OBSERVATIONS ON CERULOPLASMIN IN WILSON'S DISEASE * †

By ANDREW SASS-KORTSAK, MORRIS CHERNIAK,‡ DOUGLAS W. GEIGER § AND ROBERT J. SLATER ||

(From the Department of Pediatrics, Faculty of Medicine, University of Toronto, and The Research Institute, Hospital for Sick Children, Toronto, Canada)

(Submitted for publication January 29, 1959; accepted June 19, 1959)

In the light of present knowledge hepatolenticular degeneration is classified as a metabolic disease, probably based on an inborn error of metabolism and transmitted by an autosomal recessive gene. The biochemical manifestations of Wilson's disease have been studied extensively and were summarized in several recent reviews (1–4). The findings point to aberrations in copper and protein metabolism, but the nature of the basic metabolic defect and the mechanism of the disease are still in the realm of speculation.

The most consistent biochemical abnormality in Wilson's disease is the deficiency of the copper containing α_2 globulin, ceruloplasmin. It was suggested that a defect in the production of this protein may be the basic metabolic abnormality, and Wilson's disease has been classed accordingly as one of the pathological conditions resulting from the congenital deficiency of a specific plasma protein (5, 6).

This paper describes, for the first time as far as we are aware, two patients with Wilson's disease and normal serum concentration of ceruloplasmin. These two patients were investigated in detail, including studies with radioactive copper, in order to substantiate their diagnosis and also in the hope that the results might contribute to our knowledge of the basic mechanism of Wilson's disease. For the purpose of comparison a third patient who exhibited all the typical clinical and biochemical characteristics of Wilson's disease was also studied by the same methods.

CASE MATERIAL

Rob. M. and Ron. M. were brothers, sons of second cousins. The older (Rob. M.) had onset of symptoms at 10 years of age and was diagnosed and treated by us for some time as a case of posthepatitic cirrhosis. Two years later his younger brother (Ron. M.) presented at 11 years of age, with hepatosplenomegaly, an indurated liver and slight but definite laboratory evidence of liver dysfunction. This prompted investigation of the sibs for familial causes of cirrhosis. In the course of these studies both were found to have an increased urinary copper excretion and aminoaciduria. Kayser-Fleischer rings were detected in both by slit-lamp examination. Neither of the patients developed neurological signs and symptoms during life. They both ran a progressively downhill course as far as their liver disease was concerned, with bouts of jaundice, and gradual loss of activity and appetite, followed by the development of portal hypertension and intractable ascites. Both died in hepatic coma approximately four years after the onset of signs and symptoms of their disease.

The third patient (Mar. Ch.) exhibited all the characteristic clinical and biochemical manifestations of hepatolenticular degeneration. She was admitted at 12 years of age for the investigation of neurological symptoms suggestive of extrapyramidal tract damage. She had evidence of cirrhosis, biochemical findings typical of Wilson's disease and, in addition, Kayser-Fleischer rings. Her neurological symptoms improved following prolonged treatment with BAL (2,3-dimercaptopropanol) and penicillamine (β,β-dimethyl cysteine); her cirrhosis however progressed relentlessly. She developed portal hypertension and died in hepatic coma following portacaval anastomosis.[1]

METHODS

The method of Eden and Green was used to measure copper in the serum and quantitative 24 hour collections of urine (7). For tissue copper determinations 1 to 30 Gm. of fresh tissue was digested completely in redistilled concentrated nitric acid (with a trace of Antifoam® [2]). The resulting clear solutions were concentrated by further boiling, transferred quantitatively into volumetric flasks and made up to volume with copper-free distilled water. Suitable aliquots were taken then for copper

* This work was presented in part at the Annual Meeting of the Canadian Clinical Investigation Society at Montreal, October 17, 1957.

† Supported by a grant from the National Research Council of Canada.

‡ Elizabeth Arbuthnot Dyson Fellow of the University of Toronto.

§ Metabolic Ward Fellow, Hospital for Sick Children, Toronto.

|| Playtex Park Research Institute Grantee.

[1] Detailed case histories and clinical courses of these patients will be reported in a forthcoming publication.

[2] Dow-Corning.

determination by the method of Eden and Green (7). Duplicate analyses were performed on all urine and tissue samples and most of the sera. Pairs of blank and standard samples were carried through the digestion procedure with each set of determinations. The confidence limits of the method were calculated according to Copeland (8), on the basis of pairs of parallel determinations on 22 normal serum samples. The confidence limits for a single determination were $3\sigma = \pm 6.21$ μg. per 100 ml. in a range of total copper levels from 89 to 150 μg. per 100 ml. serum.

Ceruloplasmin in the serum was estimated by its paraphenylene-diamine-oxidase (PPD oxidase) activity using the method of Ravin (9). The enzyme activity was expressed as the optical density of the diluted reaction mixture as suggested in the original method (O.D. units).

Radioactive copper studies were carried out in two of the patients (Ron. M. and Mar. Ch.). At the time of these experiments both patients were relatively well, afebrile and stabilized on a low and constant dietary copper (1.3 mg. per day) and moderately high and constant protein and caloric intake. Ron. M. had ascites, which was resistant to various forms of treatment, but was stabilized on a diet containing less than 100 mg. of sodium per day. He had most of his ascitic fluid removed by paracentesis (4,500 ml.) four days before the radioactive copper study. Following this his weight remained stable between 32 and 33 Kg. for the next 10 days.

The studies with radioactive copper were carried out in the manner outlined by Jensen and Kamin (10). After a period of six hours, during which only clear fluids were allowed, an oral dose of copper acetate was given which contained 1 mc. activity in 1.1 mg. (Ron. M.) and 1.3 mg. (Mar. Ch.) copper. Heparinized blood samples were taken thereafter at regular intervals for 60 hours and fractional urine collections obtained for similar time intervals. Stools were pooled for 24 hour periods on three consecutive days with the aid of alternating carmine and charcoal markers.

The packed red cell volume was determined in each blood sample, and this showed no significant variation. Cu^{64} activity was assayed on suitable aliquots of plasma, urine and homogenized 24 hour stool collections in a scintillation well counter. Plasma albumin bound activity was measured in the supernate obtained by high speed centrifugation following precipitation of the globulins by 50 per cent saturation with ammonium sulphate (10, 11). Globulin bound activity was calculated as the difference between total plasma and albumin bound activity.

The method of electrophoresis in a starch supporting medium was also used to assess the relative amounts of Cu^{64} bound to albumin and ceruloplasmin (12). Samples of 5 ml. of serum were used for these experiments and after electrophoretic separation, 1 cm. wide segments of the starch block were placed in perspex tubes and packed tightly by centrifugation. Direct counts were obtained on these preparations. The protein concentration was determined by a modified Folin-Ciocalteau technique (13) and was plotted to compare with the Cu^{64} distribution.

RESULTS

1. *Urinary copper excretion*

All three patients had cupriuria of a marked degree. The highest and lowest observed 24 hour urinary copper outputs are shown for each patient in Table I. These figures can be compared to those obtained in three control groups: (*1*) children; (*2*) adults, either normal or suffering from various diseases; and (*3*) children suffering from cirrhosis due to causes other than Wilson's disease. It is evident from the figures that the three patients had cupriuria in excess of the controls and in a range reported by others in Wilson's disease (14, 15). Administration of the decoppering agents BAL, ethylenediaminetetraacetic acid (EDTA) or penicillamine produced a marked increase in urinary copper output in all three patients.

TABLE I

*Ranges of urinary copper excretion, serum paraphenylenediamine (PPD) oxidase activity and total serum copper levels in groups of controls and three patients**

	Subjects	Urinary copper excretion		Serum PPD oxidase activity		Total serum copper	
		μg./24 hr.		*O.D. units*		*μg./100 ml.*	
Controls	Children	11–37	(10)	0.385–0.670	(20)	92–150	(12)
	Adults	<40	(10)	0.345–0.555	(11)	88–124	(12)
	Cirrhosis, children	10–100	(14)	0.214–0.612	(8)	63–220	(6)
Patients	Rob. M.	160–375	(8)			123–169	(3)
	Ron. M.	110–590	(25)	0.320–0.470	(6)	116–175	(5)
	Mar. Ch.	160–920	(13)	0.021–0.054	(10)	28–69	(6)

* Figures in parentheses stand for number of individuals tested in the control groups and number of specimens analyzed in the case of the individual patients.

TABLE II

Results of serum ceruloplasmin assays by various methods in groups of controls and patients included in this study

Method	Controls*			Patients		
	Normal	Cirrhosis	Wilson's disease	Rob. M.	Ron. M.	Mar. Ch.
Manometric PPD† oxidase (16),‡ μMole O_2/ml./hr.	3.6 ± 0.73 2.14–5.06	6.7 ± 0.5	0.9 ± 0.5	2.15	2.20	0.39
PPD oxidase standardized against ceruloplasmin (4, 17),§ mg. %	24 16–33		5 0–14		26.6	
Immunochemical (18),‖ mg. %	34 ± 4 27–38	39 ± 10.3 19–56	9 2–19		22.0	4.0

* In the control groups the means and standard deviations are shown and the lowest and highest values obtained in the group, except in the case of normals by the manometric method where the range of ±2 standard deviations is shown.
† PPD, paraphenylenediamine.
‡ Courtesy of Dr. A. G. Bearn, Rockefeller Institute for Medical Research, New York.
§ Courtesy of Dr. I. H. Scheinberg, Albert Einstein College of Medicine, New York.
‖ Courtesy of Dr. G. E. Cartwright, University of Utah, College of Medicine, Salt Lake City.

2. Serum ceruloplasmin levels

It can be seen in Table I that Mar. Ch's. serum had markedly reduced PPD oxidase activity pointing to reduced serum ceruloplasmin level, a finding typical for Wilson's disease (5, 14, 15). Ron. M., the younger of the two brothers, had his serum PPD oxidase activity measured on six occasions within a period of three years. The values fell within the normal range in all except one sample, in which the activity was minimally reduced but still within the range of children suffering from cirrhosis due to causes other than Wilson's disease. The serum PPD oxidase activity of the older brother (Rob. M.) was assayed by a slightly different enzymatic method elsewhere (16).[3] The result fell within two standard deviations from the mean of normals as shown in Table II.

Because of the unusual nature of the above findings their confirmation was sought by other methods (Table II). Both by a manometric enzyme assay (16)[4] and by a sensitive PPD oxidase method calibrated in terms of spectrophotometrically determined ceruloplasmin (17)[5] Ron. M's serum proved to have a ceruloplasmin content within the range of normal. In a third sample of the same patient's serum the amount of ceruloplasmin was measured by a quantitative immunochemical procedure (18).[6] The result, 22 mg. per 100 ml. serum, was only slightly below the normal range of 27 to 38 mg. per 100 ml., obtained by the same method (Table II). In the same sample PPD oxidase activity measured in our laboratory amounted to 0.340 O.D. unit, a level just at the lower limit of our series of controls (Table I).

3. Serum copper levels

Total serum copper levels were also measured and are shown in Table I. Mar. Ch's. serum copper was tested on six occasions; the range of these figures fell below the range of normals, as expected in Wilson's disease (14, 15). The total serum copper levels of the two M. brothers, however, were within the range of normal or higher than normal. Measurements of "direct" and "indirect reacting" copper were not performed but in a single serum specimen of Ron. M. the copper partition could be calculated from the total copper and ceruloplasmin content. The sample contained 116 μg. copper and 22 mg. ceruloplasmin per 100 ml., the latter by immunochemical determination. Based on 0.34 per cent copper in ceruloplasmin, the copper bound to ceruloplasmin was 75 μg. per 100 ml. By subtracting this figure from the total serum copper content of 116 μg. per 100 ml., the derived value of 41 μg. per 100 ml. for noncerulo-plasmin bound copper was obtained, which amounted to 35 per cent of the total copper in

[3] Courtesy of Dr. A. G. Bearn, Rockefeller Institute for Medical Research, New York.

[4] Courtesy of Dr. A. G. Bearn, Rockefeller Institute for Medical Research, New York.

[5] Courtesy of Dr. I. H. Scheinberg, Albert Einstein College of Medicine, New York.

[6] Courtesy of Dr. G. E. Cartwright, University of Utah, College of Medicine, Salt Lake City.

the serum. Both these absolute and relative amounts of nonceruloplasmin bound copper in Ron. M's case were higher than Markowitz and associates found in normals (18).

4. Copper content of tissues

The results of the tissue copper analyses are shown in Table III. The copper content of tissues was determined in five children 7 to 13 years of age, who died of causes unrelated to Wilson's or liver disease. The results obtained in these children are very close to those found in adults by Gubler and colleagues (19).

In the case of Rob. M., liver was the only tissue analyzed, the specimen having been obtained as a biopsy at the time of shunting operation. The copper content was 77 μg. per Gm. of wet tissue, a more than seven fold increase over the upper limit of copper content given for normal subjects and more than twice the highest value observed in cirrhosis. In the case of Ron. M. and Mar. Ch. post mortem material was available and several tissues were analyzed. Ron. M's brain contained an excess of copper, compared to brains of both "normals" and patients suffering from cirrhosis. The copper content of his liver and kidney was far in excess of controls; these figures in fact are to our knowledge the highest ever reported in a case of Wilson's disease. The same patient's lung and spleen contained much less copper but it was still in excess of controls. Both the brain and the liver of the third patient (Mar. Ch.) contained an excess of copper, the liver far less than in the other two patients. Her kidney contained normal amounts of copper, as did her lung and spleen. This patient had extensive treatment with BAL and penicillamine in the last two years of her life, which may have influenced these results.

5. Cu^{64} studies

Radioactive copper studies were performed on two patients, Ron. M. and Mar. Ch. The results of the total plasma, the globulin and the albumin bound activity measurements, following the oral administration of Cu^{64}, are plotted against time in Figure 1 and can be compared to values obtained in four normal adults by Jensen and Kamin, in experiments of identical design (10).[7]

The response of the normals to a single oral dose of Cu^{64} had the following characteristics: (*1*) An initial rapid rise in total plasma radioactivity was followed by a steep fall. The peak was reached within the first three hours. (*2*) Starting at six to eight hours following the administration of the dose a secondary rise occurred regularly, which was gradual and was maintained for the whole length of the experiment. (*3*) In three out of the four individuals, 60 hours after the administration of the Cu^{64}, the total plasma activity rose higher than the initial peak. (*4*) The plasma globulin bound activity showed a gradual rise during the whole time of the experiment and this accounted for the secondary rise in total plasma ac-

[7] These data are reproduced with the kind permission of the authors and the Journal of Laboratory and Clinical Medicine.

TABLE III

Copper content of tissues in groups of controls and patients

	Subjects	No. of Cases	Brain	Liver	Kidney	Lung	Spleen
					μg./Gm. wet tissue		
Controls*	Children	5	2.5 1.1–3.3	5.8 3.5–9.6	2.9 1.3–4.9	1.3 1.1–1.5	1.3 0.6–2.3
	Adults†	5	6.3 5.1–8.3	5.3 3.0–9.5	2.2 1.8–3.1	1.2	1.0 0.7–1.1
	Cirrhosis† (adults)	12	6.5 3.5–10.2	9.4 3.4–29.7	2.2 1.6–3.5		1.1 0.9–1.5
Patients	Rob. M.			77			
	Ron. M.		24	482	226	11	7.4
	Mar. Ch.		57	29	2.7	1.4	0.9

* In the control groups the means and the range are shown.
† Gubler and associates (19).

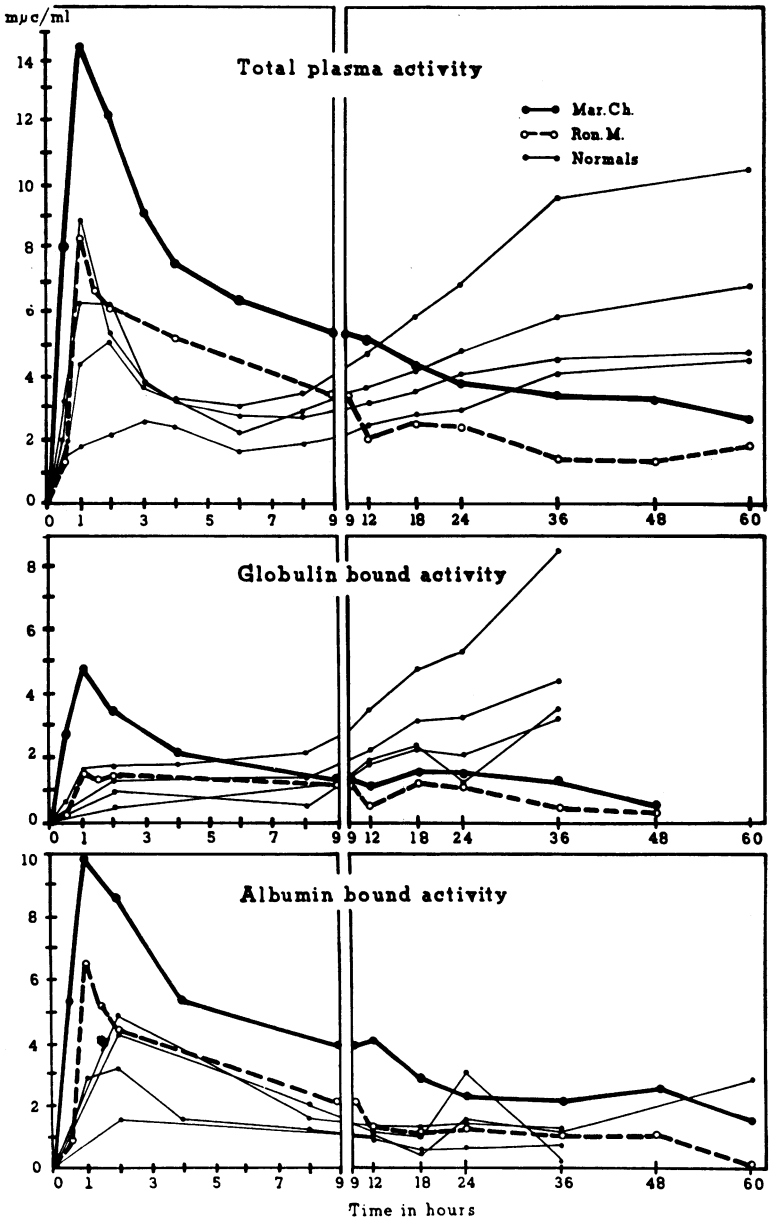

FIG. 1. TOTAL PLASMA, ALBUMIN AND GLOBULIN BOUND ACTIVITY PLOTTED AGAINST TIME FOLLOWING ADMINISTRATION OF SINGLE ORAL DOSE OF Cu^{64}

tivity. (*5*) The albumin bound activity showed an early peak which was followed by gradual decline of the activity in this fraction. The albumin bound activity accounted for the initial peak of the total plasma activity (10, 11, 20).

The patient Mar. Ch., exhibiting all the clinical and biochemical characteristics of Wilson's disease including a markedly reduced serum ceruloplasmin level, responded to the oral administration of a single dose of Cu^{64} in a manner typical for Wilson's disease (10, 11, 20). In her case the initia. peak of total plasma activity was not followed by a secondary rise, but a gradual fall. The albumin bound activity closely paralleled the curve of total plasma activity and the globulin bound activity showed an initial rise followed by a gradual fall instead of the gradual and sustained rise typical for control subjects (Figure 1). This type of re-

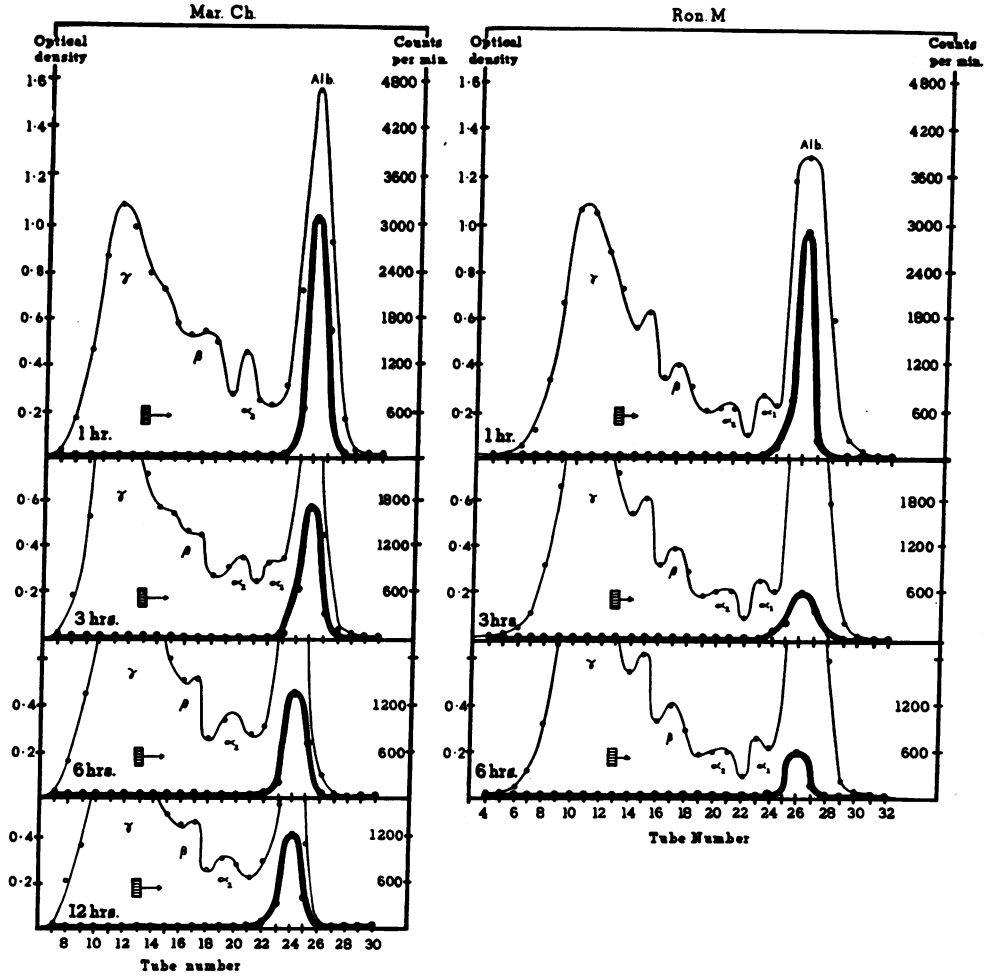

FIG. 2. ELECTROPHORETIC DISTRIBUTION OF Cu^{64} ACTIVITY FOLLOWING ADMINISTRATION OF SINGLE ORAL DOSE OF Cu^{64}

The heavy lines connecting the closed circles show the distribution of radioactivity. The protein distribution curve is represented by the light lines connecting open circles. A cross-hatched box marks the area of the origin and the arrow points in the direction of migration. The time at which the sample was drawn following the administration of Cu^{64} is shown at the lower left corner of the individual plots.

sponse in cases of Wilson's disease does not suggest incorporation of Cu^{64} into ceruloplasmin but was shown by Bush and co-workers to be due to contamination of the 50 per cent ammonium sulfate precipitate with albumin bound Cu^{64} (11).

The results obtained in the case of Ron. M. were of special interest because this patient had a normal serum ceruloplasmin level. In spite of this, he gave a response similar to Mar. Ch. and other cases of Wilson's disease. There was no secondary rise in his total plasma activity and no rise in globulin bound activity but a gradual slow fall following a slight initial rise (Figure 1).

The electrophoretic distribution of the protein bound Cu^{64} activity, observed in the course of the above two experiments, is shown in Figure 2. In the case of Mar. Ch., no measurable amount of radioactivity appeared in the α_2 globulin fraction which contains ceruloplasmin, in sera collected 1, 3, 6 and 12 hours after oral administration of Cu^{64}. Similarly in Ron. M. activity did not appear in the α_2 globulin of sera collected 1, 3, and 6

TABLE IV

Excretion of Cu^{64} in the urine and stool following oral administration

	No. of cases	Urine	Stool
		% of orally administered dose	
Controls*			
Normal†	8	0.1 0.05–0.12	67 45–94
Wilson's disease†	7	1.93 0.2–4.4	55 36–85
Patients			
Mar. Ch.	1	1.12	34
Ron. M.	1	0.83	60

* In the control groups the means and the range are shown.

† Combined data of Bush and colleagues (11) and Jensen and Kamin (10).

hours following the ingestion of Cu^{64}. On the other hand substantial amounts of activity were present in the region of albumin in all sera.

Bearn and Kunkel showed in similar experiments that in normal individuals following the administration of a single oral dose of Cu^{64} radioactivity first appeared in the region of albumin. This was soon followed by an appearance of activity in the α_2 globulin region, whereas the albumin bound activity decreased simultaneously. They found six hours following the administration of Cu^{64} approximately 50 per cent of the activity bound to α_2 globulin, presumably ceruloplasmin (21). This shift of copper from albumin into ceruloplasmin did not occur in cases of Wilson's disease studied by Bearn and Kunkel (21) nor did it occur in our two cases of Wilson's disease (Mar. Ch. and Ron. M.).

Urinary and fecal excretion of Cu^{64} was also measured in both Ron. M. and Mar. Ch. for three days following the oral administration of Cu^{64}. It was found previously by others (10, 11, 20) that patients suffering from Wilson's disease excrete a higher percentage of a single oral dose of radioactive copper in the urine and a lower percentage in the stool than normals. As can be seen in Table IV both Mar. Ch. and Ron. M. excreted a much higher percentage of the administered Cu^{64} in the urine than did normals. Their values fit into the range found in Wilson's disease (10, 11, 20). Mar. Ch. excreted less Cu^{64} in the stool than did normals in similar experiments. Ron. M., the patient with a normal serum ceruloplasmin level, excreted 60 per cent of the orally administered dose in the feces, which is within the range of both normals and Wilson's disease.

DISCUSSION

The finding of normal blood levels of ceruloplasmin is the one important feature of the biochemical picture in the two M. brothers which does not fit the characteristic pattern of Wilson's disease. While this statement is based on a single observation in one of the patients, the other was closely followed for a three year period, during which he maintained his serum ceruloplasmin level within the normal range. Since a deficiency of circulating ceruloplasmin has been considered one of the most constant biochemical abnormalities in Wilson's disease, the finding of normal levels in our two patients may throw some doubt on the validity of the diagnosis. However, we believe that with the presence of practically all other characteristic features of the disease, especially the Kayser-Fleischer rings and the great excess of copper in their tissues, the available evidence is overwhelmingly in favor of this diagnosis.

According to a currently favored theory a genetic defect in the synthesis of ceruloplasmin is the basis of pathological events in Wilson's disease (4). The constant finding of low serum ceruloplasmin levels in these patients has fitted well into this theory. Probably the most convincing evidence was furnished by Scheinberg and associates who found two young patients in whom a profound deficiency of ceruloplasmin antedated all other biochemical, pathological and clinical manifestations of Wilson's disease (22, 23, 24). One of these patients was only 10 months old when he was discovered to have no measurable amounts of ceruloplasmin in the blood. The diagnosis in this patient has not at this time been confirmed beyond doubt, but it was quite probable in view of the finding of an excessive amount of copper in his liver at four years of age and the definite diagnosis of Wilson's disease in one of his sibs (23, 24). The virtual absence of ceruloplasmin from this patient's blood at 10 months of age suggests that a deficiency of ceruloplasmin is not only a constant feature of Wilson's disease but may be present as early as during the first year of life.

There are, however, a number of observations which are difficult to fit into this theory. First, it has not been explained adequately how the deficiency in ceruloplasmin leads to an increased copper absorption in Wilson's disease. Secondly, maintenance of normal blood levels of ceruloplasmin by intravenous administration has apparently failed to produce biochemical improvement (25, 26). In addition, the administration of estrogens resulting in a rise of serum ceruloplasmin levels into the normal range did not influence the course of the disease (1). Furthermore, in several unrelated conditions such as nephrosis, sprue and in a rather ill-defined copper deficiency state in infants, ceruloplasmin deficiency of considerable duration and of a degree comparable to that of Wilson's disease has been observed (18, 27, 28). In none of these disease states does ceruloplasmin deficiency lead to manifestations of Wilson's disease. In addition, it was found that in Wilson's disease the serum ceruloplasmin levels vary quite widely from patient to patient, ranging from "none" to more than fifty per cent of the normal (4, 18). Thus it was recognized that in Wilson's disease the deficiency of ceruloplasmin was of a lesser and more variable degree than in other disease states based on a genetic defect in the formation of a plasma protein, such as congenital agammaglobulinemia, afibrinogenemia and hemophilia. Moreover, correlation could not be established between the degree of ceruloplasmin deficiency and the severity of the disease (18).

According to the findings presented in this paper, a patient suffering from Wilson's disease may even have normal amounts of ceruloplasmin in the serum. To our knowledge this has not been reported in an authentic case of Wilson's disease although Dr. Bearn has kindly communicated to us that he has made the same observation in two other proven patients (29).

The presence of normal amounts of ceruloplasmin in our two patients cannot be related to the fact that they have failed to show signs of neurological involvement. We have observed extremely low levels of ceruloplasmin in two other cases of hepatic Wilson's disease and others have made similar observations (18, 29).

In trying to explain the finding of normal blood levels of ceruloplasmin in a case of Wilson's disease the argument can be raised that environmental factors may have either stimulated a genetically defective synthetic process or slowed the rate of degradation of ceruloplasmin, leading thereby to a rise in ceruloplasmin levels into the normal range at some stage of the disease. Bearn has shown that in Wilson's disease this may occur in response to the administration of estrogen (1). Our patients had gynecomastia and a delay in male sexual maturation, which may have been the result of increase in circulating estrogens secondary to impaired ability of their liver to metabolize such compounds. Unfortunately, measurements of circulating estrogens or urinary excretion were not carried out and therefore no firm conclusions can be drawn in this respect. However, in cases of Wilson's disease with comparable degree of liver involvement, rise in ceruloplasmin levels has not been observed by other investigators. Moreover, Ron. M. had normal levels of ceruloplasmin before the appearance of the signs of estrogenic overactivity. Whatever the reasons may be for the maintenance of normal ceruloplasmin levels in these patients, there remains the question of why the disturbances of copper metabolism persisted and the disease progressed in the absence of a deficiency in ceruloplasmin.

The finding of marked delay in the incorporation of Cu^{64} into ceruloplasmin in Ron. M. may suggest that an abnormality in the metabolism of ceruloplasmin still existed in this patient, which, however, did not affect the blood levels of ceruloplasmin. In connection with this it is of interest to note that Sternlieb, Morell and Scheinberg found no increase in the rate of incorporation of Cu^{64} into ceruloplasmin in a patient with Wilson's disease in whom blood levels of ceruloplasmin were raised into the normal range by intravenous infusion (26).

It was shown previously by others that in Wilson's disease, compared to normals and other types of cirrhosis, there is a marked delay in the incorporation of copper into ceruloplasmin (10, 11, 20). Since there is no evidence to show that copper bound to ceruloplasmin exchanges with ionic copper *in vivo,* these findings could be interpreted as evidence of a defective synthesis of ceruloplasmin. This interpretation fitted in very well with the usual marked reduction in circulating ceruloplasmin in Wilson's disease. In view of these considerations the present findings in Ron. M's. case

are extremely difficult to interpret. In this patient a marked delay in the incorporation of copper into ceruloplasmin was observed in the presence of normal blood levels of ceruloplasmin. If we accept that the delay in the incorporation of Cu^{64} is evidence of a decreased rate of synthesis, the normal concentration of ceruloplasmin in the blood could only have been maintained by a simultaneous decrease of a similar degree in the rate of decay of ceruloplasmin. This seems highly unlikely and to our knowledge such a mechanism has not been proven to exist in the case of any other plasma protein.

Scheinberg and Morell (17, 30) have shown recently that contrary to previous belief, the copper-protein bond in ceruloplasmin can be reversibly dissociated under certain rather unphysiological conditions *in vitro*. Should this occur *in vivo,* indicating that ceruloplasmin may reversibly release and bind copper at various sites in the body, the rate of incorporation of Cu^{64} into ceruloplasmin may reflect the rate of this exchange process as well as synthesis of ceruloplasmin. Consequently the finding of a delay in the incorporation of Cu^{64} into ceruloplasmin in the presence of normal blood levels of ceruloplasmin could point to a disturbance of this hypothetical exchange process. This explanation, however, cannot as yet be seriously considered, since there is no proof whatsoever of the physiological occurrence of copper exchange in ceruloplasmin.

Finally, we have to consider a suggestion first made by Bush and colleagues (11). According to this the failure to measure incorporation of Cu^{64} into ceruloplasmin in Wilson's disease could be due to the fact that the administered Cu^{64} is markedly diluted into the tremendously expanded copper pool of these patients. This suggestion was made following an observation very similar to ours. They found that in a case of Wilson's disease with a moderately reduced serum ceruloplasmin level of 19 mg. per 100 ml. (normal 27 to 38 mg. per 100 ml.) the uptake of labeled copper into ceruloplasmin was just as much delayed as in patients with barely measurable amounts of ceruloplasmin in the serum. Our patient (Ron. M.) certainly had an expanded copper pool as evidenced by an excess of nonceruloplasmin bound copper in his serum and the large copper deposits found in his tissues at the time of death, four months after the radioactive copper studies were performed. Therefore it is possible that our failure to measure incorporation of copper into ceruloplasmin was primarily due to considerable dilution of the isotope.

It will be evident at this point that the observations presented in this study are difficult to interpret. The finding of normal blood levels of ceruloplasmin in two otherwise typical cases of Wilson's disease suggests that a defect in the production of ceruloplasmin may not be the basic metabolic abnormality in this condition, or at the least not in all cases. Nevertheless, the observation of a marked delay in the rate of incorporation of copper into ceruloplasmin in one of these patients may still suggest the presence of an abnormality in the metabolism or function of ceruloplasmin. Concerning this latter point, however, no firm conclusions can be drawn until the real reasons for the generally observed delay in copper uptake into ceruloplasmin in Wilson's disease are clarified by further experimental work.

The observations presented here do not have direct bearing on other theories concerning the pathogenesis of Wilson's disease, especially the theory advanced by Iber, Chalmers and Uzman (31), and therefore no comments can be made in this respect.

In spite of the wealth of new knowledge and a multitude of metabolic studies, the nature of the basic defect in Wilson's disease remains uncertain. One is increasingly impressed by the wide variation of both the clinical and metabolic findings in this condition. Are these all secondary changes and is the primary expression of the basic defect still undiscovered? Or do these variations represent a series of similar syndromes produced by a series of alleles varying among themselves? These are open questions requiring further investigation and especially more detailed family studies, including families with consanguineous parentage.

SUMMARY

1) In two brothers suffering from Wilson's disease normal blood levels of ceruloplasmin were found.

2) Detailed investigation of the two patients revealed the presence of practically all other typical features of Wilson's disease.

3) One of the patients, in spite of the presence of normal blood levels of ceruloplasmin, showed a marked delay in the rate of incorporation of Cu^{64} into ceruloplasmin.

4) The significance of these findings was discussed and related to a currently held theory concerning the pathogenesis of Wilson's disease.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Drs. A. G. Bearn and I. H. Scheinberg for valuable advice and cooperation. We also wish to thank Dr. G. E. Cartwright for the immunochemical determinations of ceruloplasmin.

The authors gratefully acknowledge the invaluable technical assistance of Mrs. Mary Louise Hose, Miss Barbara L. Brown, Miss Jaqueline Bailey and Mr. Donald Mills as well as the staff of the Biochemical Laboratories and the Metabolic Ward of the Research Institute, Hospital for Sick Children.

REFERENCES

1. Bearn, A. G. Wilson's disease. An inborn error of metabolism with multiple manifestations. Amer. J. Med. 1957, **22**, 747.
2. Walshe, J. M. Hepatolenticular degeneration (Wilson's disease). Brit. med. Bull. 1957, **13**, 132.
3. Matthews, W. B. Hepato-lenticular degeneration *in* Diseases of the Liver, Leon Schiff, Ed. Philadelphia, J. B. Lippincott Co., 1956, p. 509.
4. Scheinberg, I. H., Harris, R. S., Morell, A. G., and Dubin, D. Some aspects of the relation of ceruloplasmin to Wilson's disease. Neurology 1958, **8**, Suppl. 1, 44.
5. Scheinberg, I. H., and Gitlin, D. Deficiency of ceruloplasmin in patients with hepatolenticular degeneration (Wilson's disease). Science 1952, **116**, 484.
6. Bearn, A. G. Genetic and biochemical aspects of Wilson's disease. Amer. J. Med. 1953, **15**, 442.
7. Eden, A. K., and Green, H. H. Micro-determination of copper in biological material. Biochem. J. 1940, **34**, 1202.
8. Copeland, B. E. Standard deviation. A practical means for the measurement and control of the precision of clinical laboratory determinations. Amer. J. clin. Path. 1957, **27**, 551.
9. Ravin, H. A. Rapid test for hepatolenticular degeneration. Lancet 1956, **1**, 726.
10. Jensen, W. N., and Kamin, H. Copper transport and excretion in normal subjects and in patients with Laennec's cirrhosis and Wilson's disease: A study with Cu^{64}. J. Lab. clin. Med. 1957, **49**, 200.
11. Bush, J. A., Mahoney, J. P., Markowitz, H., Gubler, C. J., Cartwright, G. E., and Wintrobe, M. M. Studies on copper metabolism. XVI. Radioactive copper studies in normal subjects and in patients with hepatolenticular degeneration. J. clin. Invest. 1955, **34**, 1766.
12. Kunkel, H. G., and Slater, R. J. Zone electrophoresis in a starch supporting medium. Proc. Soc. exp. Biol. (N. Y.) 1952, **80**, 42.
13. Kunkel, H. G., and Tiselius, A. Electrophoresis of proteins on filter paper. J. gen. Physiol. 1951, **35**, 89.
14. Cartwright, G. E., Hodges, R. E., Gubler, C. J., Mahoney, J. P., Daum, K., Wintrobe, M. M., and Bean, W. B. Studies on copper metabolism. XIII. Hepatolenticular degeneration. J. clin. Invest. 1954, **33**, 1487.
15. Bearn, A. G. Genetic and biochemical aspects of Wilson's disease. Amer. J. Med. 1953, **15**, 442.
16. Bearn, A. G., and Kunkel, H. G. Biochemical abnormalities in Wilson's disease (abstract). J. clin. Invest. 1952, **31**, 616.
17. Scheinberg, I. H., and Morell, A. G. Exchange of ceruloplasmin copper with ionic Cu^{64} with reference to Wilson's disease. J. clin. Invest. 1957, **36**, 1193.
18. Markowitz, H., Gubler, C J., Mahoney, J. P., Cartwright, G. E., and Wintrobe, M. M. Studies on copper metabolism. XIV. Copper, ceruloplasmin and oxydase activity in sera of normal human subjects, pregnant women, and patients with infection, hepatolenticular degeneration and the nephrotic syndrome. J. clin. Invest. 1955, **34**, 1498.
19. Gubler, C. J., Brown, H., Markowitz, H., Cartwright, G. E., and Wintrobe, M. M. Studies on copper metabolism. XXIII. Portal (Laennec's) cirrhosis of the liver. J. clin. Invest. 1957, **36**, 1208.
20. Bearn, A. G., and Kunkel, H. G. Metabolic studies in Wilson's disease using Cu^{64}. J. Lab. clin. Med. 1955, **45**, 623.
21. Bearn, A. G., and Kunkel, H. G. Localization of Cu^{64} in serum fractions following oral administration. An alteration in Wilson's disease. Proc. Soc. exp. Biol. (N. Y.) 1954, **85**, 44.
22. Scheinberg, I. H. Relation of ceruloplasmin and plasma copper to hepatolenticular degeneration (Wilson's disease) *in* Progress in Neuro-biology I. Neurochemistry, S. R. Korey and J. I. Nurnberger, Eds. New York, Paul B. Hoeber, Inc., 1956, p. 52.
23. Scheinberg, I. H., Anderson, D. H., Santulli, T. V., and Harris, R. C. Hepatic structure in a child lacking ceruloplasmin (abstract). Gastroenterology 1958, **34**, 1048.
24. Harris, R. C., and Scheinberg, I. H. Hepatic function in a child lacking ceruloplasmin (abstract). Gastroenterology 1958, **34**, 1049.
25. Bickel, H., Schultze, H. E., Grüter, W., and Göllner, I. Versuche zur Coeruloplasminsubstitution bei der hepatocerebralen Degeneration (Wilsonsche Krankheit). Klin. Wschr. 1956, **34**, 961.

26. Sternlieb, I., Morell, A. G., and Scheinberg, I. H. The effect of intravenously administered ceruloplasmin on copper absorption in a patient with Wilson's disease (abstract). J. clin. Invest. 1958, **37**, 934.
27. Butterworth, C. E., Jr., Gubler, C. J., Cartwright, G. E., and Wintrobe, M. M. Studies on copper metabolism. XXVI. Plasma copper in patients with tropical sprue. Proc. Soc. exp. Biol. (N. Y.) 1958, **98**, 594.
28. Zipursky, A., Dempsey, H., Markowitz, H., Cartwright, G. E., and Wintrobe, M. M. Studies on copper metabolism. XXIV. Hypocupremia in infancy. J. Dis. Child. 1958, **96**, 148.
29. Bearn, A. G. Personal communication.
30. Morell, A. G., and Scheinberg, I. H. Preparation of an apoprotein from ceruloplasmin by reversible dissociation of copper. Science 1958, **127**, 588.
31. Iber, F. L., Chalmers, T. C., and Uzman, L. L. Studies of protein metabolism in hepatolenticular degeneration. Metabolism 1957, **6**, 388.