*CYTOCHROME OXIDASE DEFICIENCY IN WILSON'S DISEASE: A SUGGESTED CERULOPLASMIN FUNCTION**

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Abstract.—The hypothesis is advanced that ceruloplasmin functions in enzymatic transfer of copper to copper-containing enzymes, such as cytochrome oxidase. To test this hypothesis, leucocytes from Wilson's disease patients, heterozygous carriers, and normal subjects were assayed for cytochrome-oxidase activity. The data reported here show markedly reduced levels of activity in Wilson's disease cases and moderate reductions in heterozygous individuals relative to normal controls. These observations and a close correlation between the level of cytochrome-oxidase activity in the leucocytes and ceruloplasmin in the serum tend to support the hypothesis.

Hepatolenticular degeneration (Wilson's disease) is an inherited autosomal recessive disorder, first systematically described by Wilson in his monograph of 1912.[1] The manifestations of the syndrome are virtually ubiquitous. However, the liver, basal ganglia of the brain, cornea, and kidneys are most severely affected. Chronic degeneration of the basal nuclei is associated with degeneration of the hepatic parenchyma, which leads to eventual cirrhosis. The course of the disease is inexorably progressive and ultimately fatal. From the laboratory standpoint, the most consistent salient feature has been severe diminution or occasional absence of serum ceruloplasmin.

Two major hypotheses have been put forward to account for the pathological findings in the study of Wilson's disease: (1) The abnormal gene suppresses the synthesis of normal ceruloplasmin.[2–5] As a consequence of ceruloplasmin deficiency, by some unknown mechanisms, copper absorption from the gut is accelerated. Copper is then deposited in excessive quantities in the tissues. Structural damage and functional impairment ensue in the organs thus involved, particularly the liver, basal ganglia, and renal tubules. The manner in which the damage occurs is not understood. (2) An abnormal protein, presumably determined by the mutant gene, has an enhanced affinity for copper. This, in turn, deprives ceruloplasmin of its copper, blocking its formation; hence the deficiency.[6, 7] A number of objections beset both hypotheses. The first one, although valid as far as it goes, is inadequate inasmuch as it fails to provide a mechanism whereby the functional defects, including excessive absorption, are engendered by ceruloplasmin deficiency. The second hypothesis can be more readily dismissed on several grounds.[8–10]

Independently of its implication in Wilson's disease, ceruloplasmin has attracted the attention of those interested in the "trace" proteins of the serum. The protein has strong oxidase activity towards aromatic diamines,[11–13] a reaction in which copper plays the role of prosthetic group as an electron acceptor.[14] It has been suggested that ceruloplasmin carries out its function by

virtue of this oxidase property.[15] A number of compounds have been invoked as potential substrates, e.g., dihydroxyphenylalanine, epinephrine, serotonin, and ascorbic acid. To date, the evidence supporting this hypothesis has not been convincing.[16] Holmberg[17] discussed the possibility that ceruloplasmin may function as a copper transport protein.

In an effort to explain the protean manifestation in Wilson's disease, the hypothesis was formulated that ceruloplasmin functions principally in the enzymatic transfer of copper to the vital copper-containing enzymes and proteins. Particularly pertinent in this regard is the capability of ceruloplasmin to readily and reversibly dissociate at least four, and perhaps as many as six, of its eight copper atoms.[18, 19] In order to test the hypothesis, a study was made of one of those copper-containing enzymes which might be dependent for their functional integrity on delivery of copper by ceruloplasmin. Among these enzymes, cytochrome oxidase is undoubtedly the most crucial by virtue of its critical role as the ultimate electron acceptor in the oxidative respiration of the cell. It is noteworthy that the copper atoms within the cytochrome oxidase enzyme are the ultimate site of electron transfer. If transfer of copper to cytochrome oxidase is via ceruloplasmin, one might predict that in Wilson's disease, where the serum ceruloplasmin level is drastically reduced, cytochrome-oxidase activity would be correspondingly reduced.

To test this prediction, cytochrome-oxidase activity was assayed in extracts from leucocytes of Wilson's disease patients and healthy controls. Furthermore, individuals known to be heterozygous for Wilson's disease—parents or offspring of confirmed cases—were also ascertained, and their serum ceruloplasmin and leucocyte-oxidase activity were determined.

The results presented here show that individuals with Wilson's disease have extremely low levels of cytochrome oxidase in their leucocytes, comparable to the low levels of ceruloplasmin in their sera. Carrier individuals display levels of cytochrome oxidase in the leucocytes and ceruloplasmin in the serum that are intermediate between those of normal individuals and those of patients clinically affected with Wilson's disease, although these levels are closer to those of the normal individual.

Materials and Methods.—Leucocytes were used as the source of cytochrome oxidase for assay because of their ready accessibility. They were separated from whole heparinized blood by differential centrifugation[20] at 600 rpm for 2 min after the addition of 0.1 ml of phytohemagglutinin *M* per 10 ml of whole blood to agglutinate the erythrocytes. Special care was taken to avoid the inclusion of any red cells in the preparation. The leucocyte preparation was subsequently treated by sonication at 0°C for three consecutive periods of 30 sec each, separated by 30-sec intervals. An equal volume of 0.01 *M* potassium phosphate buffer, pH 7.0, was added. The preparation was thoroughly mixed and centrifuged at a speed of 1000 rpm for 2 min. The supernatant was used for assay of cytochrome-oxidase activity.

Serum was obtained from whole, clotted blood drawn in plain vacutainers. Ferrocytochrome *c* was prepared from 1% cytochrome *c* (Sigma Chemical Co., type VI) dissolved in 0.01 *M* potassium phosphate buffer, pH 7.0.[21] 20 mg of potassium ascorbate were added to 10 ml of cytochrome *c* solution. Excess was removed by dialysis against 0.01 *M* phosphate buffer, pH 7.0, for 36 hr with four changes of buffer. The solution was then frozen for future use.

A Beckman DU-2 spectrophotometer was used for assay of both leucocyte cytochrome oxidase and serum ceruloplasmin. A thermal regulating unit with circulating fluid was attached to the chamber in which the reactions were conducted.

Cytochrome-oxidase assay: The method depends upon the measurement, at 550 mμ, of the rate of decrement in absorbancy by ferrocytochrome *c*. The change in optical density per minute is indicative of the rate of oxidation of ferrocytochrome *c* by cytochrome oxidase. Two controls were used: one in which cytochrome *c* was completely oxidized by 0.1 *M* potassium ferricyanide, and the other in which, by poisoning cytochrome oxidase with 0.1 *M* sodium cyanide, no oxidation was permitted. The procedure was adapted from that described by Smith.[22] To each of three 3-ml cuvettes with a 10-mm lightpath were added 0.3 ml potassium phosphate buffer and 0.3 ml ferrocytochrome *c*. To the blank cuvette, 0.4 ml potassium ferricyanide was added; to the other control, 0.3 ml sodium cyanide was added. Volume in all cuvettes was then adjusted to 2.65 ml by the addition of distilled water. After temperature equilibration at 38°C, the reaction was initiated by the addition of 0.35 ml of the enzyme preparation. The diminution in absorbancy was measured at 550 mμ every 30 sec.

Ceruloplasmin assay: Serum ceruloplasmin-oxidase activity was measured by a method essentially as described by Cox,[23] with only minor modifications. 0.15 ml of serum was added to a mixture of 1 ml of 1.0 *M* sodium acetate buffer, pH 5.3, and 2 ml of 0.2% solution of para-phenylene diamin in the same buffer. The reaction was conducted in 3-ml cuvettes with 10 mm pathlengths in a temperature-controlled chamber at 37°C. The increase in absorbancy at wavelength 530 mμ was recorded every minute. The estimate of activity in milligram per cent was computed from the change in optical density per minute when multiplied by a conversion factor; thus, concentration of ceruloplasmin in milligram per cent = ΔOD per minute times 790.

Results.—The results of serum ceruloplasmin and leucocyte cytochromes oxidase assays on three patients with Wilson's disease and three normal controls are presented in Table 1. Patients B. M. and R. C. are males in their early

TABLE 1. *Results of assays of serum ceruloplasmin and leucocyte cytochrome oxidase of Wilson's disease patients and normal controls.*

	B. M.	Control	A. D. M.	Control	R. C.	Control
Serum ceruloplasmin (mg%)	2.2	38.7	7.7	36.12	1.7	36.98
Cytochrome oxidase (Δ OD/min)*	1.4	31.8	10.8	59.8	2.4	51.0
Cytochrome oxidase/ serum ceruloplasmin	0.636	0.821	1.40	1.65	1.41	1.38

* Mean of five determinations.

twenties. A. D. M. is a 34-year-old female (mother of K. M. and C. M. in Table 2). It is noteworthy that A. D. M.'s ceruloplasmin level is higher than that of the other two patients and that her cytochrome-oxidase activity is increased accordingly. Her relatively higher ceruloplasmin level may be due to the use of oral contraceptives.[24] All three patients present the clinical findings of hepatolenticular degeneration. The diagnosis was further corroborated for R. C. and A. D. M. by the finding of cirrhosis and excess copper deposition on liver biopsy. The controls were all normal, healthy individuals matched for age and sex to the patients with Wilson's disease.

In Table 2, results on four heterozygous carriers, one presumed carrier, and three controls are presented. Both C. M. and K. M. are the offspring of A. D. M., while M. G. and C. L. G. are the parents of an individual who died

TABLE 2. *Results of assays of serum ceruloplasmin and leucocyte cytochrome oxidase of heterozygotes for Wilson's disease and their normal controls.*

	K. M.	C. M.	Control	B. G.	M. G.	Control	C. L. G.	Control
Serum ceruloplasmin (mg%)	30.00	27.52	36.12	24.08	30.10	33.54	17.2	37.8
Cytochrome oxidase (Δ OD/min)*	46.2	40.2	59.8	32.8	42.8	47.0	25.2	53.2
Cytochrome oxidase/ serum ceruloplasmin	1.54	1.46	1.65	1.36	1.42	1.40	1.46	1.41

* Mean of five determinations.

of Wilson's disease at age 15 (autopsy confirmation of clinical diagnosis). B. G. is a sibling of the deceased patient with an *a priori* probability of 2:3 of being a heterozygote.

The results indicate that leucocytes from Wilson's disease patients contain approximately 10 per cent of the cytochrome-oxidase activity found in normal controls. As expected, their serum ceruloplasmin levels are also quite low. The most remarkable feature of these results is the fact that the cytochrome-oxidase activity of the leucocytes is very closely proportional to the ceruloplasmin level in the serum over a very wide range of values. Among heterozygous individuals, the average level of activity for both enzymes is approximately 70 per cent of normal controls. The relatively lower values obtained for cytochrome oxidase on B. M. and his control are due to a different lot of cytochrome *c* substrate used in that assay.

The possibility that an inhibitor in the leucocytes of Wilson's disease patients depressed cytochrome-oxidase activity was investigated (Table 3). Mixtures of extracts from normal and patient's leucocytes were assayed and compared with untreated normal extracts. These studies demonstrate that there is no inhibitory effect by leucocyte extracts from patients with Wilson's disease on normal cytochrome-oxidase activity.

Discussion.—The data above leave no doubt that leucocytes from patients with Wilson's disease are grossly deficient in cytochrome-oxidase activity. It is noteworthy that the cytochrome activities in the cells of patients, heterozygous individuals, and normal controls all correspond closely to the ceruloplasmin level in their sera. Note also that in heterozygotes the levels of both activities are intermediate between normal subjects and patients with Wilson's disease.

TABLE 3. *Cytochrome oxidase assay in mixtures of leucocyte extracts from patients with Wilson's disease and normal individuals.*

Enzyme preparation	Cytochrome oxidase* (Δ OD/min)
0.35 ml normal extract	55
0.175 ml normal extract + 0.175 ml Wilson's disease extract	27
0.35 ml normal extract + 0.35 ml Wilson's disease extract	56

* Mean of two determinations.

We are postulating that deficiency in serum ceruloplasmin may impair the functional activity of cytochrome oxidase by reducing the enzymatic transfer of copper from ceruloplasmin to the oxidase molecule. Although no cause-effect relationship has been demonstrated, the data presented here very strongly support this hypothesis. The situation is comparable to atransferrinemia, where deficiency of transferrin is accompanied by impaired synthesis of an iron-containing compound, hemoglobin, and a concomitant abundant deposit of iron in the tissues. This hypothesis may shed some light on a number of bewildering manifestations of Wilson's disease, which are not satisfactorily explained by an excess copper deposit alone. The energy-dependent, renal tubular reabsorption is substantially impaired in almost all cases of Wilson's disease.[25, 26, 9] Deficient activity of cytochrome oxidase in red cell precursors during erythropoiesis could account for the hemolytic tendency often observed in patients with Wilson's disease. Two further observations are inconsistent with the thought that an excess copper deposit is the sole etiological factor in hepatolenticular degeneration: (1) Administration of copper to experimental animals has failed to mimic Wilson's disease.[27] (2) A relatively poor correlation has been observed between the increased urinary excretion of copper and the clinical improvement which sometimes results from the use of BAL (dimercaprol) or penicillamine.[28]

Another enzyme for which enzymatic transfer of copper by ceruloplasmin might be required, and which may be therefore deficient in Wilson's disease, is tyrosinase. The remarkable success achieved by treating Parkinsonism, a basal ganglia affection, with L-dihydroxyphenylalanine (the immediate product of tyrosinase action on tyrosine) suggests that an impeded reaction at that step might be responsible, at least in part, for the neurological manifestations of Wilson's disease. The validation of this conjecture awaits further investigations.

This finding, of course, raises some puzzling questions concerning aerobic respiration in this disease. Cytochrome-oxidase activity may be substantially greater in other tissues than in the leucocytes. The observation of Baehner and Nathan[29] that leucocytes from patients with Wilson's disease intensively utilize the hexose monophosphate pathway raises the possibility that under these exceptional circumstances certain known shunts may expand to a remarkable degree.

In addition to the proposed function of enzymatic transfer of copper to various copper-containing proteins, ceruloplasmin may serve to control the absorption of copper from the intestine.[17] Its ability to dissociate reversibly half or more of its copper content might help in curbing excess diffusion of copper from the intestinal lumen into the plasma.

These findings illustrate an error that may arise in localizing the site of the defect in a genetically determined biochemical abnormality. In the case of Wilson's disease, had cytochrome oxidase been studied before ceruloplasmin, the genetic defect would probably have been ascribed to genes regulating the production or structure of this enzyme, rather than to those concerned with ceruloplasmin.

Finally, although the necessary prospective studies remain to be done, it

seems quite likely that in addition to the well-established deficiency in ceruloplasmin, deficiency in other enzymes may also be present from birth in patients destined to develop Wilson's disease. This speculation immediately raises questions concerning the nature of the factors that hold the onset of clinical symptoms in check, in the case of Wilson's disease, for some 15–25 years after birth.

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