma from the two *in vivo* treated rats. Three mg of cold carrier human ceruloplasmin were added to each aliquot of plasma (or serum), and all three samples were diluted approximately five times with .05 M NaAc buffer[4], pH = 5.8. The samples were applied to a .5 x 15 cm column of DEAE cellulose equilibrated with the above buffer. Before the ceruloplasmin was eluted with .3 M NaCl in the buffer, 25 ml of the buffer were washed through the column to elute contaminating

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[3] Cadmium-109 obtained from New England Nuclear as $^{109}CdCl_2$ in .5 N HCl, carrier free; 2.0 μCi/ul. Injected in .2 ml .05 N HCl, .15 M NaCl.

[4] All acetate buffers prepared by adjusting the pH of .05 M NaAc solution with concentrated acetic acid unless otherwise noted.

proteins. The ceruloplasmin was collected in one to two ml of effluent.

The ceruloplasmin fraction was applied to a Sephadex G-200 column (2 x 95 cm) equilibrated with .05 M sodium acetate buffer, pH = 5.8. Flow rate was .5 ml per minute and 100 drops were collected per tube. Eluted fractions were counted for cadmium-109 and the absorbance was measured at 280 mμ. Ceruloplasmin oxidase activity was assayed by adding .2 ml of elute to one ml of .2% PPD in assay buffer and incubating at 37°C for 30 minutes. The reaction was stopped by adding five ml of .02% sodium azide.

Results and Discussion

The results of Sephadex G-200 chromatography of the ceruloplasmin fraction from DEAE cellulose chromatography of the plasma from one of the two rats injected with cadmium-109 is shown in Figure 3. The results of identical treatment of plasma from the other injected rat were very similar. Inspection of Figure 3 reveals a cadmium-109 peak coincident with the ceruloplasmin peak as measured by PPD-oxidase activity and absorbance at 280 mμ.

No cadmium peak was present in the G-200 chromatogram of the sample containing *in vitro* added cadmium-109.

These results suggest that cadmium binding to ceruloplasmin occurs *in vivo* but not *in vitro*. The *in vivo* binding of cadmium to ceruloplasmin is indirectly substantiated

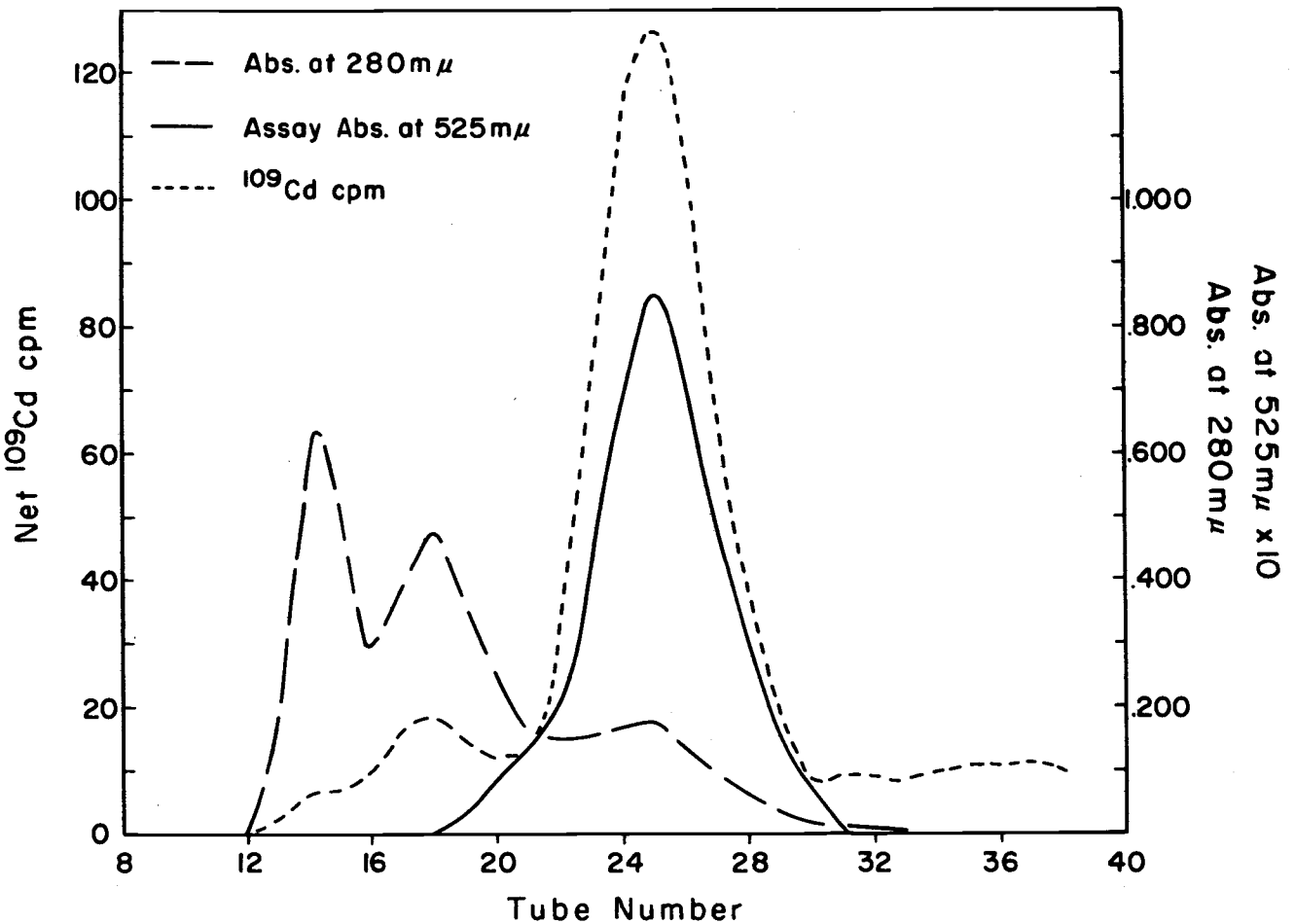

Figure 3. Sephadex G-200 chromatogram of ceruloplasmin fraction from DEAE cellulose chromatography. Rat injected I.P. with carrier-free cadmium-109 as the chloride in .05 M HCl, .15 M NaCl, five hours prior to obtaining plasma.

by the work of Shaikh and Lucis (65) who report injected (s.c.) cadmium-109 was associated mainly with the α-globulin fraction of the plasma, using paper electrophoresis as the separation technique.

A lack of *in vitro* binding is consistent with the lack of enzymatic inhibition produced by *in vitro* incubation with cadmium concentrations of up to 500 micromolar (66). In the latter study human ceruloplasmin was used.

IV. PREPARATION OF CERULOPLASMIN-^{14}C

Methodology

Two male O.S.U. Wistar rats (236 and 230 grams) raised on Purina chow and tap water were fasted overnight and injected (I.P.) with 126 μCi L-leucine-^{14}C-(U).[5] Two and a half hours following L-leucine-^{14}C injection, rats were anesthetized with ether and blood was obtained from the abdominal aorta. Approximately 18 ml of blood were collected (citrate used as an anticoagulant), from which nine ml of plasma were obtained.

The plasma was diluted five times with .05 M sodium acetate buffer, pH = 5.8, and applied to a 1 x 10 cm column of DEAE cellulose equilibrated with the same buffer. The ceruloplasmin band was washed with 100 ml of .1 M NaCl in the acetate buffer, followed by 25 ml of .2 M NaCl in the acetate buffer before being eluted as a sharply defined band by .3 M NaCl in the acetate buffer.

The ceruloplasmin was dialyzed against two 2-liter changes of .05 M phosphate buffer, pH = 6.4 (first dialysis five hours, second dialysis overnight at 2°-5°C). The dialyzed ceruloplasmin preparation was applied to a 1 x 20 cm

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[5] Obtained from Amersham/Seale Corp. Specific activity 342 and 311 mCi/mole, in sterile 2% ethanol aqueous solution, radioactive concentration 50 μCi/ml. Injection solution was made .15 M in NaCl before injection, and was 252 μCi/5.4 ml.

column of hydroxylapatite equilibrated with phosphate buffer. The ceruloplasmin was eluted by a concentration gradient similar to the method of Holtzman et al. (67). The gradient at pH 6.4 was formed by adding 80 ml .05 M phosphate buffer to the first chamber of a Technicon Varigrad apparatus, followed by 80 ml .2 M phosphate buffer in the next three chambers, with 80 ml .4 M phosphate buffer in the last chamber.

The flow rate was approximately one ml (20 drops) per minute and the eluate monitored at 280 mμ by an Isco Model 584 U.V. monitor. The protein peak corresponding to ceruloplasmin was collected, the fractions pooled, and dialyzed against .05 M sodium acetate buffer,pH = 5.8. The ceruloplasmin solution was then placed on a .7 x 8 cm column of DEAE cellulose[6] and eluted in 2.9 ml of the acetate buffer (.3 M in NaCl).

It should be noted in passing that rat ceruloplasmin appears to bind much less strongly to hydroxylapatite under these conditions than does human ceruloplasmin. This statement arises from the observation that the rat ceruloplasmin began to elute very early in the gradient as opposed to the indicated movement of human ceruloplasmin (67). Other investigators have reported a similar observation (68).

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[6]Obtained from Bio-Rad Laboratories, Richmond, California

Results and Discussion

The ratio of absorbance at 610 mμ to the absorbance at 280 mμ is .0513 for a pure preparation of rat ceruloplasmin as reported by Holtzman and Gaumnitz (69). A value of .042 for the same ratio has been reported by Vasilets et al. (68), a value close to that of human ceruloplasmin (32,70). The present data support the higher number of Holtzman and Gaumnitz so this ratio was used as a criterion for pure ceruloplasmin. Therefore, the following equation was used to determine purity of ceruloplasmin preparations:

$$100\ [A_{610m\mu}/A_{280m\mu}]/.0513 = \%\ \text{purity}$$

Holtzman and Gaumnitz also reported that the $A_{610m\mu,1\ cm,1\%} = .64$. Using this value the mg of holoceruloplasmin per ml of a solution may be obtained by the equation (using a one cm path length cuvette):

$$\frac{(10)(A_{610m\mu})}{(.64)} = \frac{\text{mg ceruloplasmin}}{\text{ml solution}}$$

It was found that 1.9 mg ceruloplasmin-^{14}C were present at the end of the purification procedure.

A 25 μl sample (16 μg ceruloplasmin) was reacted with antibody and counted generally as outlined in Appendix III. Incubation mixture was 1.0 ml .02 M phosphate buffer, .02 M EDTA, .15 M NaCl, pH = 7.2, plus 25 μl

ceruloplasmin preparation plus .75 ml antiserum and sample remained in NCS overnight without stirring. Results showed that 268 DPM ^{14}C were present which gave a specific activity of 16.8 DPM/µg. It was recognized that the figure did not provide for losses during the antibody precipitation procedure but of more interest was the DPM of immunoprecipitable ceruloplasmin one might reasonaly expect to obtain upon spiking of tritium labeled samples. A precise number was not necessary.

V. PURIFICATION OF RAT CERULOPLASMIN ANTIGEN

Methodology

Three hundred ml of pooled plasma were obtained from male and female O.S.U. Wistar and O.S.U. Brown rats (180-300 grams body weight) that were raised on Purina rat chow and tap water. The 300 ml of plasma obtained was composed partially of plasma from rats treated with varying amounts of cadmium and partially from untreated rats. This was done to avoid the possibility that ceruloplasmin from cadmium treated rats contained different immunological recognition sites than ceruloplasmin from untreated rats. Some of the plasma had been frozen for several months.

Purification of ceruloplasmin was accomplished basically by the method of Holtzman et al. (67) with a few modifications. Plasma was centrifuged at 15,000 x g at approximately 0°C for 30 minutes for flotate lipids. The resultant lipid layer was drawn off with a pipette. The plasma was then diluted to approximately 1.5 liters with .05 M sodium acetate, pH = 5.8, and applied to a 2 x 56 cm column of DEAE cellulose equilibrated with acetate buffer, at a flow rate of 2 ml per minute. After the sample was on the column, 100 ml of additional buffer were applied, followed by 200 ml of acetate buffer made .1 M in NaCl. Ceruloplasmin was

eluted with a linear sodium chloride gradient[7] in the acetate buffer.

Tubes exhibiting an $A_{610m\mu}$ of .014 or greater were pooled, chilled to 2°-5°C and added to two volumes of 90% ethanol-10% chloroform mixture which had been maintained at approximately -20°C. The mixture was stirred for 30 minutes at 2°-5°C, and then centrifuged at 26,000 x g for 25 minutes at approximately 0°C. The supernatant was discarded and the blue ceruloplasmin pellet in each tube was suspended in .05 M sodium acetate buffer, pH = 5.8, containing .3 M NaCl. The suspension was placed in polycarbonate tubes and centrifuged at 105,000 x g in a Beckman Model L2-65 refrigerated ultracentrifuge for 25 minutes at approximately 0°C. The supernatant of this step contained the ceruloplasmin and was pooled. Purity at this stage, based on the absorbance ratio, was 78%, amounting to 63.8 mg ceruloplasmin.

The pooled ceruloplasmin was dialyzed at 2°-5°C against two 2-liter changes (approximately 10 hours per change) of .05 M phosphate buffer, pH = 6.4 (containing approximately four mg $Cu(Ac)_2$). The dialyzed sample was placed on a 2 x 46 cm column of hydroxylapatite equilibrated with the phosphate buffer. Further washing with 100 ml of phosphate

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[7]Increasing linear gradient was formed by placing 500 ml of .1 M NaCl in the acetate buffer in one vessel and an equal volume of .3 M NaCl in the acetate buffer in an identical vessel.

buffer was followed by elution of ceruloplasmin with a phosphate concentration gradient as indicated previously.[8]

The effluent was monitored at 280mμ and 610mμ absorbance and the ceruloplasmin fractions were pooled and dialyzed at 2°-5°C against two 2-liter changes (approximately 10 hours per change) of .05 M sodium acetate buffer, .05 M NaCl, pH = 5.8 (containing approximately four mg $Cu(Ac)_2$). The ceruloplasmin containing dialysate was applied to a 2 x 20 cm DEAE cellulose column equilibrated at 2°-5°C with the acetate buffer, .05 M in NaCl and eluted in 10 ml by application of .5 M NaCl in the buffer. Purity at the end of the hydroxylapatite step was approximately 81% and approximately 59 mg of ceruloplasmin remained as determined by the summation of absorbances of the individual pooled fractions.

An aliquot of 5.5 ml of the ceruloplasmin solution was applied to a 2 x 90 cm column of Sephadex G-200[9] equilibrated with the acetate buffer, containing .05 M NaCl. Ceruloplasmin was eluted at a flow rate of .5 ml per minute and 5.2 ml fractions were collected. The absorbance of the fractions at 280 mμ and 610 mμ was measured and disc gel electrophoresis[10] was performed on the various fractions of

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[8] 150 ml in each chamber.

[9] Gel filtration media from Pharmacia Fine Chemicals packed with a flow rate of 1.0 ml/minute.

[10] Precise gel recipes, running buffer and electrophoresis procedure given in Appendix I.

the ceruloplasmin peak in an alkaline system described by Gabriel (71).

Results and Discussion

Analysis of each fraction of the Sephadex G-200 chromatography step for purity via the absorbance ratio indicated that the leading edge (high molecular weight side) of the ceruloplasmin peak contained the impurities and the trailing half of the peak was pure. Fractions in the trailing half of the peak indicated a purity of greater than 100% when compared with the reported absorbance ratio (69) of pure rat ceruloplasmin. Electrophoresis revealed that bands of impurity present in the hydroxylapatite fraction were also present in the leading fractions of the ceruloplasmin peak but faint or absent in the trailing half of the peak.

The electrophoresis procedure revealed two major bands in all the fractions of greater than 100% indicated purity. Previous gel permeation column work had at times revealed two peaks of ceruloplasmin activity and both these major bands stained positive for oxidase activity.[11] Others (68) have reported the existence of two species of rat ceruloplasmin and it has also been noted that ceruloplasmin will undergo changes in electrophoretic mobility and stability when exposed to Tris, or phosphate buffer (72). The fact that both bands stain positive for oxidase activity suggest the former explanation. Figure 4 shows two gels run on a

[11] Staining procedures detailed in Appendix I.

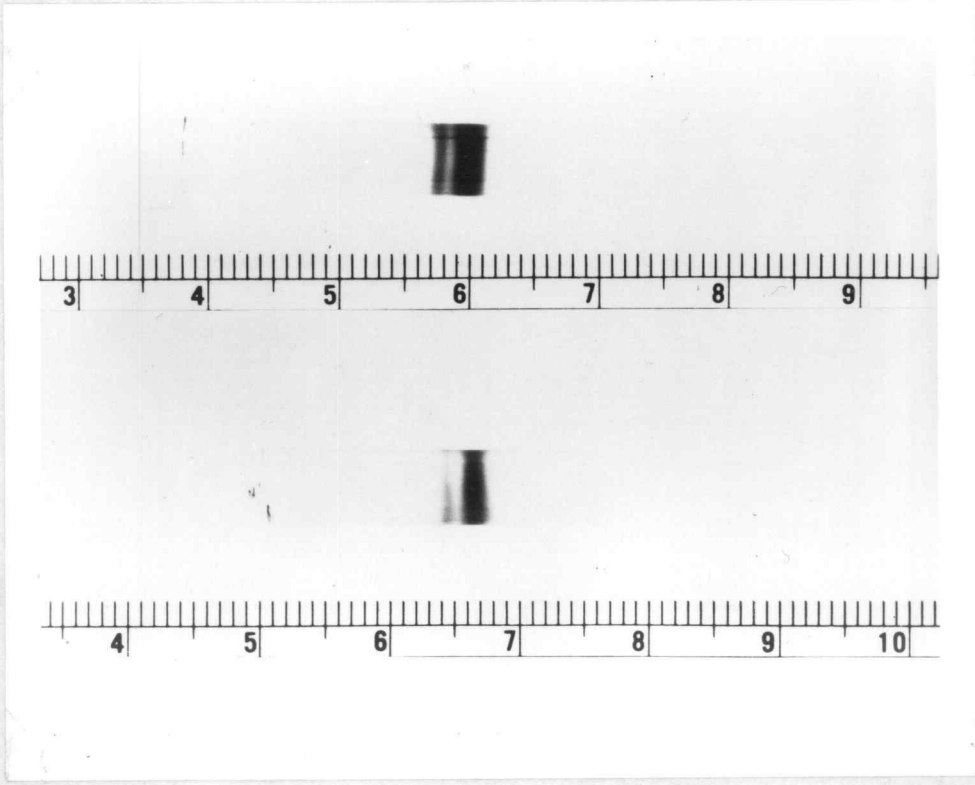

Figure 4. Disc gel electrophoresis of purified rat ceruloplasmin. Gels were stained with Amido black. Upper gel contained approximately 37 µg ceruloplasmin, lower gel contained approximately 17 µg.

fraction of ceruloplasmin of approximately 100% purity as indicated by the absorbance ratio. The gels were stained for protein via Amido black. The double band pattern is also seen when gels are stained for oxidase activity. The closeness of the two bands and the rapidity of photolytic reaction of PPD result in difficulty in viewing two distinct bands with this staining procedure. During the staining procedure, however, two distinct bands could usually be distinguished quite clearly, grading into one thick band upon exposure to light during the destaining procedure. If bands disappear upon destaining in the dark, they will usually reappear readily upon exposure to a simple incandescent light.

On some gels (of fractions indicating greater than 100% purity) faint bands were seen in addition to the two major bands discussed above. In light of the reported effect of Tris buffer in altering mobility, and the qualitative observation that a dilution of Tris running buffer by a factor of ten resulted in a lower percentage of the trailing major band, it was decided that the faint bands were artifacts. It appeared as though recycling Sephadex G-200 chromatography would result in a pure preparation if the fractions exhibiting greater than 100% purity by spectral measurements, were pooled after each run and the impure fractions concentrated and rerun.

The final ceruloplasmin solution possessed a purity of 105% as measured by the absorbance ratio of the individual pooled fractions. After concentration of the pooled fraction utilizing an ultrafiltration cell, there remained 27.8 mg of ceruloplasmin based on its absorbance at 610 mμ.

VI. IMMUNIZATION OF RABBITS AND CHARACTERIZATION OF ANTISERUM

Methodology

The purified ceruloplasmin was shipped to a company[12] who undertook a standard immunization schedule. Three rabbits were each injected in the hind footpads with approximately 2.5 mg ceruloplasmin in Freund's complete adjuvant. Injection occurred on days 0, 14, 21, and rabbits were bled on day 29. The trial bleeding on day 29 was shipped for confirmation and rabbits received a final injection on day 40 with final bleeding occurring on day 45.

Immunoelectrophoresis and immunodiffusion were performed on the antisera to determine specificity. Immunoelectrophoresis was performed on 1 x 3 inch cellulose acetate strips[13] by a method detailed by Millipore Corporation[14] (73). Strips were run in barbital buffer, pH = 8.6, μ = .075, purchased premixed from Millipore. Running voltage was 100 volts for 9-18 minutes. Slides were stained for protein using Ponceau-S, also obtained from Millipore.

Ouchterlony immunodiffusion was performed, using the general method outlined by Campbell *et al.* (74), in commercial plates.[15] Plates were incubated at room temperature in

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[12] Antibodies Incorp., Route 1, Box 1482, Davis, California.

[13] Trade name Immuno-Phoroslide

[14] Bedford, Massachusetts

[15] Obtained from Schwarz Mann, Orangeburg, New York 10962

a humid atmosphere for 24-72 hours. Protein staining was accomplished by soaking plates in saline for approximately a week with changes several times to remove unreacted protein (48-72 hours would probably be sufficient [75]). Plates were stained by immersion in ten percent acetic acid for 5-10 minutes at room temperature. Plates were destained by immersion in ten percent acetic acid for several hours at 50°-60°C followed by a change of acid and additional soaking (overnight worked well). Care must be exercised as the gels are fragile and can be easily broken. Staining and destaining should be carried out with the gel in its plate.

Results and Discussion

Both immunoelectrophoresis and immunodiffusion of the antisera indicated a non-homogeneous antibody. Apparently the faint bands in the purified ceruloplasmin were not artifacts but impurities. The impurities are apparently extremely antigenic, as they could not have been present in more than trace amounts and yet produced considerable antibody. This problem has also been reported by other investigators (76). It would appear as though removal of impurities by antibody reactions (76) or electrophoresis (69) are the only methods by which the highly persistent, antigenic impurities may be removed. Antibody removal requires a rather lengthy preparation and the requirements for mg amounts of ceruloplasmin dictate the use of preparative gel electrophoresis with its host of problems.

The actual results of the methodology employed were very gratifying. Particularly, the immunoelectrophoresis on cellulose acetate strips, as opposed to agar plates, was found to be a superior method with regard to ease of running and the speed of staining and destaining (based on author's reading of agar methods). Upon destaining, a permanent record was obtained by simply air drying the strips, thus circumventing the problems outlined below.

The staining of the Ouchterlony immunodiffusion plates in the hydrated state also appears superior to the more accepted method of drying the agar before staining and destaining. In this investigators hands, drying the plates, even after removal of salts by soaking in distilled water, resulted in curled films which distorted distances and were very brittle and easily broken.

Typical results for both immunodiffusion and immunoelectrophoresis are shown in Figures 5 and 6.

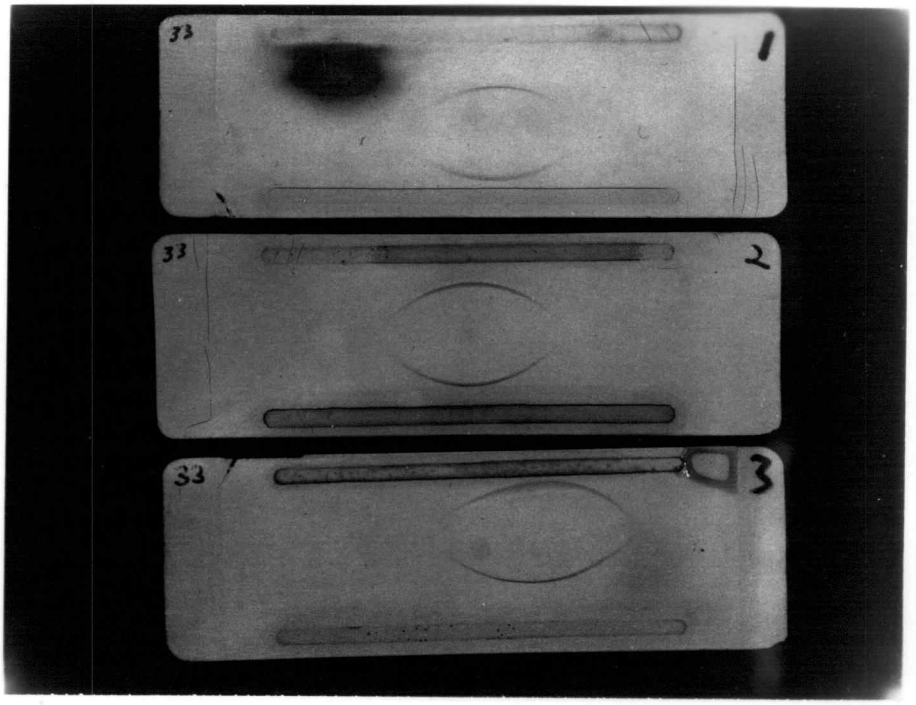

Figure 5. Immunoelectrophoresis with rabbit antiserum to rat ceruloplasmin. Slides 1 and 3 run in barbital buffer, pH = 8.6, for 9-18 minutes. Slide 2 not subjected to voltage. Center well contained purified ceruloplasmin (approximately .8 µl of 1.48 mg/ml in .05 M NaAc buffer, pH = 5.8). Side troughs contained rabbit antiserum to rat ceruloplasmin. Slides were stained with Ponceau S.

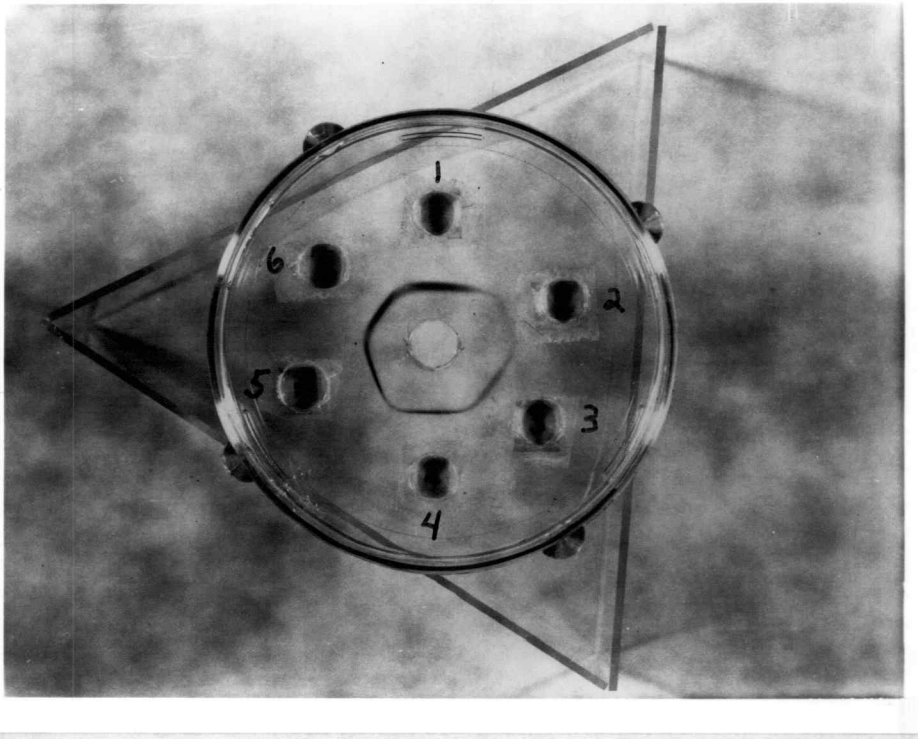

Figure 6. Ouchterlony immunodiffusion with rabbit antiserum to rat ceruloplasmin. Center well contained antiserum diluted 1/2 with saline. Outer wells contained ceruloplasmin in .05 M NaAc buffer, pH = 5.8, in the concentrations below:
1. 1.48 mg/ml
2. .74 mg/ml
3. .37 mg/ml
4. .185 mg/ml
5. .093 mg/ml
6. .046 mg/ml
Slides were stained with Amido black.

VII. PRECIPITIN RESPONSE OF RAT CERULOPLASMIN ANTISERUM PRODUCED IN RABBITS

Methodology

The effects of various pH, volume, and salt changes on the precipitin response of the antiserum were determined. Basic procedures were generally tailored after that used by Holtzman and Gaumnitz (26,69) and Campbell et al. (74).

Regardless of the incubation mixture composition, reactions were generally treated as below.

Tubes were incubated for one hour at 37°C followed by incubation at 2°-5°C for 48 hours. At the end of the incubation period the tubes were centrifuged at 2000 x g for 30 minutes at approximately 0°C in a swinging bucket Sorval, model RC 2-B, centrifuge. The supernatant was decanted into clean glass tubes for subsequent ring tests (ring test procedure below).

Rinsing of the precipitate was accomplished by adding approximately .5 ml .01 M phosphate buffer, pH approximately 7.2 and touching the tube to a vortex mixer to disperse the precipitate. Additional phosphate buffer was now added (approximately two to three ml). Precipitates were again centrifuged for 30 minutes as before. The supernatant was decanted and discarded following centrifugation and the rinsing procedure repeated two more times. All rinsing procedures were carried out at 0°-5°C.

After the last centrifugation and decantation the tubes were inverted and allowed to drain. The drained digests were now dissolved in one ml of 3% sodium hydroxide. The A_{280} of the protein digest was obtained on a spectrophotometer to establish the point of maximal precipitation.

To establish the zones of antibody and antigen excess, ring tests were performed, as described by Campbell et al. (74), on the original incubation supernatant. Antibody excess was determined by layering a solution of ceruloplasmin over the incubation supernatant. The region of antigen excess was ascertained by layering incubation supernatant over antiserum. The appearance of a ring of precipitate at the interface of the two solutions, within two hours of layering, is regarded as a positive test.

Tests were conducted in tubes approximately 2 x 50 mm fashioned from soft glass tubing. Tubing was washed with detergent and chromic acid cleaning solution prior to sealing of one end over flame. Twenty microliters of each solution were sufficient for each test.

To assist in visualizing the ring of precipitate it is necessary to have a light and a black background. A simple apparatus may be constructed by affixing a piece of carbon paper to any upright holder and positioning a fluorescent light to shine vertically down the face of the carbon sheet. The simple plexiglass holder detailed in Appendix II will assist in handling the small tubes and when held up to the

light and black background, the rings indicating a positive test are easily visualized.

Precipitin curves were obtained by setting up a series of tubes containing dilutions of ceruloplasmin from 1 to 1/128 or higher. To each tube was added the same amount of antiserum plus other ingredients. Each tube was treated as detailed above. The A_{280} values define the precipitin curve and point of maximal protein precipitation, while the ring tests define the regions of antibody excess, equivalence and antigen excess.

To evaluate the effect of pH on these responses, the following incubation mixtures were used.

For pH = 5.8, each tube received:

1. Two-tenths ml of suitable dilution of ceruloplasmin in .05 M NaAc buffer, pH = 5.8.
2. Two-tenths ml of a mixture of one part antiserum + one part .05 M NaAc buffer, .45 M NaCl, .04 M EDTA pH = 5.8-6.0.

Final incubation mixture was .01 M EDTA, .15 M NaCl, pH approximately 5.8.

For pH = 7.0, each tube received:

1. Two-tenths ml of suitable ceruloplasmin dilution in .05 M NaAc buffer, pH = 5.8.
2. Two-tenths ml of a mixture of one part antiserum + one part .02 M phosphate buffer, .45 M NaCl, .04 M EDTA, pH = 8.0.

Final incubation mixture was .01 M EDTA, .15 M NaCl, pH approximately 7.0.

For pH = 8.0, each tube received:

1. Two-tenths ml of suitable ceruloplasmin dilution in .05 M NaAc buffer, pH = 5.8.
2. Two-tenths ml of a mixture of one part antiserum + one part Borate-saline buffer[16] (74), pH = 8.3-8.5, .45 M NaCl.

Final incubation mixture was .15 M NaCl, pH approximately 8.0.

To observe the effects of dilution on the precipitin response, four tubes were prepared with 37 µg ceruloplasmin in .2 ml .05 M NaAc buffer, pH = 5.8, and to three of these tubes were added 1, 2, or 3 ml of a solution of .01 M EDTA, .05 M NaAc, .15 M NaCl, pH = 6.0. To the other tube was added .1 ml of .04 M EDTA, .45 M NaCl in .05 M NaAc, pH = 6.0. In each tube .1 ml antiserum was placed and incubated, washed, digested and the absorbance at 280 mµ determined as previously described. Ring tests were performed on the supernatants as previously described.

The effect of varying salt concentration on the precipitin reaction was investigated by the following procedure. To nine tubes were added 1.0 ml of .04 M EDTA, .04 M

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[16] Borate-saline buffer prepared by mixing 95 parts saline with five parts borate buffer (6.184 g boric acid, 9.86 g Borax [sodium tetraborate], 4.384 g sodium chloride, diluted to one liter with distilled H_2O).

phosphate buffer pH = 7.2, X M NaCl (values of X given below) plus 2.0 ml of .05 M NaAc buffer, X M NaCl, pH = 5.8, containing 50 µg ceruloplasmin, plus .75 ml of distilled water, X M NaCl plus .25 ml of antiserum. The final incubation mixture was .01 M EDTA, .01 M phosphate, .94X M in NaCl, .025 M NaAc and contained 50 mg ceruloplasmin and .25 ml of antiserum. The molarities of NaCl (X) were 0, .15, and .30. These concentrations and volumes were chosen because it was anticipated that in future experiments the ceruloplasmin to be precipitated would be the effluent from a DEAE column. The ceruloplasmin fraction would have a volume of approximately 2.0 ml in .05 M NaAc buffer, pH = 5.8, of unknown salt concentration between 0 and .3 M. Three sets of triplicate concentrations were run. Tubes were incubated, centrifuged, washed, read at 280 mµ and ring tests were performed as before. Purified ceruloplasmin was used in all procedures.

Results and Discussion

Tables 1, 2 and 3 detail the results of pH changes on both the point of maximal precipitation and the equivalence point.

As can be seen, there is a shift in the relation between the point of maximal precipitation and the point of equivalence (as evidenced by the lack of either antibody or antigen excess). The equivalence point occurs at the same

Table 1. Precipitin response, at pH = 5.8, of rat ceruloplasmin (C.P.) antiserum produced in rabbits. Each tube contained .1 ml antiserum.

Tube #	µg C.P. per Tube	Abs. at 280 mµ	Ring Tests	
			Antibody Excess	Antigen Excess
1	296	.045	-	+
2	148	.143	-	+
3	74	.475	-	+
4	37	.363	-	-
5	18.5	.375	+	-
6	9.3	.163	+	-
7	4.6	.083	+	-

Table 2. Precipitin response, at pH = 7.0, of rat ceruloplasmin (C.P.) antiserum produced in rabbits. Each tube contained .1 ml antiserum.

Tube #	µg C.P. per Tube	Abs. at 280 mµ	Ring Tests	
			Antibody Excess	Antigen Excess
1	296	.018	-	+
2	148	.149	-	+
3	74	.417	-	+
4	37	.609	-	-
5	18.5	.359	+	-
6	9.3	.200	+	-
7	4.6	.108	+	-
8	2.3	.050	+	-

point in both pH = 5.8 and pH = 7.0 groups. In the pH = 8.0 group (Table 3) two tubes (four and five) indicate equivalence. This may be an effect of pH or it may be a result of the difference in incubation medium. In any event, it would appear that even though conditions of pH have changed a

Table 3. Precipitin response, at pH = 8.0, of rat ceruloplasmin (C.P.) antiserum produced in rabbits. Each tube contained .1 ml antiserum.

Tube #	µg C.P. per Tube	Abs. at 280 mµ	Ring Tests	
			Antibody Excess	Antigen Excess
1	296	.058	-	+
2	148	.329	-	+
3	74	.582	-	+
4	37	.615	-	-
5	18.5	.390	-	-
6	9.3	.198	+	-
7	4.6	.117	+	-
8	2.3	.040	+	-

great deal, the aspect of interest, i.e. the equivalence point, has not shifted.

The effect of dilution on the equivalence point and the amount of protein precipitated is shown in Table 4.

Table 4. Effect of dilution on precipitin response of rat ceruloplasmin antiserum produced in rabbits. Each tube contained 37 µg ceruloplasmin and .1 ml antiserum.

Tube #	Volume of Incubation Mixture	Abs. at 280 mµ	Ring Tests	
			Antigen Excess	Antibody Excess
1	.4	.500	-	-
2	1.3	.504	-	-
3	2.3	.435	-	-
4	3.3	.383	-	-

As inspection indicates, a definite decrease in the amount of protein precipitated was obtained as the volume increased. However, equivalence is maintained in all cases.

The effect of changing salt concentration on the precipitin response is shown in Table 5.

Table 5. Effect of NaCl concentration on the precipitin response of rat ceruloplasmin antiserum produced in rabbits. Each tube contained 50 µg ceruloplasmin and .25 ml antiserum.

Tube #	Molarity of Added NaCl (X)	Abs. at 280 m
1	.0	.511
2	.0	.515
3	.0	.510
4	.15	.514
5	.15	.512
6	.15	.511
7	.30	.516
8	.30	.510
9	.30	.514

All tubes were found to be in the region of antibody excess. Inspection of the results reveals no significant change in the amount of precipitated protein obtained.

The results of this section indicate that a simple procedure whereby the effluent from a small DEAE column may be used directly in a precipitin reaction without undue manipulation of volume, pH, or salt concentration, would still result in quantitative precipitation of the ceruloplasmin from solution. Rather drastic volume changes are needed to effect even a change in the amount of total protein precipitated.

Due to the fact that the antiserum obtained was heterogeneous, previously planned experiments utilizing the procedures developed in Chapters IV through VII to determine the effect of cadmium injection on ceruloplasmin protein synthesis and decay were seriously compromised. Unexpected mortality among the experimental animals also hampered interpretation of the results obtained. The procedures and results of these experiments are given in Appendices III and IV.

VIII. GENERAL DISCUSSION AND CONCLUSIONS

In light of the data gathered here, it would appear as though all hypotheses except that cadmium binds to ceruloplasmin after synthesis should be discarded. The data presented are an indication of *in vivo* binding of cadmium to ceruloplasmin. It is not conclusive proof because the Sephadex G-200 ceruloplasmin peak is not 100% pure and it is conceivable, although not likely, that the cadmium present is bound to an impurity.

The question of a cause-effect relationship between observed binding and observed inhibition of ceruloplasmin by cadmium is certainly not answered by this study. If we assumed a cause-effect relationship, the important question of how this inhibition of oxidase activity occurs is still unanswered. The most commonly proposed mechanism for cadmium induced enzymatic inhibition is a binding of free sulfhydryl groups necessary for enzymatic activity (37). This mechanism is unlikely for a number of reasons. Whanger and Weswig (24) have shown that dietary mercury does not inhibit ceruloplasmin enzymatic activity and mercury forms a much stronger sulfide bond than does cadmium (11). It has been shown (77,78) that there is only one easily accessible sulfhydryl group in ceruloplasmin and its blockage with sulfhydryl reagents does not decrease PPD oxidase activity (77).

It has also been shown (77) that treatment with 7.0 M urea in the presence of EDTA resulted in exposure of three additional sulfhydryl groups. It was hypothesized that the three "masked" sulfhydryl groups are involved in the binding of copper in the ceruloplasmin molecule (77,78). It has recently been shown that histidine residues are almost certainly involved in the binding of copper to ceruloplasmin (79,80). The interaction of cadmium with these ligands is known to occur (9,11,39,81) and one may envision the replacement of copper with cadmium, resulting in an enzymatically altered protein. The fact that in vitro exchange of cadmium-109 with copper or in vitro binding did not occur in this experiment may imply that the incorporation of copper is an enzymatic process or may merely be the result of not attaining the precise conditions of pH, form of cadmium, temperature, and/or state of the enzyme, i.e., resting or active. Support for the latter is found in work by Pfordte and Roschig (82) who reported in vitro binding of cadmium to α_2-globulins. The major differences in conditions of incubation between this study and these workers was that incubation of cadmium was carried out with plasma at 37°C and tracer doses were not used. It has been reported (83) that Zn^{2+} ions bind to ceruloplasmin and competitively inhibit Fe^{2+} oxidase activity. It is important to note, however, that others report a stimulation of N,N-dimethyl-PPD and PPD oxidase activity by zinc, and cadmium has been shown to

exhibit a slight activation of iron oxidase activity (66). The existence of separate sites responsible for PPD oxidation and Fe^{2+} oxidation would assist in explaining these apparent paradoxes, but evidence mitigates against this explanation (66,83,84). It seems clear, in light of the above results and the present work, that simple in vitro binding of cadmium to ceruloplasmin is not the cause of decreased PPD oxidase activity. Further evidence that precise conditions are important is found in a study reporting the exchange of copper-64 with native ceruloplasmin copper occurred only if the incubation mixture contained ascorbic acid, oxygen, and a pH of 5-6 (85).

An exchange of ceruloplasmin copper with cadmium is an attractive hypothesis from the standpoint of the rate of decay of enzymatic activity following cadmium injection. This becomes clear if one considers the appearance of cadmium in the plasma and various organs in light of the decay of enzymatic activity. The maximum level of cadmium in the plasma occurs at eight minutes following injection while the level of cadmium tends to accumulate more slowly in other tissues and organs. Cadmium attains maximum concentration in the liver two to five hours following injection and remains virtually constant over the duration of the experiment (28.5 hours maximum) (5,86). Certainly the decay of ceruloplasmin activity more nearly follows this time sequence of events than simply that of the concentration of cadmium in the

plasma or blood. When the hypothesis that ceruloplasmin acts as a copper donor to tissues is considered (29), substitution could conceivably occur at the point of copper donation or when the molecule is in an active state.

In conclusion, therefore, cadmium appears to depress ceruloplasmin oxidase levels too quickly to interact via the pathway of impaired synthesis (tentative confirmation of this is given in Appendix III). The rate of ceruloplasmin decay also does not appear to be enhanced by the injection of cadmium (see Appendix IV). Cadmium does bind to ceruloplasmin *in vivo*, and this may be responsible for the observed decrease in PPD oxidase activity. Further experimentation is necessary to determine whether cadmium displaces copper in ceruloplasmin or binds at another site.

BIBLIOGRAPHY

1. Nordberg, G. F. Cadmium metabolism and toxicity. Environmental Physiology and Biochemistry 2:7-36. 1972.

2. Schroeder, H. A. Cadmium as a factor in hypertension. Journal of Chronic Diseases 18:647-656. 1965.

3. Carroll, R. E. The relationship of cadmium in the air to cardiovascular disease death rates. Journal of the American Medical Association 198(3):267-269. 1966.

4. Schroeder, H. A. Cadmium, chromium and cardiovascular disease. Circulation 35:570-582. 1967.

5. Friberg, L., M. Piscator, and G. Nordberg. Cadmium in the environment, Cleveland, Chemical Rubber Company Press, 1971, 166 p.

6. Albert, R. et al. Accumulation of toxic metals with special reference to their absorption, excretion and biological halftimes. Environmental Physiology and Biochemistry 3:65-107. 1973.

7. Schroeder, H. A. A sensible look at air pollution by metals. Archives of Environmental Health 21:798-806. 1970.

8. Flick, D. F., H. F. Kraybill, and J. M. Dimitroff. Toxic effects of cadmium: A review. Environmental Research 4:71-85. 1971.

9. Vallee, B. L., and D. D. Ulmer. Biochemical effects of mercury, cadmium and lead. Annual Review of Biochemistry 41:91-128. 1972.

10. McCaull, J. Building a shorter life. Environment 13(9):3-41. 1971.

11. Baes, C. F., Jr. Properties of cadmium. In: Cadmium the dissipated element, eds. W. Fulkerson, H. E. Goeller. Oak Ridge National Laboratory, 1973. pp. 29-54. (Contract no. w. 7405-eng-26, supported by National Science Foundation, ORNL NSF-EP-21)

12. Sekine, H. Neutral proteinases I and II of Aspergillus sojae. Some enzymatic properties. Agricultural and Biological Chemistry 36:207-216. 1972.

13. Klee, C. B. Metal activation of histidine ammonia-lyase. Metal ion sulfhydry group relationship. Journal of Biological Chemistry 247:1398-1406. 1972.

14. Ozawa, T., S. Saito, and I. Tomita. The coenzymic activities of 2 substituted thiamine derivatives: III. The activation of apotransketolase with various divalent cations. Vitamins (KYOTO) 44(6):308-312. 1972. (Abstracted in Biological Abstracts 54(6):no. 33828. 1972.)

15. Perttula, T. V. Aminopeptidases of rat testes. II. Effects of puberty, cryptorchidism and cadmium chloride treatment. Journal of Reproduction and Fertility 32: 45-53. 1973.

16. Perttula, T. V. and V. Nikkanen. Acid phosphatases of the rat testis in experimental conditions. Acta Endocrinologica 72(2):376-390. 1973.

17. Ogawa, E. et al. Basal study on early diagnosis of cadmium poisoning: Changes in carbonic anhydrase activity. Japanese Journal of Pharmacology 23:97-105. 1973.

18. Chank, P. and Y. Plancade. Etude comparee des effects du zinc et du cadmium sur les activtes cholinesterasiques tissulaires du rat. Biochemical Pharmacology 20(4):729-734. 1971. (Abstracted in Biological Abstracts 52:no. 101236. 1971.)

19. Landolt, R., and E. E. Gutwein. Alpha-particle autoradiography of alkaline phosphatase inhibition by cadmium. International Journal of Applied Radiation and Isotopes 22:127-128. 1971.

20. Zisapel, N., and M. Sokolovsky. Metallocarboxypeptidases: A cadmium-carboxypeptidase B with peptidase activity. Biochemistry and Biophysics Research Communications 53:722-729. 1973.

21. Kench, J. E. and P.J.D. Gubb. The activity of certain enzymes in cadmium poisoned chicks. Biochemical Journal 120:27P. 1970.

22. Harris, M. I., and J. E. Coleman. The biosynthesis of apo and metalloalkaline phosphatases of Eschericia coli. Journal of Biological Chemistry 243:5063-5073. 1968.

23. Sporn, A., I. Dinu, and L. Stoenescu. Influence of cadmium administration on carbohydrate and cellular energetic metabolism in the rat liver. Revue Roumaine de Biochimie 7:299-305. 1970.

24. Whanger, P. D., and P. H. Weswig. Effect of some copper antagonists on induction of ceruloplasmin in the rat. Journal of Nutrition 100:341-348. 1970.

25. Murthy, L., J. R. J. Sorenson, and H. G. Petering. Effect of cadmium on ceruloplasmin (copper oxidase) activity in rats. Presented at 56th Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, New Jersey, 1972. (Abstracted in Federation Proceedings 31:699 no. 2726. 1972.)

26. Holtzman, N. A., and B. M. Gaumnitz. Studies on the rate of release and turnover of ceruloplasmin and apoceruloplasmin in rat plasma. Journal of Biological Chemistry 245:2354-2358. 1970.

27. Holmberg, C. G., and C. B. Laurell. Investigations in serum copper. II. Isolation of the copper containing protein and a description of some of its properties. Acta Chemica Scandinavica 2:550-556. 1948.

28. Seal, U. S. Vertebrate distribution of serum ceruloplasmin and sialic acid and the effects of pregnancy. Comparative Biochemistry and Physiology 13:143-159. 1964.

29. Owen, C. A. Metabolism of radiocopper (Cu^{64}) in the rat. American Journal of Physiology 209:900-904. 1965.

30. Scheinberg, H. S. Ceruloplasmin, a review. In: The biochemistry of copper, eds. J. Peisach, P. Aisen, and W. E. Blumberg, New York, Academic Press 1966. p. 513-524.

31. Frieden, E. Ceruloplasmin, a link between copper and iron metabolism. Nutrition Reviews 28:87-91. 1970.

32. Osaki, S., D. A. Johnson and E. Frieden. The possible significance of the ferrous oxidase activity of ceruloplasmin in normal human serum. Journal of Biological Chemistry 241:2746-2751. 1966.

33. Topham, R. W., and E. Frieden. Identification and purification of a non-ceruloplasmin ferroxidase of human serum. Journal of Biological Chemistry 245:6698-6705. 1970.

34. Shokeir, M. H. K. Is ceruloplasmin a physiological ferroxidase? Clinical Biochemistry 5(2):115-120. 1972. (Abstracted in Chemical Abstracts 77(15):no. 99302Z. 1972.)

35. Evans, J. L., and P. A. Abraham. Anemia, iron storage and ceruloplasmin in copper nutrition in the growing rat. Journal of Nutrition 103:196-201. 1973.

36. Hampton, J. K. et al. The histaminase activity of ceruloplasmin. Proceedings of the Society for Experimental Biology and Medicine 141:974-977. 1972.

37. Vallee, B. L., and W. E. C. Wacker. The proteins. Metalloproteins. 2nd ed., vol. 5, New York, Academic Press. 1970. 192 p.

38. Wacker, W. E. C. Metalloenzymes. Federation Proceedings 29:1462-1468. 1970.

39. Vallee, B. L., and R. J. P. Williams. Enzyme action: Views derived from metalloenzyme studies. Chemistry in Britain 4:397-402. 1968.

40. Hughes, M. N. The inorganic chemistry of biological processes. London, John Wiley and Sons, 1972. 304 p.

41. Jacobs, E. E. et al. Uncoupling of oxidative phosphorylation by cadmium ion. Journal of Biological Chemistry 223:147-156. 1956.

42. Brierley, G. P. Ion transport by heart mitochondria. II. Activation of the energy linked accumulation of Mg^{++} by Zn^{++} and other cations. Journal of Biological Chemistry 242:1115-1122. 1967.

43. Brierley, G. P., and C. T. Settlemire. Ion transport by heart mitochondria. IX. Induction of the energy linked uptake of K^{+} by zinc ions. Journal of Biological Chemistry 242:4324-4328. 1967.

44. Cross, C. E. et al. Effect of cadmium ion on respiration and ATP'ase activity of pulmonary alveolar macrophage: A model for the study of environmental interference with pulmonary cell function. Environmental Research 3:512-520. 1970.

45. Mustafa, M. G., and C. E. Cross. Pulmonary alveolar macrophage. Oxidative metabolism of isolated cells and mitochondria and effect of cadmium ion on electron- and energy-transfer reactions. Biochemistry 10:4176-4185. 1971.

46. Eichorn, G. L., and Y. A. Shin. Interaction of metal ions with polynucleotides and related compounds. XII. The relative effect of various metal ions on DNA helicity. Journal of the American Chemical Society 90: 7323-7328. 1968.

47. Sundaralingharm, M., and J. A. Carrabine. Stereochemistry of nucleic acids and their constituents. XIX. Copper binding sites and mechanism of G·C selective denaturation of DNA. Crystal and molecular structures of guanine·copper II·chloride and cytosine·copper II· chloride complexes. Journal of Molecular Biology 61: 287-309. 1971.

48. Hadwiger, L. A., S. von Broembsen, and R. Eddy, Jr. Increased template activity in chromatin from cadmium chloride treated pea tissues. Biochemistry and Biophysics Research Communications 50:1120-1128. 1973.

49. Weser, U., G. Donay, and H. Rupp. Cadmium-induced synthesis of hepatic metallothionein in chicken and rats. Federation of European Biochemical Societies (FEBS) Letters 32:171-174. 1973.

50. Winge, D. R., and K. V. Rajagopalan. Purification and some properties of Cd-binding protein from rat liver. Archives of Biochemistry and Biophysics 153:755-762. 1972.

51. Lawford, D. J. An abnormal serum component in experimental poisoning by cadmium and other metals. Biochemical Pharmacology 7:109-122. 1961.

52. Axelson, B., and M. Piscator. Serum proteins in cadmium poisoned rabbits. Archives of Environmental Health 12:374-381. 1966.

53. Piscator, M., and B. Axelson. Serum proteins and kidney function after exposure to cadmium. Archives of Environmental Health 21:604-608. 1970.

54. Belmonte, A. A. et al. Biological implications of cadmium-phospholipid monolayers. Lipids 7:490-492. 1972.

55. Nishizumi, M. Electron microscopic study of cadmium nephrotoxicity in the rat. Archives of Environmental Health 24:215-225. 1972.

56. Castano, P. Chronic intoxication by cadmium, experimentally induced in rabbits: A study of kidney ultrastructure. Pathologica et Microbiologica 37:280-301. 1971.

57. Van Campen, D. R. Effects of zinc, cadmium, silver and mercury on the absorption and distribution of copper-64 in rats. Journal of Nutrition 88:125-130. 1966.

58. Whanger, P. D. Effect of dietary cadmium on intracellular distribution of hepatic iron in rats. Research Communications in Chemical Pathology and Pharmacology 5:733-739. 1973.

59. Hill, C. H. et al. In vivo interactions of cadmium with copper, zinc and iron. Journal of Nutrition 80: 227-235. 1963.

60. Houchin, O. B. A rapid colorimetric method for the quantitative determination of copper oxidase activity (ceruloplasmin). Clinical Chemistry 4:519-523. 1958.

61. Rice, E. W. Standardization of ceruloplasmin activity in terms of international enzyme units. Analytical Biochemistry 3:452-456. 1962.

62. Henry, R. J. et al. Determination of ceruloplasmin oxidase in serum. Proceedings of the Society for Experimental Biology and Medicine 104:620-624. 1960.

63. Whanger, P. D., and P. H. Weswig. Effect of age, stress, and ascorbic acid on copper levels in rats. Unpublished research, Corvallis, Oregon. Agricultural Chemistry Dept., 1972.

64. Evans, G. W., D. R. Myron, and R. E. Wiederanders. Effect of protein synthesis inhibitors on plasma ceruloplasmin in the rat. American Journal of Physiology 216:340-342. 1969.

65. Shaikh, Z. A., and O. J. Lucis. Biological difference in cadmium and zinc turnover. Archives of Environmental Health 24:410-418. 1972.

66. Huber, T. C., and E. Frieden. Substrate activation and the kinetics of ferroxidase. Journal of Biological Chemistry 245:3973-3978. 1970.

67. Holtzman, N. A. et al. Ceruloplasmin in Wilson's disease. Journal of Clinical Investigation 46:993-1002. 1967.

68. Vasilets, I. M., M. M. Shavlovskii, and G. V. Mukba. Physicochemical properties and heterogeneity of albino rat ceruloplasmin. Biochemistry 35:986-992. 1970. (Translated from Biokhimiya.)

69. Holtzman, N. A., and B. M. Gaumnitz. Identification of an apoceruloplasmin-like substance in the plasma of copper deficient rats. Journal of Biological Chemistry 245:2350-2353. 1970.

70. Osaki, S., and O. Walaas. Kinetic studies of ferrous ion oxidation with crystalline human ferroxidase. II. Rate constants at various steps and formation of a possible enzyme-substrate complex. The Journal of Biological Chemistry 242:2653-2657. 1967.

71. Gabriel, O. Analytical disc gel electrophoresis. In: Methods in enzymology. vol. XXII. Enzyme purification and related techniques, ed. W. B. Jakoby, New York, Academic Press, 1971. p. 565-578.

72. Kasper, C. B., and H. F. Deutsch. Physicochemical studies of human ceruloplasmin. Journal of Biological Chemistry 238:2325-2337. 1963.

73. Millipore Corporation. The millipore immuno-phoroslide system. Bedford, Massachusetts. 1969. (Bulletin IEP, catalog no. LTPB 068 BA.)

74. Campbell, D. H. et al. Methods in immunology. 2nd ed. New York, W. A. Benjamin, 1970. p. 235-267.

75. Carrico, R. J. et al. Some properties of an apoceruloplasmin-like protein in human serum. The Journal of Biological Chemistry 244:4141-4146. 1969.

76. Kasper, C. B., and H. F. Deutsch. Immunochemical studies of crystalline human ceruloplasmin and derivatives. Journal of Biological Chemistry 238:2343-2350. 1970.

77. Witwicki, J., and K. Zakrzewski. Sulfhydryl groups in human ceruloplasmin. European Journal of Biochemistry 10:284-290. 1969.

78. Erickson, J. O., R. D. Gray, and E. Frieden. Sulfhydryl groups in the reconstitution of ceruloplasmin. Proceedings of the Society for Experimental Biology and Medicine 134:117-121. 1970.

79. Shavlovskii, M. M., and I. M. Vasilets. Binding of copper in the catalytic center of ceruloplasmin. Photooxidation of human ceruloplasmin. Biochemistry 37:414-420. 1972. (Translated from Biokhimia.)

80. Nylen, U., and G. Petterson. The effect of diethylpyrocarbonate on some physical and chemical properties of ceruloplasmin. European Journal of Biochemistry 27:578-584. 1972.

81. Perkins, D. J. Studies on the interaction of zinc, cadmium and mercuric ions with native and chemically modified human serum albumin. Biochemical Journal 80: 668-672. 1961.

82. Pfordte, K., and M. Roschig. Uber die cadmium-serum-proteinbindung. (Serum protein-cadmium binding). Revue Roumaine de Biochimie 9(4):317-320. 1972. (Abstracted in Biological Abstracts 56(8):no. 41338. 1973.)

83. McKee, D. J., and E. Frieden. Binding of transition metals by ceruloplasmin (ferroxidase). Biochemistry 10:3880-3883. 1971.

84. Osaki, S. Kinetic studies of ferrous ion oxidation with crystalline human ferroxidase (ceruloplasmin). Journal of Biological Chemistry 241:5053-5059. 1966.

85. Scheinberg, I. H., and A. G. Morell. Exchange of ceruloplasmin copper with ionic copper-64 with reference to Wilson's disease. Journal of Clinical Investigation 36:1193-1201. 1957.

86. Perry, H. M., Jr., and M. Erlanger. Hypertension and tissue metal levels after intraperitoneal cadmium, mercury, and zinc. American Journal of Physiology 220:808-811. 1971.

87. Kalbhen, D. A. Chemilumínescence as a problem in liquid scintillation counting. In: The current status of liquid scintillation counting. ed. E. D. Bransome, Jr., New York, Grune and Stratton, 1970. p. 337-341.

88. Lenten, L. V., and G. Ashwell. Studies on the chemical modification of glycoproteins. A general method for the tritiation of sialic acid-containing glycoproteins. Journal of Biological Chemistry 242:1889-1894. 1971.

89. Hamer, V. D. et al. Physical and chemical studies on ceruloplasmin. IX. The role of galactosyl residues in the clearance of ceruloplasmin from the circulation. Journal of Biological Chemistry 245:4397-4402. 1970.

90. Curzon, G., and J. N. Cumings. Some Interactions of Ceruloplasmin with Inhibitors. In: The biochemistry of copper. eds. J. Peisach, P. Aisen, and W. E. Blumberg, New York, Academic Press. 1966. p. 545-557.

91. Levine, W. G., and J. Peisach. Ethylenediaminetetraacetate, iron, and ceruloplasmin activity. Biochimica et Biophysica Acta 77:602-614. 1963.

92. Evans, G. W., N. F. Cornatzer, and W. E. Cornatzer. Mechanism for hormone-induced alterations in serum ceruloplasmin. American Journal of Physiology 218: 613-615. 1970.

93. Starcher, B., and C. H. Hill. Hormonal induction of ceruloplasmin in chicken serum. Comparative Biochemistry and Physiology 15:429-434. 1965.

94. Sunderman, F. W., Jr., et al. Effect of estrogen administration upon ceruloplasmin and copper concentrations in rat serum. Toxicology and Applied Pharmacology 20:588-598. 1971.

APPENDICES

APPENDIX I

Electrophoresis Recipes and Procedures

Recipes

Stock solutions for 7.5% acrylamide separating gel, and large pore stacking gel. Each solution (A through E) diluted to 100 ml with double distilled water. Recipes modeled after those of Gabriel (71).

A	1.0 N HCl	48.0 ml
	Tris[17]	36.3 grams
	TEMED[18]	.10 ml
B	Acrylamide[19]	30.0 grams
	Bisacrylamide[20]	.735 grams
C	Ammonium persulfate[19]	.14 grams

Working solution for 7.5% separating gel: one part A + one part B + two parts C.

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[17] Tris (hydroxymethyl) aminomethane obtained from Sigma Chemical Company. Trade name Trizma Base.

[18] N,N,N',N'-tetramethylenediamine. Obtained from Bio-Rad Laboratories, Richmond, California.

[19] Obtained from Bio-Rad Laboratories.

[20] N,N'-methylenebisacrylamide obtained from Bio-Rad Laboratories.

D	Tris	5.98 grams
	TEMED	.10 ml
	1.0 N HCl	48.0 ml
E	Acrylamide	10.0 grams
	Bisacrylamide	2.5 grams

Working solution for stacking gel: one part D + two parts E + four parts C + one part H_2O.

Running buffer: Tris 2.0 grams, Glycine 14.4 grams, dilute to 1000 ml with H_2O.

Procedure for Pouring Gels

Glass gel-electrophoresis tubes (5 x 120 mm) were washed with detergent, rinsed with distilled water and boiled in concentrated nitric acid. Tubes were then rinsed extensively with distilled water and allowed to air dry. Dried tubes were immersed in a 1/200 dilution of Kodak Photoflo 600[21] in water and allowed to drain dry. Parafilm squares were placed over one end of the tube to form a water tight seal, and the tubes placed in a rack in a vertical position with the sealed end down.

Stock solutions A through E were warmed to room temperature and degassed under aspirator vacuum for around 30 minutes. Separation gel was mixed as indicated above and gently agitated to mix components without undue introduction

[21]Eastman Kodak Company.

of oxygen into the solution. An amount of mixed solution necessary to form a gel of the desired height was introduced into the electrophoresis tube and then quickly overlaid with a three to four mm layer of degassed water. Total time between mixing of solutions and overlaying should not exceed ten minutes, as a meniscus may form as the gel polymerizes. Gelation was complete in 30 minutes and at that time the water layer was removed by aspiration with a syringe (or by inversion of tubes). The area above the solidified gel should be washed two times with stacking gel solution prepared as above but with the persulfate (solution C) left out. This will prevent the formation of what appears to be swirls of inhomogeneity due to rising water in the stacking gel. While these inhomogeneities are disturbing, no effect could be ascertained. Following the second rinse, the stacking gel was mixed, poured and layered in exactly the same manner as the separating gel. Polymerization was complete within 20-30 minutes and was easily noted because the stacking gel became opaque upon gelation. Gels should be used within one hour of gelation (71), although no ill effect was noted if greater than one hour elapsed.

The sample[22] was placed in .3-.5 ml Tris-Glycine running buffer that was 15% in sucrose to facilitate subsequent layering. Tris-Glycine running buffer (minus sucrose) was now layered above the sample until the tube was full.

[22]Usually 1 to 50 µl.

The upper reservoir was filled with running buffer (lower reservoir had been previously filled) and a few drops of one percent bromophenol blue in ethanol were added to the upper reservoir and mixed to serve as a tracking dye. Current flow was now initiated with the cathode being the upper reservoir and the anode the lower reservoir. Current was maintained at one milliampere per tube for the first 90 minutes or until the tracking dye front entered the separating gel and then increased to two milliamperes per tube for the remainder of the run (usually one to three hours).

At the end of a run gels were quickly rimmed with ten percent glycerol in water and extruded, gels were stained as detailed below.

Staining Procedures

Protein staining was accomplished with .5% Amido black in ten percent acetic acid. Gels were placed in this solution for one hour, then removed and placed in ten percent acetic acid and soaked overnight at 60°C-75°C with a change of acetic acid wash and further soaking as needed.

Ceruloplasmin oxidase staining was accomplished by immersing gels in one percent PPD in ceruloplasmin assay buffer for one hour at 36°-39°C. Either recrystallized or crude PPD may be used although recrystallized seemed to give less nonspecific background staining. Destaining was accomplished by soaking gel in ceruloplasmin assay buffer until

the bands became visible or until virtually all color disappeared. Both staining and destaining were carried out in the dark as much as possible to minimize spontaneous oxidation of PPD.

Ceruloplasmin oxidase staining was also carried out as above at room temperature on Ouchterlony plates and seemed to work well, therefore temperature would not appear to be critical.

APPENDIX II

Construction of Small Tube Holder

Materials

1. Two 1/8 x 1.5 inch bolts
2. Six nuts to fit bolts above
3. Two 1 x 6 inch pieces of 1/8 inch plexiglass or acrylic plastic
4. Two 1 x 2.5 inch pieces of 1/8 inch plexiglass or acrylic plastic

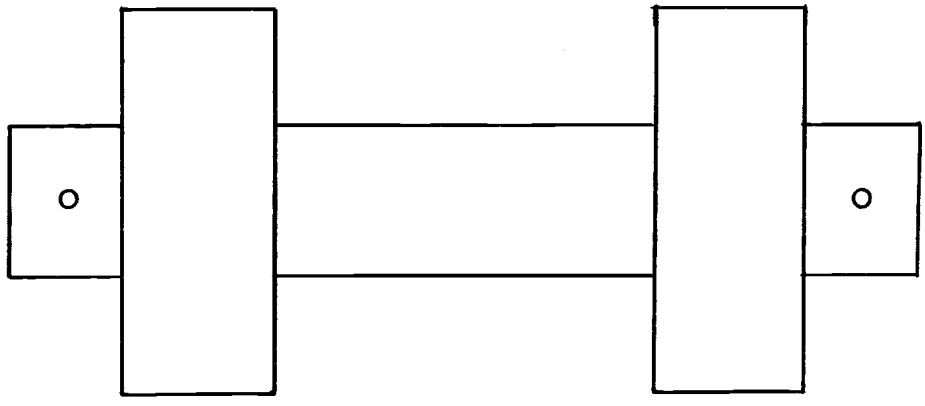
Method

Tape the two 1 x 6 inch pieces together firmly and drill a series of holes as follows. On each end drill one hole (to receive bolts above) entirely through both pieces. Holes should be 1/4 to 1/2 inch from each end, on the longitudinal center of the strip. At approximately 3/8 inch intervals between these two end holes, again on the longitudinal center of the strips, drill a series of holes such that the upper strip is completely pierced and the lower strip is drilled about one-half the way through. These holes are to receive small tubes and should be sized accordingly.

The strips may now be separated and to the undrilled side of the one strip are glued the two 1 x 2.5 inch

strips as shown in Figure 7a. The bolts, nuts, and upper strip are then assembled as in Figure 7b to form the completed holder.

(a)



(b)

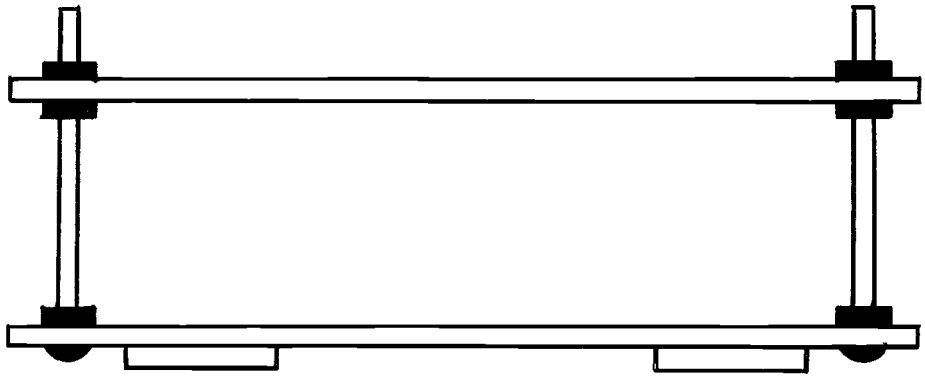


Figure 7. Assembly of a small tube holder. (a) Bottom view of base section, (b) front view of finished holder. Scale, 2 cm = 1 inch.

APPENDIX III

Effect of Cadmium on Incorporation of L-leucine-^{3}H into Ceruloplasmin Protein

Twenty male O.S.U. Wistar rats (weighing approximately 250 grams, raised on Purina rat chow and tap water) were fasted overnight. Two groups of five rats each were injected (I.P.) with 2.0 mg Cd^{2+}/kg B.W. as $CdCl_2$ in .05 M NaAc, .15 M NaCl, pH = 6.9-7.2. Two other groups of five rats each were injected with 1.0 ml/kg B.W. control solution (composition as above except minus $CdCl_2$). Five hours following cadmium (or control) solution injection, three groups of rats were injected with 80 µCi L-leucine-^{3}H/100 grams B.W.[23] The fourth group of rats were injected with 58% of this intended dose. This fourth group of rats had been injected with cadmium. At 40 minutes and again at two hours following L-leucine-^{3}H injection, one group of cadmium treated rats (given correct dose) and one group of control rats were sampled. At 40 minutes approximately one ml of blood was obtained from the tail when possible or via heart puncture when tail bleeding was unsuccessful. At two hours blood was obtained via heart puncture.

The remaining group of control rats was sampled as above at two hours and five hours. The group of cadmium injected

[23] L-leucine-4,5-^{3}H (N), specific activity 35.5 Ci/mM. Injected as 467 µCi/ml in .01 N HCl, .15 M NaCl. Obtained from New England Nuclear Corp., Boston, Massachusetts.

rats remaining had received 58% of the intended L-leucine-^{3}H dose. It was felt that if increased clearance from plasma were the cause of the precipitous drops in PPD oxidase activity that the decrease in incorporated L-leucine-^{3}H circulating in ceruloplasmin might be so great by five hours that measurement would be impossible. Therefore, this group of rats was sampled at two hours and three hours following L-leucine-^{3}H injection. Blood was centrifuged to obtain plasma which was frozen until later analysis.

Samples were thawed in water at room temperature (approximately four months later). Incorporated L-leucine-^{3}H in ceruloplasmin was measured on aliquots of plasma.

Preparation and Counting of Ceruloplasmin

A .4 ml aliquot of plasma was spiked with ceruloplasmin-^{14}C and diluted to approximately 2.0 ml with .05 M sodium acetate buffer, pH = 5.8, and applied to a short (approximately .7 x 4 cm) column of DEAE cellulose equilibrated with the same buffer. After washing the samples on the column, the absorbed ceruloplasmin band was washed with an additional portion of roughly 15 ml of the same buffer. The ceruloplasmin was eluted by .3 M NaCl in the above buffer. The first few drops (usually 20-30) of eluate after the application of the .3 M NaCl were discarded as the ceruloplasmin band had moved approximately one-half of the way down the column at

that point. Approximately the next three ml of effluent were collected and contained the ceruloplasmin band.

To the effluent was added .2 ml .2 M EDTA, pH = 7.2 and .8 ml of rat ceruloplasmin antiserum produced in rabbits. The final incubation mixture was .01 M EDTA, .15 M NaCl (approximate), .0375 M NaAc, pH approximately 7.1. Tubes were incubated at 37°C for one hour, followed by incubation at 2°-5°C for 66 hours (26). At the end of the 67 hour incubation, the tubes were centrifuged at 2000 x g for 30 minutes at 0°-4°C in a Sorval model RC2-B centrifuge with a swinging bucket head. The supernatant was carefully decanted into clean four ml glass tubes and stored at 2°-5°C for subsequent ring tests to establish the presence of excess antibody. Rinsing of the precipitate was accomplished as detailed in the body of the thesis.

Selected samples were shown to be in the region of antibody excess, as anticipated, but a rather strange phenomenon occurred, and this was the indication of excess antigen in the same samples. The occurrance of both excess antibody and excess antigen in the same mixture is not likely. Since excess antibody was tested with spectroscopically pure ceruloplasmin and excess antigen is tested by layering incubation mixture over antisera it is thought that the positive ring tests indicating antigen excess were the reaction of the antibodies produced to the impurities in the original ceruloplasmin antigen. This view is substantiated

by the fact that the equivalence point of the antisera used in the experiment occurred at about 2.0 µg ceruloplasmin per µl antiserum. In the above incubation mixture the calculated ratio was .25 µg ceruloplasmin per µl of antiserum, placing the sample well into the region of antibody excess.

After the last centrifugation and decantation, the tubes were inverted over absorbant paper and allowed to drain overnight. The drained precipitates were then removed from the cold and .2 ml of NCS[24] was added to each tube. Digestion was speeded by touching the tube to a vortex mixer for 30-90 seconds, followed by an additional .2 ml of NCS to wash down any particles that were deposited above the level of the liquid. At this point no visible particles remained in the solution. Samples were allowed to set for a period of time at room temperature at which time they were again stirred.

The resultant digest was transferred to a scintillation vial containing eight ml PCS[25] scintillation fluor. Two ml additional fluor were then added to the empty sample tube and touched to the vortex mixer for a few seconds. This fluor wash was also transferred to the scintillation vial. It should be noted that in some samples fine particles

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[24]Trademark, tissue solubilizer from Amersham/Searle Corp. 2636 S. Clearbrook Drive, Arlington Heights, Illinois.

[25]Trademark, emulsion fluor obtained from Amersham/Searle Corp.

appeared on the sides of the tube upon transfer of the NCS digest. These particles adhered very tightly to the glass surface and did not seem to be dislodged even by high rates of stirring. It was felt that the amount of activity remaining on such particles was negligible. All of the above cocktail preparation took place under indirect incandescent light, as fluorescent light could cause chemiluminescence difficulties (87). Samples were counted, along with suitable standards and blanks on a Packard Tri-Carb model 3375 liquid scintillation counter (counting temperature 15°C). Counts were corrected for percent recovery and converted to DPM.

Results and Discussion

Table 6 tabulates the usable data obtained from this experiment. The data is very sketchy and, of course, since a homogeneous antibody was not obtained, the DPM reported can only be considered an indication of the behavior of ceruloplasmin. However, no trends are apparent to suggest that cadmium prevents the synthesis of ceruloplasmin. This tends to support the conclusion drawn previously in the body of the thesis (page 12), that decreased rate of synthesis is not the cause of decreased PPD oxidase activity in ceruloplasmin. The maximum rate of incorporation of L-leucine-^{3}H is thought to occur at 2 hours following injection (26), therefore, the 40 minute and two hour samples are indicative of the rate of protein synthesis.

Table 6. Effect of cadmium on the DPM of L-leucine-^{3}H incorporated in ceruloplasmin protein. Five hours following cadmium or control injection rats received 80 μCi L-leucine-^{3}H/100 grams B.W. Data presented as average DPM in ceruloplasmin from .4 ml plasma ± one standard deviation (S.D.).

Group #	mg Cd^{2+}/kg B.W. Injected	Time (H)[a]			
		.67	2	3	5
1	0.0	245± 50[b]	551± 57[c]		
2	2.0	178±106[d]	513± 33[d]		
3	0.0		327± 59[c]		447±200[c]
4	2.0[f]		414±145[e]	422±216[c]	

[a] Time from L-leucine-^{3}H injection.

[b] Average of three rats.

[c] Average of four rats.

[d] Average of two rats.

[e] Average of five rats.

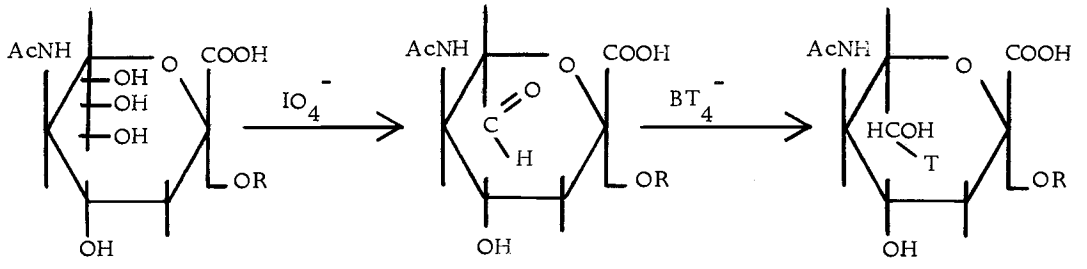
[f] Rats received 58% of the L-leucine-^{3}H injected in groups one through three.

It is interesting to note that group four rats received only 58% as much L-leucine-^{3}H as did group three, yet virtually no difference in the amount of incorporated activity is seen.

APPENDIX IV

Effect of Cadmium on Ceruloplasmin Degradation

Ceruloplasmin was purified as described in Chapter V[26] and labeled by the method of Lenton and Ashwell (88). Briefly, the method involved conversion of glycosidically-bound sialic acid to the tritiated 5-acetamido-3,5-dideoxy-L-arabino-2-heptulosonic acid derivative by periodic acid oxidation of terminal carbons and subsequent reduction with tritiated sodium borohydride[27] as shown below.



One group of four rats was injected (I.P.) with 2.0 mg Cd^{2+}/kg B.W. as $CdCl_2$ in .05 M sodium acetate, .15 M in NaCl, pH = 6.9-7.2 and another group of four rats served as controls. All rats were males of the O.S.U. Wistar strain raised on Purina rat chow and tap water and weighing 202-244 grams. Five hours after cadmium injections, .1 ml ceruloplasmin-^3H was injected in the jugular vein of each

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[26] Final Sephadex G-200 filtration omitted.

[27] Tritiated sodium borohydride obtained from New England Nuclear Corp. Specific activity 120 mCi/mmole.

rat.[28] At 1, 4, and 7 hours following ceruloplasmin-^{3}H injection, blood (approximately 1.0 ml) was obtained from the jugular vein or via heart puncture. At 11 or 12 hours blood was obtained by heart puncture. These samples were prepared for assay and counting as indicated previously.

Due to unexpected mortality among the rats used, only one rat from the cadmium group and one rat from the control group survived the entire experiment.

Figure 8 shows the data collected from these two rats. It is apparent that with this level of cadmium injection, no significant difference in the clearance of ceruloplasmin from the plasma between cadmium treated and control rats exists. The rapidity with which the ceruloplasmin disappears from the plasma is quite puzzling. The half-life of the more rapid component of the decay curve is about three hours and that of the long component of the decay curve is about six or eight hours. This is much shorter than the 25 hours reported by Lenton and Ashwell (88) for an identical preparation of human ceruloplasmin injected in rats. Hamer et al. (89) reported a half-life of 27 hours for rat ceruloplasmin labeled in vivo with ^{64}Cu and injected into the rat. From data presented by Holtzman and Gaumnitz (26), rat ceruloplasmin, labeled in vivo with L-leucine-^{3}H and

[28] Approximately 80,000 DPM in .05 M sodium acetate buffer, pH = 5.8, approximately .15 M NaCl.

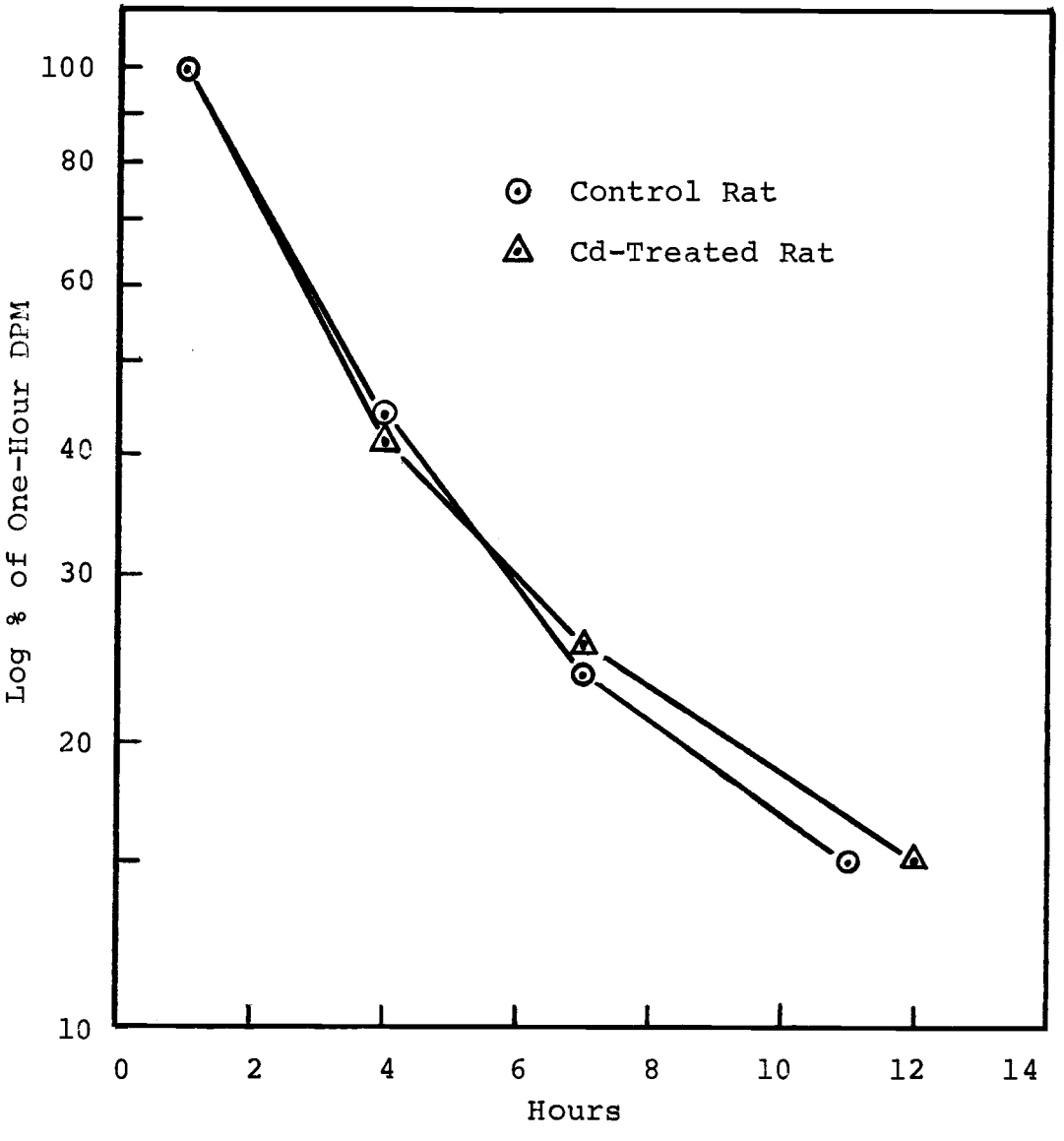

Figure 8. Effect of cadmium on clearance of injected (I.V.) ceruloplasmin-^{3}H from the plasma of rats. Five hours prior to ceruloplasmin-^{3}H injection Cd-treated rat received I.P. injection of 2.0 mg Cd^{2+}/kg B.W. as $CdCl_2$ in .05 M NaAc buffer, .15 M NaCl, pH = 6.9-7.2.

subsequently injected in rats, possessed a half life of approximately 12 hours.

While it is true that the removal of only two sialic acid residues (89) from ceruloplasmin results in rapid (30-60 minutes) clearance of ceruloplasmin from the plasma, the persistence of this preparation (>12 hours) indicates that removal or serious disruption of sialic acid residues did not occur.

The appearance of a two-phase decay curve leads one to believe that possibly the major portion of the ceruloplasmin injected was apoceruloplasmin. This would bring the first component more nearly in line with the reported five hours of Holtzman and Gaumnitz (26). The second component would correspond, then, to the holoceruloplasmin left in the injected solution. The observed half lives would still be only 60% and 50-60% of their predicted values for the short and long components respectively. However, as several months elapsed between the time of labeling of ceruloplasmin and its use, this may be the most logical explanation.

In any event, one may say that there is no significant difference in the observed rate of decay of injected activity between cadmium treated and control rats.

It should be noted that, while the procedure used to label ceruloplasmin <u>in vitro</u> is rather simple and results in a high specific activity product (1.6 μCi/mg), there is

an oddity observed. Following passage of the preparation of 1.16 μCi/mg through a .22 micron millipore filter a reduction of specific activity to .415 μC/mg occurs. Also, electrophoresis of the preparation resulted in a recovery of about 50% in the ceruloplasmin bands. The other 50% was largely recovered at the junction between the spacer-gel and small pore separation gel. This would seem to indicate the presence of colloidal or polymeric particles, however, this is only conjecture. Careful scrutiny of ceruloplasmin thus prepared is necessary.

It should also be noted that there is some uncertainty involved in the sampling times in Figure 8. Specifically, the final two sample times could conceivably be six or seven hours and eleven or twelve hours. The conclusions previously enumerated are not compromised by this, however. If one assumes the Cd-treated rats to have been sampled at 1, 4, 7 and 11 hours and the control rats at 1, 4, 6 and 12 hours from ceruloplasmin-^{3}H injection (the largest possible discrepancy), then ceruloplasmin decay could result in no more than a 15% difference in ceruloplasmin PPD oxidase activity in three hours. The previous statement assumes a direct relation between ceruloplasmin protein and PPD oxidase activity. Inspection of Figures 1 and 2 reveal a much more rapid drop.

APPENDIX V

Attempted Purification Methods

Before questions involving synthesis and decay could be answered or even attempted, several procedural problems had to be resolved. Of course, the major task was to find a method of purifying the ceruloplasmin in .2-.5 ml aliquot of plasma. Because of the number of samples involved it was important that the method remain simple as well as effective.

Due to lack of experience in producing and using antibodies, it was felt that work with the more familiar DEAE cellulose and gel permeation chromatography would be more profitable.

The first major task undertaken was to establish the conditions under which ceruloplasmin could be eluted as a well-defined peak in the sensitive portion of a gel permeation column (separation by molecular size).

A 1.3 x 48 cm column of Bio-Rad's[29] P-200 polyacrylamide gel showed ceruloplasmin to elute quite close to the front. As resolution of a gel column falls off rather sharply near its exclusion limit, use of this gel was not indicated.

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[29] Obtained from Bio-Rad Laboratories.

A different type of column packing to be tried was also a product of Bio-Rad. Bio Gel A-0.5m is an agarose gel with an exclusion limit of approximately 500,000 daltons. A 2 x 60 cm column of the agarose run at approximately 6 drops per minute (roughly .3 ml) revealed ceruloplasmin to occur farther away from the exclusion front than it did with P-200 and indeed appeared quite promising.

A rather interesting observation was that human ceruloplasmin purchased commercially[30], and supposedly pure, was resolved into two components as was the blue dextran, a high molecular weight marker used to indicate the void volume of the column. The lack of two clearly resolved components for either blue dextran or ceruloplasmin in later columns would seem to indicate the interaction of blue dextran and ceruloplasmin with the agarose.

Further agarose runs revealed what appeared to be a very low level of contaminants in the ceruloplasmin peaks when the ceruloplasmin was previously purified from plasma by a simple three-step NaCl gradient from DEAE cellulose.[31]

Attention was now turned to DEAE cellulose in the hope that a more refined purification of ceruloplasmin would remove the contaminants not removed by the agarose column.

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[30]Sigma Chemical Company, P. O. Box 14508, St. Louis, Missouri

[31]Obtained from Bio-Rad.

A 1.5 x 16 cm column of DEAE equilibrated with .05 M NaAc buffer[32], pH = 5.8, was prepared and to this was added an eight ml aliquot of rat plasma which had been diluted by approximately five times with the above buffer.

Elution was effected with a continuous gradient of NaCl. The exact shape of the gradient is not known, but is hypothesized to approach .2 M NaCl in a generally linear fashion and then remain constant for the final 250 ml (500 ml total gradient). The ceruloplasmin peak was not perfectly defined but it was generally separated from other major impurities and the absorbance rates indicated 68% purity. This purity is quite suspect because it was subsequently found that cloudiness occurring in similar samples gave erroneously high readings. This cloudiness was removed by filtration through .22 micron millipore filters.

Elution of ceruloplasmin with a linear NaCl gradient from 0 to .3 M NaCl, revealed it to be well separated from the main body of contaminants although the peak was rather spread out. Purity was only a disappointing 40% however.

Hydroxylapatite, a support of calcium phosphate, whose mode of interaction with proteins is not well understood, was also evaluated. A column of hydroxylapatite was prepared using the conditions reported by Holtzman et al. (67)

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[32]In this section the pH of .05 M NaAc solutions were adjusted by addition of concentrated HCl to obtain a buffer of the desired pH.

for human ceruloplasmin. This column achieved poor results both from the standpoint of a clean chromatogram (peak was spread out) or purification. Indeed it appeared as though the ceruloplasmin bound to the column only slightly and tended to denature. The purity of a sample of human ceruloplasmin decreased after elution, thereby discouraging further investigation in this direction. A later run attempted at low temperatures (2°-5°C) revealed that ceruloplasmin did not bind to the column at these temperatures. Others have reported that rat ceruloplasmin binds less strongly to hydroxylapatite under these conditions than does human ceruloplasmin (68) and it has been suggested that chromatography on hydroxylapatite (72) may alter the protein structure of ceruloplasmin.

A new column of agarose A-0.5 m was now poured and a attempt was made to repeat the earlier agarose chromatograms. Perhaps because the agarose was from a new batch, perhaps from other causes unknown, the clean resolution previously obtained could not be duplicated even on samples prepared in almost identical fashion. Column size, flow rate, and buffer were nearly identical so the investigator can only assume that the agarose was somehow different.

In any event, it became apparent that continued work in the direction of columns was fruitless for several reasons:

1. The procedures were not simple and short.
2. Satisfactory, repetitive purification was not being achieved.

3. In light of the experience gained, it became apparent that even if a satisfactory purification scheme could be developed for five to ten ml samples, it could not be realistically scaled down by an order of magnitude to accommodate the anticipated experiments.

Considerable time and effort was spent in evaluating the possible use of disc gel electrophoresis as the separating technique. Initial problems in this approach resulted from the high levels of chemiluminescence present after the digestion of polyacrylamide gels. Using the basic procedure detailed by Kalbhen (87), chemiluminescence can be minimized. The major remaining obstacle was the specific activity of the ceruloplasmin which was anticipated (and later confirmed) to be so low that the micrograms of ceruloplasmin that could be placed on a gel became the limiting factor. At the time of evaluation another block was that purification of the ceruloplasmin on a DEAE cellulose column, most likely a one-step elution with NaCl, was deemed necessary. This would result in volume too large to be placed on one electrophoresis gel (a 5 mm diameter gel was being used with maximum capacity of about .5 ml sample volume). A concentration step would be necessary and the apparatus available were not routinely operable at this small volume. It was learned much later that a new product

produced by Amicon[33], namely small disposable units for concentrating small volumes, was available. In light of this observation, it is felt that if electrophoresis tubes of 10-15 mm were available, thus increasing the load possible by a factor of four to nine times, the method would be feasible and probably one of the most simple and direct to use.

One should not rely upon merely increasing the dose of radioactive amino acids to increase the specific activity of the labeled protein as a 42% difference in injected dose resulted in no discernable difference in the ceruloplasmin DPM per ml of plasma obtained.

If facilities and expertise are available, the most desirable appearing method is that of antibodies described previously. However, as indicated both by this communication and other investigators (76), the preparation of a homogeneous antibody is a major chore. Certainly, when one considers the time required to purify the ceruloplasmin antigen, immunize the rabbits, characterize the antiserum as well as following the procedure for precipitating out the ceruloplasmin, electrophoresis is a faster method.

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[33]Amicon Corporation, 21 Hartwell Avenue, Lexington, Massachusetts.

APPENDIX VI

Miscellaneous Observations and Admonitions

Probably one of the most important details to be cognizant of is that the pH of injected cadmium solutions is quite important with regard to the response of ceruloplasmin activity following injection. In general, the lower the pH the greater the effect of a given cadmium dose. A striking illustration of this is the fact that rats injected with 2.0 mg Cd^{2+}/kg B.W. in saline, pH approximately 5.5, died within eight hours while no mortalities directly attributable to cadmium injection were found in rats given the same dose in either saline adjusted to a pH of approximately 7.2 or in 0.5 M NaAc, .15 M NaCl, pH = 6.9-7.2.

A cadmium-109 incorporation experiment similar to that reported in this communication revealed no incorporation of cadmium-109 into ceruloplasmin when the injection solution was buffered to pH 7.2 with phosphate buffer. More concentrated solutions of nonradioactive cadmium revealed a white gelatinous precipitate, possibly a hydroxide or phosphate complex. It is suspected that these observations relate to the cadmium absorption rates from the intraperitoneal cavity.

Heparin should not be used as an anticoagulant because its use results in a fine cloudy precipitate in the ceruloplasmin assay mixture resulting in very erratic results.

Saturated sodium citrate solution proved satisfactory in this investigation. However, it is reported (90) that citrate is an inhibitor of ceruloplasmin activity and that EDTA will also inhibit PPD oxidase activity (91) so it would seem that any anticoagulant commonly used has shortcomings.

The sensitivity of ceruloplasmin levels to environmental factors has been reported by others (63,64) and was also noted in the present work. The presence of any skin disruptions, such as those associated with an itching-like dermatitus contracted by the rats here at O.S.U., result in elevated ceruloplasmin activities. Pneumonia also resulted in elevated ceruloplasmin levels. Surgery (cannulation) resulted in ceruloplasmin levels of 1.5 to 2 times higher than normal levels and persisted for up to 17 days at which time monitoring was discontinued. Two other rats sampled at the time of cannulation and subsequently five days later (citrate as anticoagulent) showed ceruloplasmin oxidase activity two to four times their original levels. Unexplainedly, the ceruloplasmin levels at cannulation were abnormally low (46% to 63% of expected values).

The report by Whanger and Weswig (63) that heat stress elevates ceruloplasmin levels was confirmed by observations of an increase of up to twice normal reported levels with the onset of hot summer temperatures. The variation in temperature for the colony is thought to be less than 10°F but a general rise in ceruloplasmin activity

was noted on two consecutive summers. Therefore, strict environmental and stress control is indicated to obtain meaningful data when studying ceruloplasmin.

Male rats were used throughout this investigation because it has been reported (92-94) that hormone levels affect ceruloplasmin levels and it was feared that estrus cycles in the female rat could conceivably result in cyclic fluctuations in its activity.