amounts into 125-ml Erlenmeyer flasks and sterilized by autoclaving at 10 psi for 10 min. The inoculum consisted of approximately 0.01 ml of a washed cell suspension, and the incubation period was 48 hr at 35° C. Growth was measured turbidimetrically with a Klett-Summerson photoelectric colorimeter, using the blue filter. No growth occurred in the absence of putrescine, and maximum growth response was obtained at a concentration of approximately 0.5 μg/ml. Growth response of the organism was almost linear between 0.05 and 0.25 μg putrescine/ml. The presence of putrescine did not alter the biotin requirement for this organism. Three additional strains of *N. perflava* have been investigated, and all demonstrate a requirement for putrescine similar to that exhibited by culture #876.

Other diaminic compounds[2] which we have tested for growth response with *N. perflava* (876) indicate that spermidine, agmatine, and cadavarine may be substituted for putrescine. Of these compounds, cadavarine was the least active, and it is possible that its low activity might be due to contamination with putrescine.

Experiments are in progress at this time to determine the extent to which the amino acid content of the medium, as shown in Table 1, can be reduced without materially affecting growth, and to determine what effect these alterations may have on the vitamin requirements of this organism.

References

1. HERBST, E. J., and SNELL, E. E. *J. Bact.*, **58**, 379 (1949).
2. NEMES, J. L., PELCZAR, M. J., and DOETSCH, R. N. *J. Infectious Diseases*, **88**, 156 (1951).

Manuscript received April 23, 1952.

[2] We wish to thank E. E. Snell, Department of Biochemistry, University of Texas, for his kindness in supplying several of these compounds.

Deficiency of Ceruloplasmin in Patients with Hepatolenticular Degeneration (Wilson's Disease)[1]

I. Herbert Scheinberg and David Gitlin[2, 3]

Departments of Medicine and Pediatrics, Harvard Medical School, and The Children's Medical Center, Boston, Massachusetts

Wilson's disease (hepatolenticular degeneration) is associated with abnormalities in copper metabolism (*1*). The liver and the lenticular nucleus of the brain contain abnormally large amounts of copper (*2*), urinary excretion of copper is excessive (*1, 3*), the pigmented Kayser-Fleischer corneal ring, which is characteristic of the disease, presumably contains copper (*1*), and the level of serum copper has been reported to be abnormal (*4, 5*). Since most, if not all, of the copper normally present in serum is bound to ceruloplasmin (*6*), an investigation of this protein in Wilson's disease was undertaken. This paper reports the first results of the study, which show that there is a deficiency of ceruloplasmin in patients with this disease.

Ceruloplasmin is a blue α-globulin, with a molecular weight of 151,000, containing 0.34% copper, or 8 atoms copper/molecule (*7*). Normal plasma contains about 30 mg of the protein/100 ml so that ceruloplasmin constitutes roughly 0.5% of the plasma proteins. The copper appears to be an integral part of the molecule and is presumably responsible for its blue color, the absorption peak of which is at 6100 A.

Spectrophotometric. This method is based on the observation that the blue color of ceruloplasmin disappears on the addition of reducing reagents (*6*), such as ascorbic acid. Blood from fasting subjects was drawn, with precautions to avoid hemolysis. After centrifuging the blood twice and clarifying the plasma by Seitz filtration, light absorption was measured in a Beckman spectrophotometer from 5400 to 6600 A. A cell with a 5.0 cm path length was required. When successive measurements of the spectral curve showed no change with time, a solution of buffered sodium ascorbate was added in sufficient amount to make the plasma concentration 0.27%. Spectra were measured hourly for at least 5 hr at room temperature, by which time (although not much sooner), the maximum decrease in optical density at 6100 A had occurred. The concentration of ceruloplasmin was estimated by dividing this decrease in optical density, corrected for dilution, by the extinction coefficient $\varepsilon^{5\text{ cm}}_{1\%,\ 6100\ \text{A}} = 3.4$.

Immunochemical. Rabbits were immunized with either crystallized or purified ceruloplasmin (prepared and kindly supplied by D. R. Kominz and J. L. Oncley, of the University Laboratory of Physical Chemistry). The antisera obtained, even when crystallized ceruloplasmin was the antigen, gave precipitin tests with other purified plasma proteins, as well as with ceruloplasmin. The antiserum could be rendered specific for ceruloplasmin by repeated absorption with the serum of a patient with Wilson's disease which was particularly low in ceruloplasmin content. The method for the quantitative determination of ceruloplasmin in serum was similar to methods previously published (*8*). Copper determinations were made according to the method of Cartwright, Jones, and Wintrobe (*9*).

The results are shown in Table 1. It is apparent that in each individual the immunochemical method yields consistently higher values for ceruloplasmin than the spectrophotometric method. This is true

[1] This study was supported in part by a grant from the National Institutes of Health, USPHS.

[2] Postdoctoral research fellow of the National Institutes of Health, USPHS.

[3] We are very grateful to D. Denny-Brown, who kindly gave us access to all his patients with Wilson's disease, to J. A. V. Davies, who kindly allowed us to study his patient, D. B., and to L. K. Diamond for generous cooperation in the study of his patients E. M. and M. M. E. L. Rolls and J. A. Murphy provided expert and invaluable technical assistance.

TABLE 1

CERULOPLASMIN LEVELS IN PLASMA AND SERUM OF NORMAL SUBJECTS AND PATIENTS WITH WILSON'S DISEASE

	Spectrophotometric method (mg ceruloplasmin/100 ml plasma)		Immunochemical method (mg ceruloplasmin/100 ml serum)	
Normal subjects	H. M.	16.5		
	H. S.	18.1	H. S.	33.5
	" "	15.4		
	" "	14.2		
	E. B.	15.4	E. B.	25.8
	L. A.	19.0	L. A.	37.6
	N. N.	13.2	N. N.	30.1
	W. Bo.	10.7	W. Bo.	23.6
	W. Be.	12.1	W. Be.	38.7
	Av	15.0	Av	31.6
Patients with Wilson's disease	M. M.	< 2.0	M. M.	4.0
	" "	< 2.0		
	E. M.	< 2.0	E. M.	12.8
	D. B.	< 2.0	D. B.	11.8
	" "	< 2.0		
	" "	2.0		
			E. R.	7.5
			R. K.	6.4
			D. P.	4.3
			M. G.	18.3
			J. G.	9.7
		Av < 2.0	Av.	9.4

both for normal subjects and for patients with Wilson's disease. It is probable that the immunochemical results are a more reliable index of the plasma ceruloplasmin than the spectrophotometric values. In two instances, copper analyses of the specific precipitate were used to calculate the ceruloplasmin content of the serum. In one normal subject, the value obtained, 39.4 mg %, compares with 33.5 mg % by the immunochemical method and 18.1 mg % by the spectrophotometric method. In the other, a patient with Wilson's disease, copper analysis of the specific precipitate gave a value of 4.6 mg % ceruloplasmin. The immunochemical method gave a value of 4.0 mg %, and the spectrophotometric method gave a value of less than 2 mg %.

The reasons for the inaccuracy of the spectrophotometric method are not clear. The mechanism of the reduction of ceruloplasmin by ascorbic acid in plasma is probably complex, as shown by the fact that the time required to reach equilibrium is so long and that optical densities may rise at a given wavelength before finally falling. The low values of ceruloplasmin obtained by this method may in part reflect the increase in optical density at 6100 A, which hemoglobin solutions give on being treated with ascorbic acid, thus diminishing the net decrease due to reduction of ceruloplasmin. Our plasmas usually contained between 1 and 4 mg hemoglobin/100 ml (*10*).

Nevertheless, we have included the spectrophotometric values for two reasons. First, when the decreases in optical density produced in plasma by ascorbic acid at 6100 A and longer wavelengths are plotted against wavelength, the shape of the curve resembles closely curves obtained with solutions of purified ceruloplasmin. This suggests that ceruloplasmin is responsible for the optical density decrease, although not all the ceruloplasmin appears to react. Second, the differences obtained between normal subjects and patients with Wilson's disease are striking enough to make this method a practical way of studying relative plasma ceruloplasmin levels. The immunochemical method, unfortunately, requires purified ceruloplasmin to produce the antisera, and at present very little ceruloplasmin is available.

Patients with Wilson's disease have much less ceruloplasmin in their serum than normal subjects despite the fact that their copper levels may be either higher or lower than normal (*4, 5*). The ceruloplasmin content of their sera is consistently too low to account for the serum copper present. The excess nonceruloplasmin serum copper is not freely dialyzable since measurement of the cerebrospinal fluid copper of three patients with Wilson's disease showed it to be from one tenth to one third the serum level. This is not surprising in view of the fact that other plasma proteins, including albumin and iron-combining globulin, are capable of binding copper, although not as tightly as ceruloplasmin (*6, 11, 12*).

In order to determine whether hepatic cirrhosis, which is characteristic of Wilson's disease, is associated with deficiency of ceruloplasmin, the serum ceruloplasmin content was measured immunochemically in three patients with Laennec's cirrhosis. The values obtained were 27.9, 47.3, and 51.5 mg/100 ml.

Further studies are in progress to determine the role of ceruloplasmin in normal physiology and Wilson's disease. The familial character (*1*) of the disease suggests that it is another example of a pathologic condition related to a congenital deficiency of a specific plasma protein, such as is seen in hemophilia.

References

1. DENNY-BROWN, D., and PORTER, H. *New Engl. J. Med.*, **245**, 917 (1951).
2. CUMINGS, J. N. *Brain*, **71**, 410 (1948).
3. MANDELBROTE, B. M., *et al. Ibid.*, 212.
4. GLAZEBROOK, A. J. *Edinburgh Med. J.*, **52**, 83 (1945).
5. BEARN, A. G., and KUNKEL, H. G. Abstr. 44th Ann. Meeting Soc. for Clin. Invest., Atlantic City, N. J. (1952).
6. HOLMBERG, C. G., and LAURELL, C.-B. *Acta Chem. Scand.*, **1**, 944 (1947).
7. ———. Abstr. No. 291/8, 1st Intern. Congr. Biochem., Cambridge, Eng. (Aug. 19–25, 1949).
8. GITLIN, D. *J. Immunol.*, **62**, 437 (1949).
9. CARTWRIGHT, G. E., JONES, P. J., and WINTROBE, M. M. *J. Biol. Chem.*, **160**, 593 (1945).
10. CROSBY, W. H., and DAMESHEK, W. *J. Lab. Clin. Med.*, **38**, 829 (1951).
11. KLOTZ, I. M., and CURME, H. G. *J. Am. Chem. Soc.*, **70**, 939 (1948).
12. KLOTZ, I. M., FALLER, I. L., and URQUHART, J. M. *J. Phys. & Colloid Chem.*, **54**, 18 (1950).

Manuscript received May 8, 1952.