Identification of an Apoceruloplasmin-like Substance in the Plasma of Copper-deficient Rats*

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SUMMARY

Male Sprague-Dawley rats were raised on a copper-deficient diet for 4 weeks. At that time their plasma contained little or no ceruloplasmin oxidase activity but did form a precipitate with a rabbit antibody to rat ceruloplasmin. Immunoelectrophoresis of plasma from copper-deficient rats revealed that the substance precipitating with the anticeruloplasmin antibody had the same properties as apoceruloplasmin. In addition, partial purification of the substance indicated that it contained less than 10% of the copper content of holoceruloplasmin. Quantitative precipitin assays of rat plasma showed that the apoceruloplasmin-like substance in copper-deficient blood was present in a concentration equivalent to 25% of the ceruloplasmin concentration in control rats raised on a regular diet.

The intraperitoneal injection of copper to the copper-deficient rats resulted in restoration of normal plasma concentrations of ceruloplasmin within 24 to 48 hours.

Ceruloplasmin is the main copper-containing protein in mammalian plasma. In humans and experimental animals fed copper-deficient diets its concentration, measured by oxidase activity, is markedly reduced (1-3). Previous studies have not eliminated the possibility that in copper deficiency apoceruloplasmin, which is devoid of oxidase activity, might still circulate. In this paper we report the presence of an apoceruloplasmin-like substance in the plasma of copper-deficient rats and in the accompanying paper (4) we consider the mechanisms by which ceruloplasmin oxidase activity is increased following the administration of copper to copper-deficient rats. These results have been reported previously in preliminary form.*

EXPERIMENTAL PROCEDURE

Immunoelectrophoresis and Immunodiffusion—Purified agar (Baltimore Biological Laboratory, BioQuest) was gelled, cut, and rinsed with water for 1 to 2 days. Immunoelectrophoresis was performed by the method of Scheidigger (5) with 0.05 M sodium barbital adjusted to pH 8.6 with HCl in the buffer vessels and 2% agar gels in 0.04 M barbital also at pH 8.6. Immunoelectrophoresis was also performed with 0.15 M sodium acetate buffer, pH 5.8, in the buffer vessels and 2% agar in 0.075 M sodium acetate buffer, pH 5.8. Immunodiffusion was performed by the method of Ouchterlony (6). The agar slides were rinsed in 0.9% NaCl overnight before staining with a filtered solution of benzidine dihydrochloride, 0.75% in 0.3 M sodium acetate buffer, pH 5.2. The ceruloplasmin bands were visible within 15 to 30 min at 37°. The gels were then rinsed with water for 24 to 48 hours, dried, and stained with Amido black.

Copper Content of Protein—Copper was determined by the method of Felsenfeld (7) following passage of the preparation over a column of Chelex-100 (Bio-Rad), 0.5 x 4 cm, equilibrated with 0.5 M sodium acetate buffer, pH 5.8.

Preparation of Ceruloplasmin—One hundred milliliters of plasma, obtained from 20 adult Sprague-Dawley rats raised on regular laboratory chow, were diluted to 300 ml with 0.3 M sodium acetate buffer, pH 5.8, and applied to a column of DEAE-Sephadex (A-50), 5 x 20 cm, equilibrated with the same buffer at room temperature. The sample was followed by application of the same buffer until no further protein, measured by A280, eluted from the column. A single, blue peak was obtained by the addition of 0.5 M sodium acetate buffer, pH 5.8. This effluent was cooled to 4°, precipitated with 90% ethanol-10% chloroform, and treated as previously described for human ceruloplasmin (8).

On acrylamide gel electrophoresis (9) the rat ceruloplasmin preparation showed one major band which stained either with Coomassie blue for protein or benzidine for oxidase activity. In some preparations a faint minor band which did not stain with benzidine was also found. On analytical ultracentrifugation one symmetrical peak with an s20, w 0.3 M acetate, pH 5.4 of 5.43 S was obtained. This was in agreement with an s20, 0.3 M acetate, pH 5.8 of 5.40 S for human ceruloplasmin.2 This has a molecular weight of 160,000 (10), that of human ceruloplasmin, was assumed for the rat protein. Calculation of the concentration of ceruloplasmin was based on the assumption of 8 copper atoms per 160,000 molecular weight. Recent studies

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1 HOLTZMAN, N. AND GAUMNITZ, B. M., Annual Meeting of the Society for Pediatric Research, 1969, p. 72.
Apoceruloplasmin was prepared by dialysis of the ceruloplasmin preparation against changes of 0.05 M NaCN-0.075 M potassium phosphate buffer, pH 7.6, for 2 to 3 days. For precipitin studies both the apo- and holoceruloplasmin were dialyzed against 0.5 M acetate, pH 5.8. The apoceruloplasmin preparation contained less than 2% of the copper and less than 1% of the oxidase activity of the ceruloplasmin preparation.

Preparation of Anticeruloplasmin Antibody—Immunoelectrophoresis and immunodiffusion of rat plasma, with antisera obtained from rabbits immunized with the ceruloplasmin preparation described above, revealed additional precipitin bands which did not spur with the ceruloplasmin band. The contaminating antigen was eliminated by further purification of the ceruloplasmin by acrylamide gel electrophoresis. The ceruloplasmin was diluted with upper gel buffer containing 30% sucrose to give a final ceruloplasmin concentration of 1 mg per ml. To each 6-mm diameter gel 0.1 ml was applied. At the conclusion of electrophoresis the gels were placed in benzidine solution until faint bands began to appear. Each gel was rinsed with 0.9% NaCl and the stained band, approximately 2 mm thick, was cut out of each gel and homogenized with 0.25 ml of 0.9% NaCl per gel. The homogenate was emulsified with an equal volume of complete Freund’s adjuvant (BioQuest). Rabbits were immunized by the injection of 0.4 ml of the emulsion into each foot pad. Two weeks later an additional 0.4 ml was injected intramuscularly in each leg. The rabbits were bled by intracardiac aspiration 3 weeks later and at 2- to 3-week intervals subsequently. Five to 7 days before each bleeding the rabbits received 0.1 ml of complete Freund’s adjuvant. Rabbits were immunized by the injection of 25 pg of ceruloplasmin that had been eluted from acrylamide gels with 0.9% NaCl. The homogenate of plasma from copper-deficient rats revealed the presence of a contaminating antigen that was eliminated by further purification of the ceruloplasmin. The Aslo mp, 1 wn, I%, = 280 mp to that at 610 mp was 19 to 20, slightly lower than that obtained for human ceruloplasmin. The $A_{410}$ for human ceruloplasmin $= 0.64$, compared to 0.68 for human ceruloplasmin. The oxidase activity was 1.4 times as high per mg for rat as for human ceruloplasmin.

Ceruloplasmin Oxidase Assay—The reaction mixture contained 0.02 to 0.05 ml of rat plasma, 0.5 ml of 0.5% p-phenylenediamine dihydrochloride (K and K Laboratories), and 0.5 ml sodium acetate buffer, pH 5.8, to bring the reaction mixture to 1.0 ml. The p-phenylenediamine was prepared by adjusting 0.6 g of Chelex 100, sodium form, and 0.1 g of p-phenylenediamine to a final volume of 20 ml with 1% NaCl-1.2 M sodium acetate buffer, pH 6.0, stirring at room temperature for 10 min, and filtering through a sintered glass funnel. The concentration of ceruloplasmin was calculated from the change in optical density at 530 mp, 37°, with purified rat ceruloplasmin as standard. The reaction was followed in a Gilford model 2000 absorbance meter.

Production of Copper Deficiency—Twenty-five day old male Sprague-Dawley rats were raised on a copper-deficient diet (Nutritional Biochemicals). The animals were housed in stainless steel cages and received deionized distilled water. Control rats were similarly housed but received either regular laboratory chow or the copper-deficient diet, supplemented with 18 ppm of copper as CuSO4·5H2O and tap water. Blood was obtained periodically from the tail for ceruloplasmin assay.

RESULTS

The concentration of plasma ceruloplasmin in the 25-day-old rats was similar to adult values. When these rats were raised on a copper-deficient diet plasma ceruloplasmin oxidase activity frequently fell to undetectable levels within 4 weeks (Fig. 1). In control rats no decline in oxidase activity was seen. When copper-deficient rats received 10 to 75 $\mu$g/100 g, body weight, of copper as cupric acetate intraperitoneally ceruloplasmin oxidase activity was restored to normal within 21 to 48 hours (Fig. 2). This dose had no effect on the concentration of oxidase activity in the control rats. An equivalent dose of sodium acetate did not alter the ceruloplasmin concentration in copper-deficient rats.

Despite the absence of oxidase activity, immunoelectrophoresis of plasma from copper-deficient rats revealed the presence of a precipitin following the addition of anti-rat ceruloplasmin.
Fig. 2. Response of plasma ceruloplasmin to intraperitoneal injection of cupric acetate or sodium acetate. •—•, rats raised on a copper-deficient diet who received either 10 or 75 µg/100 g of copper as 0.008 M cupric acetate; O—O, rats raised on the same diet who received 0.008 M sodium acetate instead of cupric acetate; ■—■, rats raised on regular laboratory chow who received 75 µg/100 g of copper as 0.008 M cupric acetate. Data are presented as mean ± standard deviation of determinations in four to 12 different animals.

Fig. 3 (top). Immunoelectrophoresis with rabbit antibody to rat ceruloplasmin. Left, pH 8.6; right, pH 5.8. Upper slides: upper portion, plasma from a copper-deficient rat; lower portion, plasma from a control rat. Lower slides: upper portion, plasma from a copper-deficient rat; lower portion, apoceruloplasmin. The slides were stained with Amido black.

Fig. 4 (bottom). Ouchterlony immunodiffusion with rabbit antibody to rat ceruloplasmin in the center well. Starting at 12 o’clock and proceeding clockwise the outer wells contained a mixture of apo- and holoceruloplasmin (1:1), a mixture of plasma from a copper-deficient and control rat (3:1), holoceruloplasmin, plasma from a copper-deficient rat, apoceruloplasmin, and plasma from a control rat. The slide was stained with Amido black.

Fig. 5. Results of quantitative precipitin reactions of rabbit antibody to rat ceruloplasmin with ceruloplasmin (•—•) and apoceruloplasmin (O—O). Assay was carried out in 0.01 M EDTA, pH 7.2, under the same incubation conditions described under “Ceruloplasmin Precipitin Assay.”

Table I

<table>
<thead>
<tr>
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<th>Oxidase assay ceruloplasmin (µg/ml)</th>
<th>Precipitin assay ceruloplasmin (µg/ml)</th>
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<tbody>
<tr>
<td>Control rats</td>
<td>380</td>
<td>390</td>
</tr>
<tr>
<td></td>
<td>260</td>
<td>275</td>
</tr>
<tr>
<td></td>
<td>260</td>
<td>285</td>
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<tr>
<td></td>
<td>340</td>
<td>365</td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td>310 ± 60</td>
<td>329 ± 57</td>
</tr>
<tr>
<td>Copper-deficient rats</td>
<td>12</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>&lt;4</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>100</td>
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<tr>
<td></td>
<td>&lt;4</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>&lt;4</td>
<td>94</td>
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<tr>
<td></td>
<td>4</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>&lt;4</td>
<td>65</td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td>&lt;7 ± 4</td>
<td>82 ± 13</td>
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Antisera to the central trough (Fig. 3, upper slides). In contrast to the arc observed following immunoelectrophoresis of control plasma the arc was oxidase-negative and had a greater mobility toward the anode.

Although the antibody to ceruloplasmin was prepared with highly purified ceruloplasmin the possibility remained that the arc seen with plasma from copper-deficient rats was due to an antigenic contaminant unrelated to ceruloplasmin. If this were so then absorption of the antisera with plasma from copper-deficient rats should not affect the ability of the antisera to precipitate ceruloplasmin from control rats. To test this possibility plasma from a copper-deficient rat was added to the antisera until no further precipitate was formed. The precipitate was removed by centrifugation. When the absorbed antisera were used in immunoelectrophoresis, immunodiffusion, or quantitative precipitin reactions with plasma from control rats or purified rat ceruloplasmin as the source of antigen, no precipitate was formed. Unabsorbed antisera, diluted to the same extent, gave the expected precipitate by each of these methods.

The relation of the material in plasma from copper-deficient rats to ceruloplasmin remains to be defined.
rats to apoceruloplasmin was investigated. Immunoelectrophoresis of apoceruloplasmin prepared from purified rat ceruloplasmin by dialysis against cyanide revealed an oxidase negative arc of mobility identical with that of the arc found in plasma from copper-deficient rats at both pH 5.8 and pH 8.6 (Fig. 3, bottom slides). On immunodiffusion, with the antibody to ceruloplasmin, the precipitate which formed with the plasma of copper-deficient rats merged smoothly with that formed between the antibody and control plasma or with purified holo- or apoceruloplasmin (Fig. 4).

Although the cross-reacting material in copper-deficient plasma appeared qualitatively identical with apoceruloplasmin it remained to establish whether apoceruloplasmin was quantitatively equivalent to holoceruloplasmin in its ability to precipitate the anticeruloplasmin antibody. This was confirmed by showing that the precipitin curves for hol- and apoceruloplasmin were identical (Fig. 5). The concentration of apoceruloplasmin-like material in the plasma of copper-deficient rats was found to be approximately 25% that of immunologically detectable ceruloplasmin in control rats (Table I). The discrepancy between the amount of ceruloplasmin detected by oxizide activity and that detected by the precipitin assay is significantly greater for plasma from copper-deficient rats than for control plasma.

**Partial Purification of Apoceruloplasmin-like Material—** Plasma, obtained from copper-deficient rats, was applied to a DEAE-Sephadex column equilibrated with 0.3 M sodium acetate buffer, pH 5.8. The fraction eluting with 0.5 M sodium acetate buffer, pH 5.8, was concentrated by ultrafiltration. Virtually all of the material which precipitated with the anticeruloplasmin antibody was recovered in this fraction. Apoceruloplasmin, prepared by the dialysis of purified holoceruloplasmin against cyanide, had identical chromatographic properties. Although electrophoretically impure, the fraction from the copper-deficient rats contained less than 1 copper atom per molecule of ceruloplasmin-like substance present. Ceruloplasmin detectable by oxizide activity accounted for approximately 10% of the immunochromatically detectable ceruloplasmin.

The further purification of holoceruloplasmin following DEAE-Sephadex chromatography and precipitation with ethanol-chloroform depends on its solubility in acetate buffer since the contaminating proteins remain insoluble. Neither apoceruloplasmin prepared by dialysis against cyanide nor the apoceruloplasmin-like material in plasma from copper-deficient rats was soluble following precipitation with ethanol-chloroform.

**DISCUSSION**

On the basis of immunoelectrophoresis at pH 5.8 and 8.6 and Ouchterlonny immunodiffusion, plasma from rats raised on a copper-deficient diet appears to contain a substance resembling apoceruloplasmin. This substance precipitates with antibody to rat ceruloplasmin, has the same electrophoretic mobility as purified apoceruloplasmin, is devoid of oxidase activity, and contains less than 10% of the copper content of holoceruloplasmin. Absorption of the antisera with this substance removed the ability of the antisera to precipitate ceruloplasmin. The inclusion of copper into the polypeptide moieties of ceruloplasmin is not, therefore, a prerequisite for either the completion of synthesis of the protein or for its release into the circulation.

The occurrence of apoceruloplasmin in the plasma of copper-deficient rats is similar to the occurrence of apotransferrin in the plasma of iron-deficient animals. However, while the concentration of apotransferrin in iron deficiency is increased (12), the concentration of apoceruloplasmin in plasma from copper-deficient rats is only 25% of the normal ceruloplasmin concentration. This reduction could be due to either a slower rate of release or a more rapid decay of the apoprotein. Studies designed to distinguish between these possibilities and to explain the rise in ceruloplasmin concentration following the administration of copper are presented in the accompanying paper (4). Recently, Carrico et al. (13), with an antibody to human apoceruloplasmin, demonstrated amounts of apoceruloplasmin in the serum of normal human subjects equivalent to 10 to 20% of the total ceruloplasmin concentration. Our studies do not exclude the presence of small amounts of apoceruloplasmin in the plasma of normocupremic rats. The observation that the precipitin assay consistently yielded slightly higher ceruloplasmin concentrations in the plasma of control rats than the oxidase assay (Table I) suggests that apoceruloplasmin is present in plasma from normocupremic rats. The assays were always performed on fresh plasma. Although immunoelectrophoresis partially separates apo- from holoceruloplasmin (Fig. 3), immunoelectrophoresis of mixtures of 25% apoceruloplasmin:75% holoceruloplasmin yielded an arc which could not be clearly distinguished from that formed when either pure holoceruloplasmin, pure apoceruloplasmin, or control plasma was used as the source of antigen. Although immunoelectrophoresis was performed on plasma obtained at eight intervals between 0 and 24 hours following the intraperitoneal administration of copper to copper-deficient rats, we could never unequivocally demonstrate the coexistence of both apo- and ceruloplasmin. Immunoelectrophoresis of normal rat plasma kept at 4°C for 5 days or more frequently showed two confluent arcs; the faster limb had the same mobility as apoceruloplasmin whereas the slower, which stained with benzidine, appeared to be holoceruloplasmin.

**REFERENCES**

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