Ceruloplasmin in Wilson’s Disease *

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Summary. Ceruloplasmin was highly purified from one patient with Wilson’s disease and partially purified from a second unrelated patient. The highly purified ceruloplasmin was indistinguishable from normal ceruloplasmin by electrophoresis, tryptic peptide map, oxidase activity, and copper, amino acid, and sugar composition. The partially purified ceruloplasmin was indistinguishable electrophoretically from normal ceruloplasmin. With penicillamine therapy, ceruloplasmin disappeared from the serum of the first patient; it reappeared after the drug was discontinued. The significance of this observation in regard to the basic defect in Wilson’s disease is discussed.

Introduction

Although the concentration of the serum copper protein ceruloplasmin is decreased in most patients with Wilson’s disease (1), there are several objections to the theory that decreased ceruloplasmin synthesis (2) is the underlying defect. There are, for instance, several patients with unquestionable Wilson’s disease who have normal ceruloplasmin levels (3–5). On the other hand, normal adults unrelated to patients with Wilson’s disease, as well as some proven heterozygous persons, have persistently low ceruloplasmin levels without any clinical or chemical manifestations of the disease (6).

These findings are consistent with the hypothesis that the ceruloplasmin in patients with Wilson’s disease is structurally abnormal (7). An amino acid substitution in a polypeptide chain of ceruloplasmin, as in other proteins, could alter the function, the rate of synthesis or destruction of the protein, or both. The genetic heterogeneity in

Wilson’s disease (8) could be explained if, in one pedigree, an amino acid substitution occurs at a point in one of the polypeptide chains, resulting in a limited alteration of function, whereas in another pedigree an amino acid substitution at a different point results in a more profound disturbance.

Ceruloplasmin accounts for the oxidase activity of serum towards several biological aromatic amines in vitro (9). However, its function in vivo is still unknown. In previous studies (1, 10, 11), insufficient data were presented to determine if the oxidase activity per milligram of ceruloplasmin in sera from patients with Wilson’s disease differs significantly from normal. Furthermore, these studies were performed on serum, in which inhibitory factors occur in variable amounts (12), and therefore may not accurately compare the oxidase activity per mole of pure ceruloplasmin in Wilson’s disease to that of ceruloplasmin in normal individuals.

Structural differences of ceruloplasmin in Wilson’ disease sera have not been detected either by one dimensional electrophoresis (13–15) or by immunoelectrophoresis (16). Hirschman, Morell, and Scheinberg (17), however, demonstrated a difference in the electrophoretic mobility of the minor component of ceruloplasmin in one patient with Wilson’s disease. The methods employed in
the other studies cited would not have detected such a difference.

This report describes a patient with the neurological manifestations of Wilson's disease whose serum ceruloplasmin concentration was normal when he was first seen. To investigate the possibility that ceruloplasmin was abnormal in this patient, we crystallized ceruloplasmin from his serum and from the sera of normal individuals. The structure of each was extensively studied, and no differences were detected. Ceruloplasmin was partially purified from another unrelated patient with Wilson's disease with persistently low levels of ceruloplasmin. It too was indistinguishable from normal ceruloplasmin by electrophoresis. Preliminary results of this study were previously reported (18).

Case report. E.C. (JHH no. 114-56-16), a 31-year-old Negro male, was first admitted to The Johns Hopkins Hospital because of tremor of 2 years' duration. There was no family history of hepatic or neurologic disease or consanguinity. Serum ceruloplasmin concentration in all seven of the patient's siblings, as well as his mother, was normal.

The patient was employed as a nail maker until he developed intention tremor of his hands. It became progressively more severe, even at rest, and was halted only when he slept or sat on his hands. Before his referral to The Johns Hopkins Hospital, one physician noted glycosuria. The patient also complained of back pain of several years' duration, relieved by lying down. There was no history of anemia, jaundice, ascites, hematemesis, melena, or fractures.

On physical examination, the patient had severe, flapping, intention tremor of the upper extremities and a pill-rolling resting tremor of the hands. Facial expression was fixed and speech was slow and monotonous with some scanning. Cog wheel rigidity of the upper extremities was present. Reflexes were brisk and symmetrical in the lower extremities and present but difficult to evaluate in the upper extremities because of the tremor. Muscle strength was not grossly diminished. Except for the fine tremors of the tongue, the cranial nerves were intact. There was no nystagmus. The heel to knee test and tandem gait were normal. On slit lamp examination, prominent golden brown deposits were present in Descemet's mem-

brane, most dense superiorly, but encircling the limbus bilaterally.

No organs or masses were palpable in the abdomen. There was no icterus, telangiectasia, gynecomastia, clubbing, or palmar erythema. Testes were of normal size.

Admission routine hemogram and urinalysis revealed no abnormality. Serum bilirubin was less than 0.8 mg per 100 ml, serum glutamic oxaloacetic transaminase 26 U, serum glutamic pyruvic transaminase 14 U, alkaline phosphatase 2.8 Bodansky U, albumin/globulin 3.8/4.7 g per 100 ml plasma, uric acid 2.5 mg per 100 ml, and sulfobromophthalein retention 3% 45 minutes after a dose of 5 mg per kg. Prothrombin time was 50% of normal but responded to intramuscular vitamin K. A low copper diet was started. Two days after admission, a 24-hour urine sample contained 400 μg copper (normal value for this laboratory 62 ± 28 μg per 24 hours). Serum ceruloplasmin (measured by its oxidase activity) was 25 mg per 100 ml, low normal for this laboratory (19). An increase in urinary amino acids, consistent with a generalized renal tubular defect, was found.

One g per day of D-penicillamine was begun. In the second 24 hours, the patient excreted 4,502 μg copper in the urine. This cupresis was maintained over the next 9 weeks, after which urine collections were discontinued. The daily dosage was increased to 4 g. After 3½ months of penicillamine, the serum ceruloplasmin concentration had fallen to 8 mg per 100 ml. Three months later none was detectable.

After the patient had been receiving penicillamine for 2 months, a closed liver biopsy was performed. Liver copper was 114 μg per g wet weight (normal value = less than 15 μg per g wet weight). The biopsy showed moderate fibrosis and regenerating nodules. There was no necrosis.

There was no clinical improvement for the first 9 months on penicillamine therapy, but over the next month progressive improvement, with almost complete disappearance of tremor and speech changes, occurred. The patient felt so well that he failed to return for medicine until approximately 1 year after his first admission, when he noted some recurrence of tremor. He was readmitted at this time and aside from some mild intention tremor of the upper extremities, where the deep tendon reflexes were hypoactive, and the persis-
tent Kayser–Fleischer rings, physical examination was unremarkable. His ceruloplasmin was now 4 mg per 100 ml. d-Penicillamine, 4 g per day, was again started, resulting in the renal excretion of 3,710 μg copper on the second day.

A plasmapheresis, yielding 600 ml of heparinized plasma, was performed on the patient 1 week after he was started on penicillamine for the first time. A second plasmapheresis, yielding 800 ml of plasma, was performed about 1 year later before penicillamine therapy was re instituted. There were no ill effects from the procedure. Ceruloplasmin was purified from the plasma obtained.

In summary, this 31-year-old male presented with severe intention and resting tremor, fixed facies, and monotonous speech and Kayser–Fleischer rings. He had some hepatic fibrosis and elevated liver and urinary copper. His serum ceruloplasmin concentration was normal but, concomitant with penicillamine therapy, fell to undetectable levels. A small amount reappeared after the drug was discontinued. He had an excellent clinical remission after taking the drug for several months.

**Methods**

*Purification of ceruloplasmin.* Plasma was dialyzed against 0.33 M sodium acetate, pH 5.8, overnight at 0 to 4°C. The precipitate was removed by centrifugation, and the supernatant fluid was warmed and applied to a column of DEAE–Sephadex A 50 approximately 30 × 4 cm equilibrated with 0.33 M sodium acetate, pH 5.8, at room temperature. The column was rinsed with this buffer at a rate of 3 ml per minute until the OD_{280} of the effluent was less than 0.2. A variable gradient multichamber device was employed for elution with 400 ml of the 0.33 M acetate buffer in the first chamber and 0.5 M sodium acetate, pH 5.8, in the second to fourth chambers. Elution was monitored at 280 μm with a Gilford model 2000 absorbance meter coupled with a circulating water bath at 20°C and a Radiometer CDM-2 conductivity meter for continuous measurement of the conductivity of the effluent (Figure 1, top). Tubes containing ceruloplasmin were pooled, chilled to 0 to 4°C, and precipitated with 2 vol of 90% ethanol: 10% chloroform stored at −20°C. After 30 minutes of stirring at 0 to 4°C, the blue precipitate was sedimented at 36,000 × g for 20 minutes at 0 to 4°C. The pellets were partially dissolved in a small volume of 0.35 M sodium acetate, pH 5.8, and the suspension centrifuged in a Spinco model L for 20 minutes at 105,000 × g. The intensely blue, but occasionally slightly turbid, supernatant was finally clarified by passage through a 0.45–μ Millipore filter, yielding fraction cer_{e, e}.

**Fig. 1. Top:** Elution of ceruloplasmin from DEAE–Sephadex by an increasing ionic strength gradient of sodium acetate, pH 5.8. Further purification was carried out on the “pooled ceruloplasmin” fractions. **Bottom:** Elution of the two ceruloplasmin components, HA I and HA II, from hydroxylapatite by an increasing ionic strength gradient of potassium phosphate, pH 6.4.

Crystallization from this material was accomplished by the method of Deutsch (20), except that 0.05 M sodium acetate, pH 5.25 to 5.30, rather than 0.025 M sodium acetate, pH 5.20 to 5.25, was used.

**Separation on hydroxylapatite.** Cer_{e, e} was dialyzed against 0.05 M potassium phosphate, pH 6.4, and applied to a 20 × 1-cm column of hydroxylapatite (21). The variable gradient multichamber device was employed for elution with 80 ml of 0.05 M potassium phosphate, pH 6.4, in the first chamber, 80 ml of 0.20 M phosphate, pH 6.4, in the next three chambers, and 80 ml of 0.40 M phosphate, pH 6.4, in the last chamber. Two ceruloplasmin components, HA I and HA II, were eluted (Figure 1, bottom). Each of these was concentrated to a gelatinous pellet in a Schleicher and Schuell collodium bag under water vacuum overnight at 0 to 4°C. The pellets were dissolved in 0.35 M sodium acetate, pH 5.8, and passed through 0.45–μ Millipore filters.

**Assays.** Ceruloplasmin concentration was determined either by assay of oxidase activity (19) or by its OD_{400} using E_{400} of 1 cm, % = 0.68 (20). Copper
was determined by the biquinoline method of Felsenfeld (22). There was excellent agreement between the concentration of ceruloplasmin obtained by the extinction coefficient and that obtained from the copper assay based on the assumption of 8 moles copper per 160,000. Spectrophotometric measurements were performed on either a Zeiss PMQ II or Beckman DU coupled to a Gilford model 2000 absorbance meter. Spectral curves were determined on the Bausch and Lomb 505 spectrophotometer.

Composition studies. Amino acid analyses on duplicate samples of ceruloplasmin hydrolyzed for 20 hours in 6 N HCl were performed on a Technicon five column analyzer.

Sialic acid was determined (23) after hydrolysis for 1 hour at 80° C at pH 1.7. The remaining sugars were determined after hydrolysis in 2 N HCl for 4 hours at 100° C. At the conclusion of hydrolysis, tracer amounts of glucosamine, fucose, mannose, and galactose, each containing a known amount of 14C, were added to the hydrolyzate. All counting was performed in a Tri-Carb liquid scintillation counter. Repeat counting on samples of each of the sugars after chromatographic purification permitted the chemically determined values to be corrected for losses. Glucosamine was purified by the method of Boas (24) and determined by a modified Elson-Morgan procedure (25). The neutral sugars contained in the wash from the Dowex 50 column used in the preparation of glucosamine were further purified by the addition of Dowex 1 to the wash until no chloride was detectable. The resin was removed by filtration. The filtrate was concentrated and applied over a 2-inch band to Whatman 1 chromatography paper and run by the descending method overnight in n-butanol: acetic acid: water (12:3:5). A 1-inch guide strip from each sample was stained by the silver nitrate method (26), and the bands corresponding to the stained areas were cut out of the unstained paper and eluted with water. The stained areas had Rf identical to fucose, mannose, and galactose.

A blank area of paper was eluted for use as an internal background in the determination of 14C in each of the eluted fractions and in the determination of fucose by the method of Dicshe and Shettles (27). Mannose and galactose were determined on the eluents from the paper by the Nelson method (28) with standards of mannose and galactose, respectively.

Electrophoresis. Vertical starch gel electrophoresis (29) was performed in a continuous system at pH 5.6 (0.03 M sodium acetate) and at pH 6.4 (0.0054 M sodium phosphate) with the wicks immersed in 0.04 M sodium phosphate, pH 6.4. Electrophoretic comparisons were also made in acrylamide in a discontinuous system at pH 8.9 (30) in a new device in which permitted samples to be run in one slab (31). The gels were stained either for protein (29) or oxidase (ceruloplasmin) with 0.1% benzidine in 0.2 M sodium acetate, pH 5.2.

Tryptic digests. To reduce the size of the trypsin-resistant core, we aminooethylated the purified protein (32). After reduction for 3 hours with 0.04 M mercaptoethanol in 8 M urea and 1 M Tris-HCl, pH 9.1, ethylene imine was added to a final concentration of 0.5 moles per L. After 2 hours the nitroprusside test (33) for free sulfhydryl was still positive, and ethylene imine was again added to a final concentration of 1 mole per L. The incubation continued for a total of 16 to 20 hours at room temperature, at which time the free sulfhydryl was detectable. The samples were desalted by passage through Sephadex G-25 columns equilibrated with 0.025 N NH4OH and lyophilized.

The dried protein was suspended in 1% (NH4)2CO3 and 20 μg trypsin per mg protein was added. Digestion at 30° C with 0.005 ml toluene present continued for 16 hours and was terminated by repeated lyophilization to remove (NH4)2CO3 (34). Tryptic peptide maps were performed on 2.5 to 3 mg of digest by the method of Ingram (35), except that chromatographic separation employed a solvent of n-butanol: acetic acid: water: pyridine (12:3:12:10) (36) in either the ascending or descending direction. The resulting maps were stained with Ninhydrin and with stains specific for histidine or methionine (37).

Results

Chromatographic purification of ceruloplasmin from the plasma of our patient with Wilson's disease when his ceruloplasmin concentration was 4 mg per 100 ml is shown in Figure 1, top, together with a purification of ceruloplasmin from plasma of normal subjects. Because of the reduced ceruloplasmin concentration in the patient's plasma, the protein eluting just after ceruloplasmin became a major contaminant. The protein was completely

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<th>TABLE I</th>
<th>Comparison of ceruloplasmin purified from normal plasma and Wilson's disease plasma</th>
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<tr>
<td>Ceruloplasmin preparation</td>
<td>OD410 mg/μg/OD410 mg</td>
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<td>Normal subjects</td>
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<td>Ceras1</td>
<td>26</td>
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<tr>
<td>Crystals</td>
<td>23</td>
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<td>HA I</td>
<td>23</td>
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<td>HA II</td>
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<td>Wilson's disease (E.C.)</td>
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<tr>
<td>Ceras1</td>
<td>28</td>
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<tr>
<td>Crystals</td>
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<td>HA I</td>
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<td>HA II</td>
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* Michaelis constant, determined in 0.17 M NaCl, 0.9 M sodium acetate, pH 5.8, at 30° C with α-phenylenediamine dihydrochloride. The oxidation of 1 mmole of this substrate causes a rise in absorbance at 530 μm of 0.75. The differences shown are within the range of experimental error.

† Maximal velocity determined under the same conditions as KM in 0.017 M α-phenylenediamine dihydrochloride and expressed as OD410 mg/μg/OD410 mg per milligram ceruloplasmin per minute.

† Values in this line refer to the preparation when the patient had a ceruloplasmin concentration of 25 mg per 100 ml plasma.
removed from the ceruloplasmin fraction, however, by passage over hydroxylapatite (Figure 1, bottom). As shown, the ceruloplasmin from the patient with Wilson’s disease was eluted from both DEAE–Sephadex and hydroxylapatite at the same ionic strength, measured by conductivity, as ceruloplasmin from normal subjects. Blue crystals, consisting of tetragonal needles and rosettes, were obtained from both Wilson’s disease and normal ceruloplasmin.

The ratio of absorbance at 280 mμ to 610 mμ of crystalline electrophoretically pure ceruloplasmin is 22 to 23 (38). In Table I the 280:610 ratios of the various preparations are shown together with their copper content and oxidase activity. There is no significant difference among any of the preparations. Spectral curves of the major component eluted from hydroxylapatite (HA I) from normal individuals and from the patient with Wilson’s disease are identical between 220 and 730 mμ, with peaks at 280 and 612.

The results of the electrophoretic studies are shown in Figures 2 to 4. Only those preparations which were at least 3 months old (stored at 4 to 10° C) at the time of electrophoresis and which had been prepared slightly differently (sample 1 in Figure 2, samples 4 and 5 in Figure 3) showed a mobility of ceruloplasmin different from freshly prepared ceruloplasmin from either normal or Wilson’s disease subjects. Such alterations of aged preparations were reported previously (39).

Sample 8 in Figure 2 represents partially purified ceruloplasmin from a second unrelated patient with Wilson’s disease who, so far as known, always had a low serum ceruloplasmin concentration. His ceruloplasmin chromatographed

![Fig. 2. Acrylamide gel electrophoresis, pH 8.9, of ceruloplasmin preparations stained for protein. Samples were applied to a large pore gel (not shown) above the small pore gel. The slowest band is ceruloplasmin. Slot 1 = aged ceruloplasmin from a normal subject. Slots 2, 3, 4, and 9 = fresh preparations from different normal subjects in various stages of purification. Slot 5 = ceruloplasmin from E.C. with Wilson’s disease, partially purified. Slot 6 = E.C.’s crystallized ceruloplasmin. Slot 7 = Ceruloplasmin from E.C. partially converted to apoceruloplasmin with CN⁻ (41). Slot 8 = Ceruloplasmin from the second patient with Wilson’s disease (see text).]
normally on DEAE–Sephadex and had a normal electrophoretic mobility at pH 6.4.

We have confirmed the slower electrophoretic mobility at pH 5.6 (17) of the purified minor ceruloplasmin component eluted from hydroxylapatite (HA II), but we could not show any difference between the mobility of this component from our patient with Wilson’s disease and that from normal subjects (Figure 4). In both cases the minor component comprised approximately 20% of the total ceruloplasmin.

The sugar composition of HA I is shown in Table II together with values reported by others (40–42). The purified ceruloplasmin from our patient with Wilson’s disease is not significantly different from the ceruloplasmin from normal subjects. The sialic acid content of our patient’s ceruloplasmin can be assumed to be normal, although it was not determined because the electrophoretic mobility of his ceruloplasmin was normal. The amino acid compositions of the ceruloplasmin from E.C. and normal subjects do not differ significantly from each other or from the values reported by Kasper and Deutsch (38).

Figure 5 represents the Ninhydrin–stained tryptic peptide map of ceruloplasmin (cer 5.5) from two normal individuals (2U) and from our patient E.C. with Wilson’s disease. There are no differences. Peptide maps of HA I and HA II from E.C. are identical to normal HA I and II. The peptide maps of HA II each have one additional peptide compared to HA I (43). This is the only known difference in chemical composition between HA I and II. Those spots which stain for histi-

TABLE II

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<thead>
<tr>
<th>Carbohydrate composition of ceruloplasmin*</th>
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<td>----------------------------------------</td>
</tr>
<tr>
<td>Sialic acid</td>
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<td>Hexosamine</td>
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<td>Hexose</td>
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<td>Galactose</td>
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<td>Fucose</td>
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* Column I is data reported by Jamieson (40); column II is data reported by Schultze (41, 42); column III is our data.
**Fig. 4.** Starch gel electrophoresis, pH 5.6, of ceruloplasmin preparations stained for protein. Slots 1 and 5 = cer_{s4} from E.C., with Wilson's disease (two different preparations). Slots 2 and 6 = cer_{s8} from normal subjects. Slot 3 = HA II from E.C. Slot 4 = HA I from E.C. Slot 7 = HA II from normal subjects. Slot 8 = HA I from normal subjects. For explanation of cer_{s4}, HA I and II, see text.

**Fig. 5.** Tryptic peptide maps of ceruloplasmin prepared from two normal subjects (2U) and from the patient with Wilson's disease (E.C.). Chromatography was run in the ascending direction.
dine and methionine are the same on the maps from E. C. as on maps from normal subjects. As can be seen, there is a Ninhydrin-positive spot at the origin of the map. Ninhydrin assay (44) of the tryptic digest before and after removal of this core by centrifugation indicated that it comprises approximately 10% of the total protein.

Discussion

When first seen, E.C., a patient with Wilson's disease, had a normal concentration of ceruloplasmin. This has been explained as a consequence of advanced liver failure with increased amounts of estrogen stimulating ceruloplasmin synthesis (45). This patient had remarkably little hepatic disease; consequently, this explanation cannot account for his normal ceruloplasmin concentration.

A structural abnormality in his ceruloplasmin, which altered the essential function of the protein without altering its rate of synthesis, seemed an attractive possibility. On the basis of the tryptic peptide map, electrophoretic analysis, amino acid, and sugar composition reported here, this patient's ceruloplasmin appears normal. Although we do not know the physiological function, if any, of ceruloplasmin, this patient's was normal in its copper content and oxidase activity.

While he was on penicillamine, the patient's ceruloplasmin completely disappeared. A similar effect has been observed in other patients with Wilson's disease (46), but not in patients with schizophrenia (47) or cystinosis (48) who received the drug for long periods of time. If ceruloplasmin synthesis is stimulated by copper (19), it is possible that the defect in this patient is a diminished—but not absent—sensitivity of the ceruloplasmin-synthesizing system to copper. As body copper was lowered with penicillamine, the attenuated stimulus for synthesis was removed. The reappearance of some ceruloplasmin after penicillamine was discontinued is consistent with this theory.

It would appear even less fruitful to look for an amino acid alteration in ceruloplasmin as the underlying defect in those patients with Wilson's disease in whom the ceruloplasmin is persistently low. In one such patient from whom ceruloplasmin was partially purified, we could detect no abnormality electrophoretically. When penicillamine was temporarily discontinued in this patient, ceruloplasmin rose from less than 2 to 6.5 mg per 100 ml after 3½ months.

Without establishing the sequence of amino acids of the entire ceruloplasmin molecule we cannot completely eliminate the possibility of an alteration in amino acid composition. Such an alteration, however, would have to be an ionic substitution and would most likely be in the insoluble core, which is less than 10% of the total protein.

This study failed to confirm a report (17) of an increased electrophoretic mobility of the minor ceruloplasmin component (HA II) purified on hydroxyapatite from a patient with Wilson's disease. In that study HA II purified from normal umbilical cord blood had the same electrophoretic mobility as HA II from a patient with Wilson's disease. Both were electrophoretically faster than HA II from normal adults. In that study, however, one step in purification used in preparing ceruloplasmin from normal adults was omitted in the preparation of ceruloplasmin from the patient with Wilson's disease and also from normal cord blood. This difference in procedure may account for the different mobilities of the minor component. We have observed alteration in mobility associated with minor changes in purification and after refrigeration (4 to 10° C) for periods of more than 3 months.

The current study, together with those of Hirschman and associates (17) and Broman (49), fails to confirm Richterich, Gautier, Stillhart, and Rossi's report (50) that patients with Wilson's disease have only the minor ceruloplasmin component. The relation of the minor ceruloplasmin component to the major one is unclear. Although it has an additional peptide, it is uncertain if its structure is determined by a different gene from that which determines the structure of the major component (43). The tryptic peptide map of the minor component in our patient with Wilson's disease is indistinguishable from the normal minor component. There was, unfortunately, insufficient quantity to determine copper or sugar composition of this component.

Acknowledgment

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