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A Genetic Study of Wilson's Disease: Evidence for Heterogeneity

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Wilson's disease (hepatolenticular degeneration) is an inherited disorder of copper metabolism. Common clinical manifestations are cirrhosis of the liver, extrapyramidal symptoms, and Kayser-Fleischer corneal rings. The clinical, biochemical, and genetic aspects of copper metabolism and Wilson's disease are summarized in several reviews [1–3].

The basic metabolic defect causing the accumulation of copper, mainly in the liver, brain, cornea, and kidney, is unknown. However, serum ceruloplasmin is deficient in most patients. In one series of 111 patients, the ceruloplasmin concentration was below the normal limit in 96.4%; only one of 53 asymptomatic patients had a normal ceruloplasmin concentration [4]. The concentration of serum ceruloplasmin in heterozygotes is usually normal but occasionally reduced [5, 6]. Patients with Wilson's disease do not incorporate radioactive copper into ceruloplasmin [6–9] even when their concentration of serum ceruloplasmin is normal [10]. Heterozygotes given radioactive copper by mouth [5, 6] or intravenously [11, 12] incorporate the isotope into ceruloplasmin at a rate between that of normal individuals and patients. These findings suggest that defective synthesis of ceruloplasmin is a rather fundamental manifestation of the disease. In addition, patients, and to a lesser degree heterozygotes, show an increased total body retention of copper [13].

Clinical and biochemical differences between Jewish patients from eastern Europe and non-Jewish patients from other geographical regions were reported by Bearn [14], suggesting possible genetic heterogeneity.

We have investigated families in which Wilson's disease has occurred. The serum ceruloplasmin concentration has been measured in many known heterozygotes and other relatives. We have examined the relation of a decreasd serum ceruloplasmin concentration to the heterozygous state. Our biochemical, clinical, and radioisotope

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studies of patients and their relatives suggest that there may be at least three genetic types of Wilson's disease.

MATERIALS AND METHODS

Probands

A total of 44 patients with Wilson's disease from 28 families were studied. Fifteen probands were patients at The Hospital for Sick Children, Toronto, and nine were referred there for special investigation. Three were patients at The Montreal Children's Hospital. One was ascertained through a search of hospital records in the larger hospitals in Montreal and Toronto. This series probably includes all patients in Toronto, Montreal, and the larger cities of southern Ontario in whom a diagnosis of Wilson's disease had been made up to mid-1970. Two known patients (French-Canadian females, age of onset 25 years and 16 years) were not included because data were very incomplete for relatives. The 28 probands had 16 affected sibs. The diagnosis was based on clinical features, including in most instances the presence of Kayser-Fleischer rings, and on biochemical assays including measurements of serum copper and ceruloplasmin concentrations, urinary copper excretion, and tests of liver and renal function. To confirm the diagnosis, the incorporation of ^{64}Cu or ^{67}Cu into ceruloplasmin was studied in 22 patients, including all those lacking Kayser-Fleischer rings.

Included among the 44 patients were eight presymptomatic patients and five sibs who died before the proband was diagnosed and who, in retrospect, probably had Wilson's disease. All five sibs died of hepatic failure following relatively brief illnesses. Their clinical course was compatible with the hepatic form of the disease, so that they can be included with reasonable certainty.

Relatives of Patients

Extensive family pedigrees were obtained from each of the 28 families, tracing in almost all cases back to ancestry in Europe and occasionally Asia. Patients were grouped according to geographic and ethnic origin as follows: eastern European (Polish, Russian, Czechoslovak), the region of the Slavic languages, subdivided into Jewish and non-Jewish because of Bearn's previous findings [14]; central European (Austrian, Hungarian, Romanian), in general non-Slavic regions, although Austria particularly has people of mixed origins; western European (British, German, French); Mediterranean (Italian); and Oriental (Chinese). In families residing in Canada or the United States for several generations, the ethnic origin given is that of their nearest foreign-born ancestors.

Four sibs were excluded from the study: one had died in infancy from an unknown cause, one died at 6 years of age of nephrosis, one died accidentally at 12 years, and one apparently healthy brother 31 years of age refused to be tested. A physical examination, measurements of serum ceruloplasmin and copper, and slit-lamp examination for Kayser-Fleischer rings were carried out on all living sibs of the probands, with the one exception noted above. In addition, some had liver function tests, measurement of urinary copper excretion, and studies with radioactive copper. In all sibs in which the disease was suspected, all of the above tests were carried out. Clinically normal sibs were considered to be patients if they showed no incorporation of radioactive copper into ceruloplasmin in the test described below. All but one of these presymptomatic sibs also had a pronounced deficiency of serum ceruloplasmin; one presymptomatic sister and her affected brother both had normal ceruloplasmin concentrations.

Eleven genetically proven heterozygotes (10 parents of children with Wilson's disease and the monozygotic twin of a parent) were studied over periods of 6–7 months to determine the variation in concentration of serum ceruloplasmin and copper. Blood was obtained by venipuncture at monthly intervals on six or seven occasions, and serum sam-

ples were frozen and stored at −14° C. Determinations of serum ceruloplasmin and copper were made on all samples from an individual at the end of the test period to eliminate variation due to technique.

The serum ceruloplasmin and copper concentrations were determined on blood samples obtained (usually on two or more occasions) from 37 other parents of patients with Wilson's disease and from five offspring of patients.

The serum ceruloplasmin concentration was measured in serum samples from 60 sibs in 19 families. In 15 of these families, the ceruloplasmin was assayed in sera from 102 other relatives, including 37 aunts and uncles, seven grandparents, 50 first cousins, seven nieces and nephews, and one first cousin once removed. Quantitative assay was carried out on a total of 214 relatives.

A capillary blood sample for screening for a decreased serum ceruloplasmin concentration was obtained from an additional 167 relatives from 10 of the above 15 kindreds. The relatives included two grandparents, 19 aunts and uncles, 145 first cousins, and one nephew.

Any relatives pregnant or taking oral contraceptives at the time of the study were eliminated from the analysis, since either of these factors approximately doubles the ceruloplasmin concentration [15, 16]. Children less than 3 years of age were also excluded because normal values for this age are not established.

Biochemical Techniques

Blood samples were collected, and serum ceruloplasmin concentration was determined as described previously [17]. The ceruloplasmin concentration was determined by measuring the rate of oxidation of paraphenylenediamine dihydrochloride (PPD) by a 0.15 ml aliquot of serum. The reaction was recorded on a Beckman DK-2 spectrophotometer with a time-drive attachment and temperature regulator. The range of values for normal adults derived by this method are shown in table 1. Values for female relatives less than 12 years

TABLE 1

CERULOPLASMIN CONCENTRATION IN NORMAL ADULTS

	mg/100 ml
Mean ± 1 SD	30.4 ± 5.0
95% confidence limits: (mean ± $t_{.05}$ SD)	20.7 — 40.2
99% confidence limits: (mean ± $t_{.01}$ SD)	17.6 — 43.3

SOURCE.—From a study of 163 adults [17].

of age and male relatives less than 15 years of age were adjusted to adult values as follows [17]: adjusted concentration = observed concentration − 1.17 × (12 − age in years). For males 16 to 19 years of age, the adjusted ceruloplasmin concentration is 1.17 × the observed concentration [17].

Using plasma from capillary blood collected in a hematocrit tube, a screening test based on the oxidation of PPD was performed to identify individuals with a decreased concentration of serum ceruloplasmin [18]. The control serum for each test was appropriate for the age of the individual tested, so that a concentration of ceruloplasmin greater than 2 SD below the mean for each age could be detected. The control sera used had ceruloplas-

min concentrations as follows: age 2–3 years, 30 mg/100 ml; age 4–6 years, 27 mg/100 ml; age 7–9 years, 23 mg/100 ml; age 11 years and older, 20.7 mg/100 ml.

Total serum copper was measured by a modification [10] of the method of Eden and Green [19].

Studies with Copper-64 and Copper-67

The rate of incorporation of ^{64}Cu or ^{67}Cu was studied in 22 patients, 13 parents, 33 other relatives, and five controls. Techniques reported previously for the oral administration of a loading dose [5] and intravenous administration of a tracer dose [11] of ^{64}Cu were used. The total copper administered intravenously was less than 10 μg. The described method of mixing radioactive copper with the patient's own serum was altered for most tests: $CuCl_2$ was mixed with 1% purified human serum albumin just prior to administration. Aliquots of blood were drawn at intervals of 1 hr for the first 6 hr, then at 8-hr intervals over a 3-day period. Counts in plasma and in the ceruloplasmin fraction [6] were converted to percentage of total counts using plasma volumes obtained with ^{131}I-labeled albumin or obtained from tables [20, 21]. The rate of reappearance of radioactive copper in plasma and its incorporation into ceruloplasmin were plotted. Uptake of copper by the liver and its rate of excretion in urine and feces were usually determined. The ^{67}Cu, preferred for its longer half-life, was used with the same techniques but in reduced doses when it became available [22].

RESULTS

Clinical Features of the Probands and Their Affected Sibs

The age of onset of symptoms in the 36 clinically affected patients, classified according to the ethnic origin of their parents, is shown in figure 1. Five patients are shown twice because their parents' ethnic origins differ. The age of onset was later in patients whose parents were both of eastern European origin (mean 27.0 $\pm$ 8.6 years) than in patients whose parents were both of other origins (11.5 $\pm$ 3.6 years). The difference was barely significant ($t = 2.01$; $P = .05$).

The clinical course of the disease differed with age of onset or ethnic origin or both. Early symptoms of the disease were primarily hepatic in 14 of 15 patients with an age of onset 10 years or less and in 11 of 15 patients with age of onset 10–16 years, and primarily neurological in all six patients with age of onset greater than 23 years.

A similarity of age of onset of symptoms within a sibship is apparent in figure 1. Considering only clinically affected individuals, the correlation for age of onset in seven patients with their later-born affected sib is 0.958, which is highly significant ($t = 7.48$; $P < .001$). The age of onset of symptoms, sex, and type of onset are listed in table 2 for the six sibships in which more than one patient was clinically affected or in which a presymptomatic sib has reached the age of the youngest clinically affected patient. The features of the disease are similar in all except W6. (The presymptomatic sister had a low serum ceruloplasmin, 3 mg/100 ml, and high hepatic copper, 150 μg/g wet liver, and handled ^{67}Cu like clinically affected patients.)

The sex ratio of the affected individuals was 1.45:1 (26 males, 18 females), which does not differ significantly from a 1:1 ratio ($\chi^2 = 1.1$). If the excess of males is real, it is probably because, for unknown reasons, there is an excess of

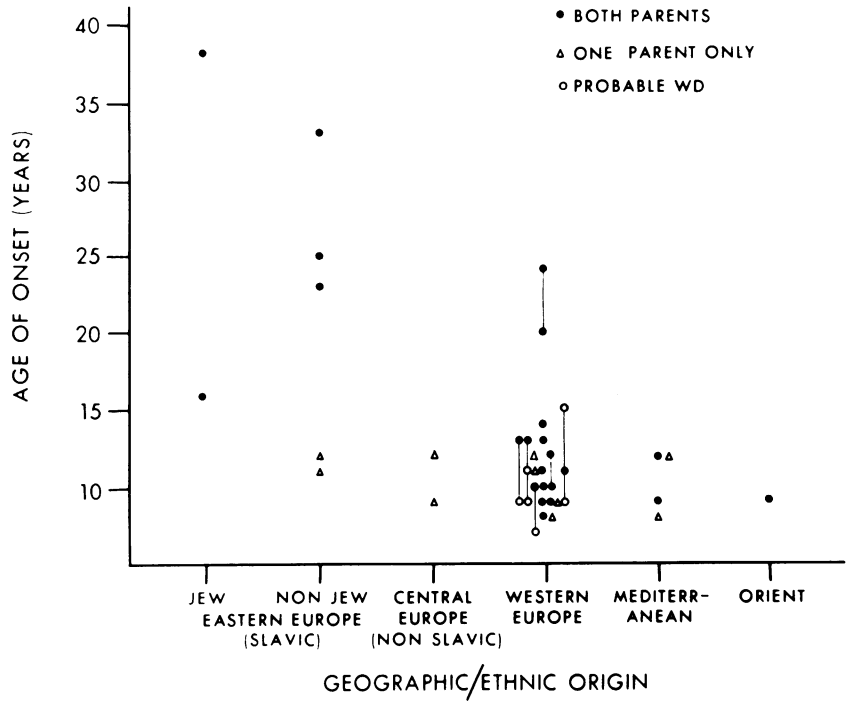

FIG. 1.—Age of onset of Wilson's disease plotted against ethnic origin of patient. Sibs are joined by vertical lines. (Eight presymptomatic patients are not included.)

TABLE 2

ONSET OF DISEASE WITHIN SIBSHIPS

PEDIGREE	PROBAND			SIBS		
	Age of Onset (Years)	Sex	Type	Age of Onset (Years)	Sex	Type
W3	10	M	H	12	M	H
W6	9	M	H	20	F	P
	...	...	...	15*	F	H
	...	...	...	11	M	H
W7	24	M	N	20	F	N
W13	10	M	H	7*	M	H
W24	13	M	H	9*	M	H
	...	...	...	9	M	P
W26	13	M	N	11*	F	H
	...	...	...	9*	F	H

NOTE.—N = predominantly neurologic; H = predominantly hepatic; P = presymptomatic.
* Probable Wilson's disease.

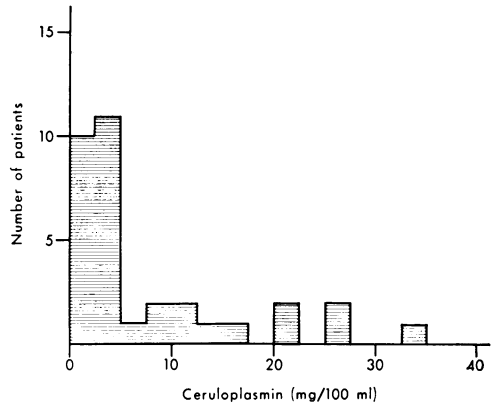

FIG. 2.—Serum ceruloplasmin concentration in 33 patients prior to treatment

males in the patients' sibships (sex ratio 1.65:1; 74 males, 45 females; $\chi^2 = 6.6$; $P < .025$).

The serum ceruloplasmin concentrations in 33 patients prior to treatment are shown in figure 2. Most patients had a pronounced deficiency. A concentration within the normal range was found in one patient (eastern European Jew) whose symptoms began at 38 years of age, in two sibs (western European) with early onset and severe hepatic involvement, and in two sibs (western European), one with severe liver damage and one clinically asymptomatic.

Mode of Inheritance

Our series consisted predominantly of patients whose disease began early in life and was primarily hepatic. In 23 sibships with the age of onset 16 years or less, 38 of 101 individuals were affected. In five sibships with the age of onset greater than 23 years, six of 14 individuals were affected. Both types of sibships were included in the segregation analysis.

Assuming complete ascertainment, the a priori method as outlined by Li [23] yielded an estimated proportion of affected individuals, $\hat{p} = 0.260$, SE $= 0.0509$. The method of discarding the singles [24], which also assumes complete ascertainment, yielded the estimated proportion $\hat{p} = 0.275$, SE $= 0.0526$. The method for very incomplete ascertainment and single selection of probands [23] yielded an estimate of $\hat{p} = 0.184$, SE $= 0.0415$. The value $p = 0.25$ lies between the estimates assuming complete and incomplete ascertainment, respectively, but close to the values for complete ascertainment. Since our survey did indeed approach complete ascertainment, the data support the hypothesis of autosomal recessive inheritance.

The frequency of first-cousin marriages among the parents in this series is three in 28 or 10.7%. One pair of parents were second cousins and one pair third cousins. This is considerably higher than the frequency in the general population and also

supports autosomal recessive inheritance. No known consanguineous marriages had occurred among the five pairs of parents of eastern European origin.

Variability of Ceruloplasmin and Copper Concentrations in Heterozygotes

The ceruloplasmin concentrations were remarkably constant in the 11 obligate heterozygotes over the 7-month period of study, with a mean standard deviation for the 11 heterozygotes of 2.1 ± 0.8 mg/100 ml. The individual values, means, and standard deviations are shown for each heterozygote in figure 3. Two parents

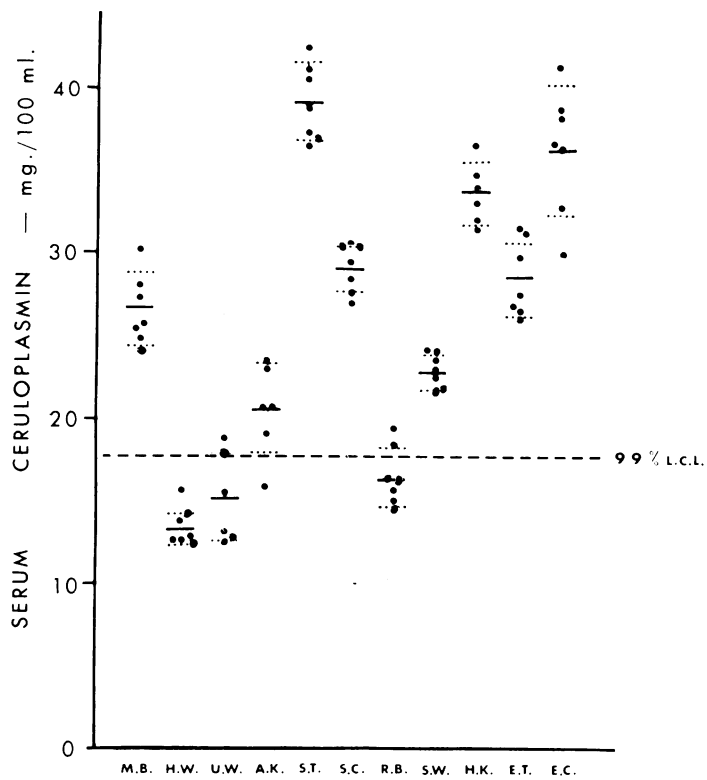

FIG. 3.—Serial determinations of serum ceruloplasmin levels in obligate heterozygotes (10 parents, one monozygotic twin of a parent). Solid bars = mean; dotted bars = 1 SD above and below mean; dashed line = lower confidence limit for normal population.

and the identical twin of one of these had concentrations below the 99% lower confidence limit for normals. The mean ceruloplasmin concentrations for the remaining eight parents studied repeatedly were normal. The serum ceruloplasmin concentration appears to be quite characteristic for any given heterozygote. The serum copper concentrations determined in the same serum samples showed slightly greater variability: the mean standard deviation for the 11 heterozygotes was 9.0 ± 6.9 μg/100 ml. The serum copper concentration was even less effective than the

ceruloplasmin concentration in distinguishing the heterozygote: all parents had a mean serum copper concentration within the normal limits (normal mean, 108 μg/100 ml; 99% confidence limits, 54–162 μg/100 ml [17]).

Mild respiratory infections that occurred during the test period in most of these heterozygotes had no apparent effect on serum ceruloplasmin or copper concentrations. No female heterozygotes were pregnant or taking oral contraceptives at the time of the study.

The serum ceruloplasmin concentration was measured in an additional 37 parents of patients with Wilson's disease, thus including a total of 23 fathers (monozygotic twin excluded) and 24 mothers from 26 families. In addition to the above two parents with low serum ceruloplasmin concentrations, one father (family W23) had a mean ceruloplasmin concentration of 17.6 mg/100 ml (16.4, 17.5, 19.8 mg/100 ml). The ceruloplasmin concentration of all other parents was within normal limits. Of all parents tested, 6.1% had a ceruloplasmin concentration below the 99% confidence limits for the normal population. The parents with abnormally low concentrations were from three different unrelated parent pairs.

The mean serum ceruloplasmin concentration of all obligate heterozygotes tested (47 parents, five offspring) was 30.8 ± 6.87 mg/100 ml, not significantly different from the normal mean.

Ceruloplasmin Concentrations in Other Relatives

The distribution of ceruloplasmin concentrations in the sera of 214 relatives (47 parents, five offspring, and 162 other relatives) is shown in figure 4 compared with the expected normal population distribution. More relatives had a low serum ceruloplasmin concentration than would be expected in the normal population. The mean ceruloplasmin concentration for all 214 relatives was 30.8 ± 8.54 mg/100 ml, not significantly different from that of the normal population.

Of 168 relatives from 10 kindreds tested by the screening test for decreased ceruloplasmin concentration, one had a positive test result. This was in a 3-year-old first cousin once removed in pedigree W11 (fig. 5). Venous blood was subsequently obtained, and his serum ceruloplasmin concentration was 11.9 mg/100 ml. He had been included in the group of relatives whose serum ceruloplasmin was assayed quantitatively. The remaining 167 tests were negative.

In the 26 kindreds in which the ceruloplasmin concentrations of some relatives were determined by quantitative assay or screening, 16 relatives (including parents) with concentrations below the 99% confidence limits were found in four families. None of these relatives appear to be affected. Studies of five sibs, three parents, one uncle, and one aunt, all with low ceruloplasmin concentrations, showed normal urinary excretion of copper, normal tests of liver function, and no abnormal clinical findings, and tested as heterozygotes with ^{67}Cu (see below). The two cousins and the first cousin once removed in pedigree W11 had normal tests of liver function and were clinically normal. They are being followed closely. The young first cousin once removed should be investigated with ^{67}Cu, but permission for this

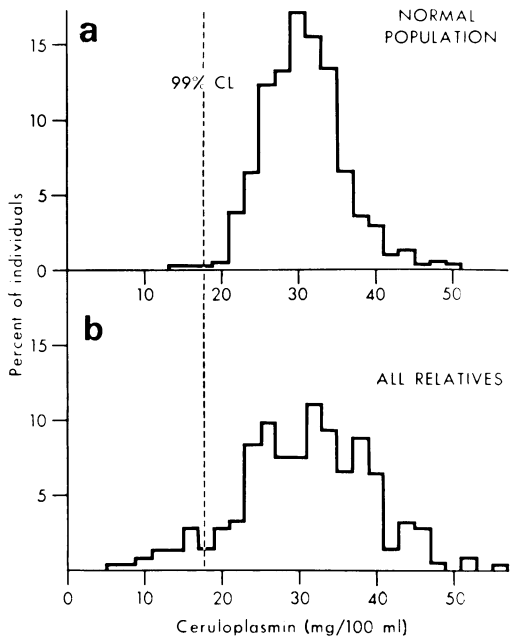

FIG. 4.—*a*, Distribution of serum ceruloplasmin concentration in 309 normal individuals [12]. *b*, Distribution of serum ceruloplasmin concentration in 214 relatives of patients with Wilson's disease.

has been withheld. The three relatives not investigated further were all 44 years of age or more and were clinically normal.

Fifteen of the 16 relatives with low serum ceruloplasmin concentrations cluster in either maternal or paternal relatives in the three families shown in figure 5. Studies outlined below indicate that such individuals are heterozygotes. The remaining relative was a 6-year-old sib in whom the ceruloplasmin concentration was low only after correction for age.

Identification of Heterozygotes with ^{64}Cu or ^{67}Cu

After an *oral* loading dose of ^{64}Cu, a very slow rate of incorporation of ^{64}Cu into ceruloplasmin was shown [5] by the following individuals: III-8 in pedigree W11; III-5, II-1, and II-3 in pedigree W10 (fig. 5). These individuals are probably heterozygous for the gene for Wilson's disease.

All other relatives studied were given an *intravenous* tracer dose of ^{64}Cu or ^{67}Cu. Thirteen patients, eight parents, 12 other relatives, and five normal controls were studied with ^{64}Cu. Nine patients, five parents, and 17 other relatives were studied with ^{67}Cu. Results of these studies have been reported [25] and will be presented in detail in a later publication. The rates of reappearance of copper isotope in plasma and incorporation into ceruloplasmin were the most useful measurements for distinguishing heterozygotes from normal homozygotes and from patients, includ-

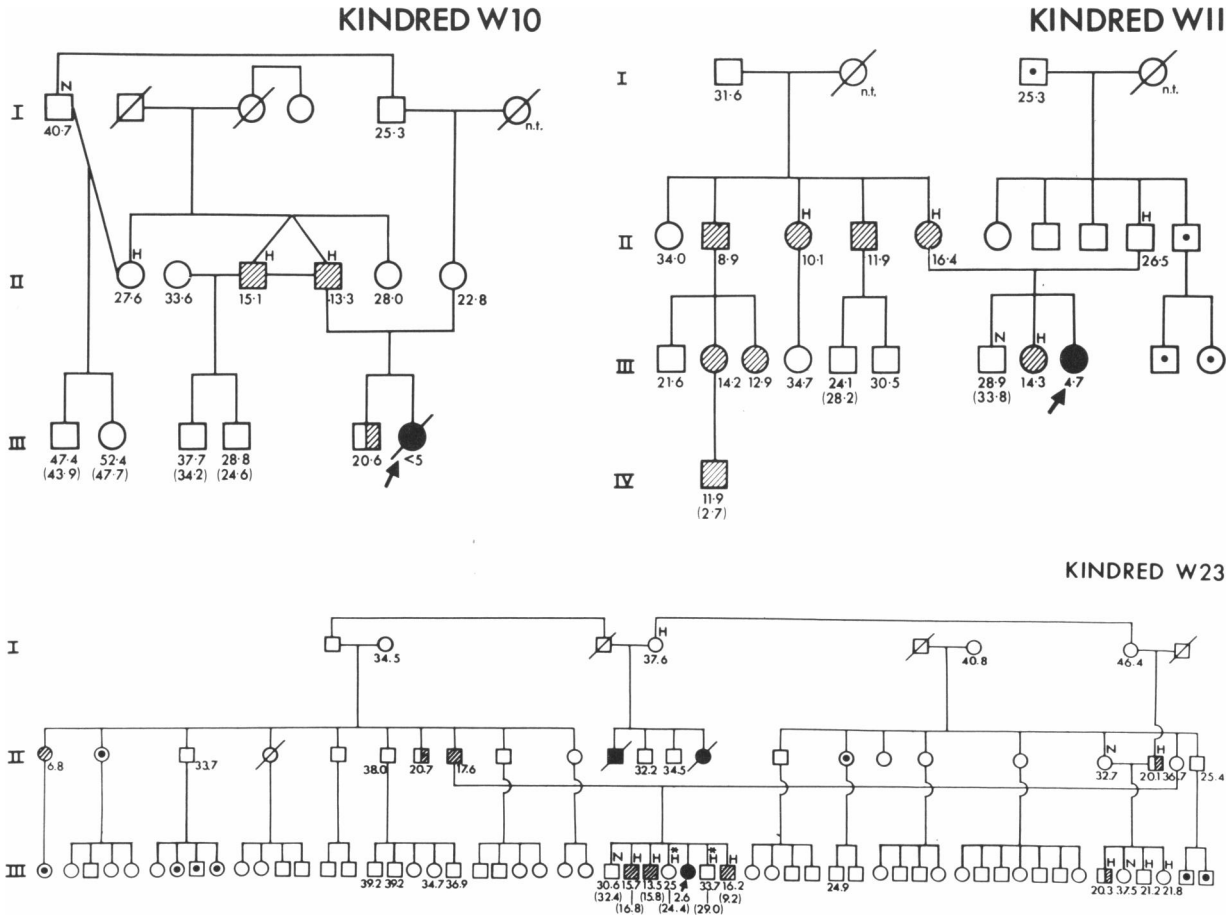

FIG. 5.—Pedigrees of "atypical" kindreds. Figures are ceruloplasmin concentrations in mg/100 ml; values adjusted to adult values, when appropriate, are shown in parentheses. Solid symbols = affected individuals; shaded symbols = individuals with ceruloplasmin concentration below 99% normal population limits; half-shaded = below 95% normal limits; H = heterozygote, N = normal, by ^{64}Cu or ^{67}Cu tests; * = paternal exclusion.

ing those who were asymptomatic. All untreated patients tested to date have not shown any reappearance of copper isotope in plasma or any incorporation into ceruloplasmin. The extent of reappearance of copper isotope in plasma and incorporation into ceruloplasmin in controls, obligate heterozygotes (parents), and patients is shown in figure 6. Copper reappeared in plasma and in ceruloplasmin more quickly in normal individuals than in the obligate heterozygotes. Results for two parents are shown separately. These were the highest values for heterozygotes and partially fell in the normal range. Using the range of values obtained for obligate heterozygotes, it was possible to classify relatives as normal homozygotes, heterozygotes, borderline (not clearly either of these), and affected homozygotes.

Classification of Wilson's Disease

We propose that the three families in which most heterozygotes can be distinguished by their low ceruloplasmin concentrations be called "atypical." (Actually only the maternal or paternal side, not both, is atypical.) The remaining 25 families, which we shall call "typical" can be further subdivided on clinical and ethnic grounds. The late-onset type of disease occurs predominantly among individuals from the regions of the Slavic languages of eastern Europe and we propose calling this the "Slavic" form. The early-onset type of disease occurs predominantly in western Europeans in our series but appears similar to the form found in Orientals [26, 27]. Since this early-onset type is not unique to a particular ethnic group, we propose to call it the "juvenile" form.

Ceruloplasmin Concentrations and Incorporation Studies by Family Type

All three patients with one atypical parent had a ceruloplasmin concentration less than 5 mg/100 ml (mean = 3.5). Values for five Slavic- and 26 juvenile-type patients were 10.9 and 8.0 mg/100 ml, respectively.

The ceruloplasmin concentrations in sera from individuals in kindreds designated as atypical (pedigrees W10, W11, and W23) are shown in figure 7. In all three cases, the parents of the proband represent an "atypical" $\times$ "typical" mating. Only the relatives from the appropriate atypical parent are considered atypical; relatives from the spouse are included among the typical relatives. Sibs of patients from these matings are included, although some of these would be expected to be typical heterozygotes. Heterozygotes, identified by pedigree analysis or radioactive copper studies, are indicated. The mean ceruloplasmin concentrations for obligate heterozygotes, all heterozygotes (obligate plus those classified by tests as heterozygotes), and all relatives in these families are presented in table 3. The mean ceruloplasmin concentrations for both categories of heterozygotes (15.8 ± 2.2 and 14.9 ± 3.4 mg/100 ml, respectively) were significantly lower than the normal mean ($t = 14.6$, $P < .001$; $t = 9.7$, $P < .001$, respectively). The mean for all atypical relatives is below the normal mean but was not analyzed statistically because of the bimodal distribution of the values.

The ceruloplasmin concentrations found in sera of individuals in the typical kindreds are also shown in figure 7. Heterozygotes are indicated. Of the 214 rela-

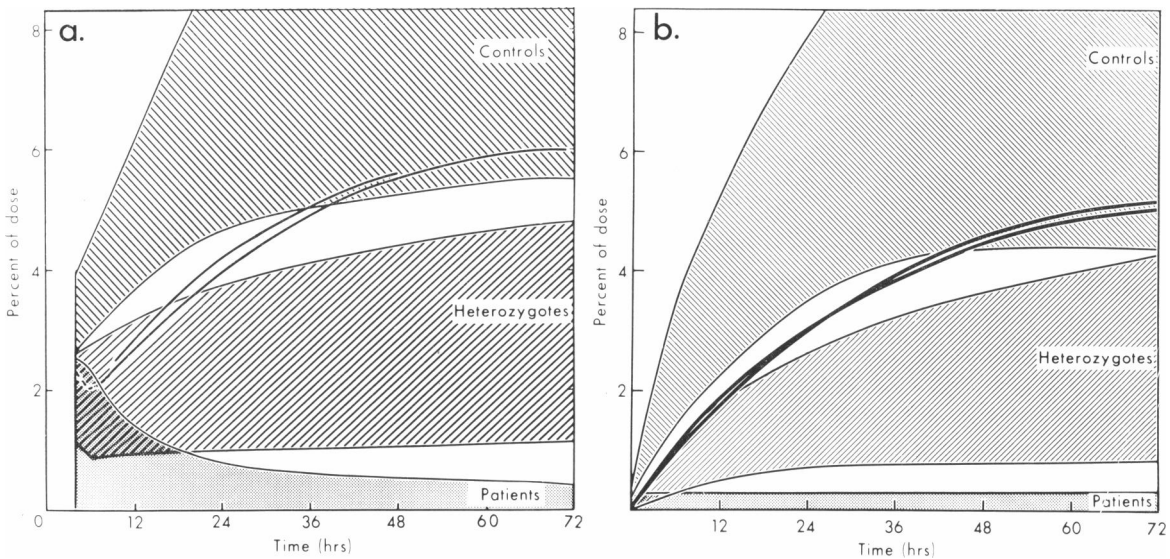

FIG. 6.—*a*, Range of values for rate of reappearance of ^{64}Cu or ^{67}Cu in plasma after intravenous injection in five controls, 16 heterozygotes (parents), and 26 patients. *b*, Range of values for rate of incorporation of ^{64}Cu or ^{67}Cu into ceruloplasmin after intravenous injection in five controls, 19 heterozygotes (parents), and 26 patients. (Two heterozygotes are shown separately.)

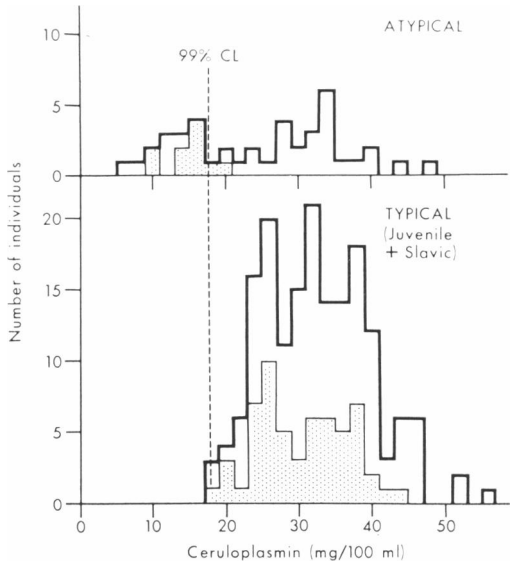

FIG. 7.—Distribution of serum ceruloplasmin concentrations in all relatives of patients with Wilson's disease in "atypical" and "typical" kindreds. Shaded = heterozygotes, by pedigree or by studies with ^{64}Cu or ^{67}Cu.

tives included, 19 are from seven kindreds in which fewer than five relatives were tested, and their classification as typical families is less certain than for families studied extensively. Relatives from families of both the juvenile and Slavic types are included. Mean ceruloplasmin concentrations for these relatives are shown in table 3. None of the mean values in these categories differs significantly from the normal mean.

Interesting differences are noted in the results of the ^{64}Cu and ^{67}Cu incorpora-

TABLE 3

CERULOPLASMIN CONCENTRATION (mg/100 ml) IN RELATIVES

GROUP	TYPICAL		ATYPICAL
	Juvenile $m \pm$ SD (N)	Slavic $m \pm$ SD (N)	$m \pm$ SD (N)
Parents	31.6 ± 5.84 (37)	32.2 ± 6.39 (12)	15.8 ± 2.22*** (3)
All heterozygotes	30.0 ± 6.21 (48)	32.2 ± 6.39 (12)	14.9 ± 3.40*** (10)
All relatives	32.3 ± 7.56 (146)	31.6 ± 5.58 (30)	24.7 ± 11.1 (38)

*** $P < .001$.

tion studies when subjects are classified by type of Wilson's disease. The lowest incorporation rates in heterozygotes occurred in those from the three kindreds designated atypical. Heterozygotes of the Slavic type were unremarkable. However, after 1 or more years of decoppering with penicillamine, both of two Slavic-type patients showed a slight incorporation of ^{67}Cu into ceruloplasmin (up to 0.7% of the administered dose). All of the three patients of the juvenile type still did not show incorporation after at least 1 year of treatment.

DISCUSSION

Our data are compatible with an autosomal recessive mode of inheritance for Wilson's disease, as suggested by others [7, 28–30]. Analyses were carried out assuming both complete and incomplete ascertainment of probands. Ascertainment of probands is probably almost complete for the largest urban centers, from which about 65% of our families originated. In other areas, there is probably a tendency to refer patients to larger centers after the disease has been diagnosed in at least one sib or a sib has died of liver disease. In the present study, criteria for diagnosis have been biochemical as well as clinical. Twenty-five patients in our series had the predominantly hepatic form of Wilson's disease, a form that Levi and co-workers suggested was not inherited in a recessive manner [31]. The contradiction may result from the uncorrected sib ratio in the five families reported by these authors.

The age of onset and presenting symptoms were generally similar within sibships. The correlation between age of onset in a patient and in his later-born affected sib was statistically significant.

In our series, there were more affected males than females, not because of greater risk of males being affected but because of an increase in the total number of males born in the families under study. Bearn [28] reported a significant excess of male patients and also an increased number of males (sex ratio 1.3:1) in the sibships studied. These differences may be chance deviations or may reflect a real difference in these sibships.

The frequency (0.107) of consanguineous marriages among the parents we studied was similar to that in a series reported from England [32], but much lower than in series from New York [14, 28] and Japan [30]. These differences probably reflect a disparity not in gene frequency but in the consanguinity rate of the populations from which the parents originated.

The serum ceruloplasmin concentration in an individual heterozygote fluctuates within a relatively narrow range when technical variation is controlled. The greater variability reported [26] may have been due to technical factors or to a difference in Japanese heterozygotes.

The rate of reappearance of injected ^{64}Cu and ^{67}Cu in plasma and its incorporation into ceruloplasmin have been used to separate heterozygotes from both normal and affected homozygotes. Oral administration of ^{64}Cu, as carried out in our earliest studies, was less satisfactory. In all parents given an intravenous dose of ^{64}Cu or ^{67}Cu, the rate of reappearance of copper in plasma and ceruloplasmin was below the values presumed to be normal; two parents, as indicated, had close to

normal rates. Heterozygotes with exceptionally low rates of copper isotope incorporation may, in addition, have a high concentration of liver copper [33], which would dilute the copper isotope when it entered the liver copper pool. In such cases, incorporation of ^{64}Cu or ^{67}Cu should increase after decoppering of the heterozygotes. This has not yet been investigated.

Biochemical and clinical data from our 44 patients with Wilson's disease and their families suggest that this disease can be classified into three types. In two typical types, the heterozygotes have a normal serum concentration of ceruloplasmin. Of these two types, the Slavic type occurs predominantly among eastern Europeans of the Slavic language group (including both Jews and non-Jews), has a late age of onset, and is primarily a neurological disease. The juvenile type occurs in several ethnic groups, has an age of onset of less than 16 years (lower than in the Slavic type), and the predominant presenting symptoms are usually hepatic, although both hepatic and neurological symptoms occur between the ages of 10 and 16 years. Twenty-nine (81%) of our symptomatic patients are of this juvenile type.

Occasionally, late onset of symptoms occurs in individuals not from eastern Europe—for example, one of our probands of Irish-English origin (fig. 1) and one patient of French-Canadian origin not included in the study, as previously stated. Environmental factors, such as an exceptionally low copper dietary intake, or genetic modifiers, may contribute to the delayed onset of symptoms. Alternatively, if a different allele is involved in the Slavic type, it may be present but in a much lower frequency in other ethnic groups. In a series from England (probably mostly western Europeans but ethnic origin unstated) the median age of onset of symptoms was about 12 years of age for 55 patients [32], similar to our juvenile type. On the other hand, 14 patients (43.8%) from New York City were from eastern Poland and the Ukraine, in the Slavic category we have designated. The increased frequency of the Slavic type in New York compared with our series probably results from differences in the source population. Our patients are drawn from a population which, according to the 1961 Census of Canada, includes 6%–10% of individuals from countries of the Slavic languages. Eastern European patients in New York were all Jewish; however, we find that Jewish and non-Jewish patients from this area are similar. Bearn, recognizing the distinct group of eastern European Jewish patients, suggested that a different mutant gene or a modifier gene might produce clinically different types. Our series has two patients from a Slavic $\times$ juvenile (i.e., not slavic, not atypical) mating, with both having the early-onset type of disease. This could result from different alleles producing the two types, with the juvenile allele dominant to the Slavic allele. More data are required to determine the genetic mechanism producing these two types, or whether there is in fact a genetic basis for this difference. Unfortunately, other published series have not given the ethnic origin of the patients.

The third type of Wilson's disease has been designated as atypical. The patients are clinically similar to those with the juvenile type. The unique feature of this type is that heterozygotes have a serum ceruloplasmin concentration below the

99% confidence limit for the general population, usually about one-half of the normal mean. About 6% of the heterozygous parents in the present series are apparently of this atypical type. Normal healthy individuals seldom have such a low ceruloplasmin concentration: we have unpublished evidence from ^{64}Cu studies that at least one of our previously reported families with individuals with low serum ceruloplasmin concentrations [17] may carry the Wilson's disease gene. Ceruloplasmin concentrations of relatives in the atypical families are bimodally distributed, apparently separating homozygous normals from heterozygotes. In two atypical families, we have evidence from isotope studies that individuals with reduced ceruloplasmin concentrations are in fact heterozygotes. In atypical families, the distribution of relatives with a low ceruloplasmin concentration suggests that the gene for Wilson's disease segregates with the reduced ceruloplasmin concentration. Further evidence for a genetic basis is provided by one father and his monozygotic twin (kindred W10), both of whom have similarly low ceruloplasmin concentrations. The occurrence of affected offspring from matings of typical $\times$ atypical heterozygotes indicates that if different genes for Wilson's disease are present, they must be allelic. The atypical gene could be of German Mennonite origin: all three atypical parents have some German Mennonite, although also British, ancestry. No other families had any German Mennonite ancestors. Several possibly atypical families were found in the literature: families Sm and Q [6], family A [34], and family D. R. W. [35]. Their ethnic origins are not stated.

The recognition of the atypical type of Wilson's disease, perhaps associated with a distinct allele for the disease, is of great clinical importance. In such families, it is extremely difficult to separate the asymptomatic patient from the heterozygote. Every available test procedure, including studies with ^{64}Cu or ^{67}Cu, will be necessary for these families before treatment is initiated. The rate of incorporation of ^{64}Cu or ^{67}Cu into ceruloplasmin is extremely low in these atypical heterozygotes, and we feel that the longer half-life of ^{67}Cu is important for making a valid discrimination. The low serum concentration of ceruloplasmin is probably a consequence of the limited rate of incorporation of copper into ceruloplasmin.

Some hypotheses put forward to explain the clinical and biochemical findings in Wilson's disease have been reviewed [2]. Ceruloplasmin deficiency, suggested as the cause of the disease [36], is probably a secondary manifestation [2]. The physiological importance of ceruloplasmin is not clearly established. It appears to be active in the mobilization of iron stores from the liver [37]. Broman's proposal that ceruloplasmin is involved in the transfer of copper into cytochrome oxidase [38] has some supporting experimental evidence [39, 40]. A defect in Wilson's disease in an amino acid–mediated copper transport system across membranes has been suggested [41]; however, abnormalities of membrane transport can be produced as a secondary effect of excess copper in the tissues [42]. Osborn and Walshe [43] found no defect in copper-concentrating capacity of the liver but proposed a primary defect in the incorporation of copper into ceruloplasmin, which is a prerequisite for biliary excretion of copper.

The most constantly occurring defect found to date in all patients, including

those who are asymptomatic, and to a lesser degree in heterozygotes, is a decreased rate of incorporation of radioactive copper into ceruloplasmin. In addition, copper accumulates in the liver, apparently because of defective biliary excretion. Our hypothesis is that the basic defect lies in a specific intracellular pathway for copper which leads to its incorporation into ceruloplasmin and into the mechanism of biliary excretion not necessarily via ceruloplasmin. An essential carrier may be missing. This carrier may be an enzyme which incorporates copper preferentially into ceruloplasmin and is also required in the pathway of biliary excretion of copper. Other metals require such enzymes for their preferential incorporation into protein or protein precursors—for example, the incorporation of iron into protoheme to form heme mediated by the enzyme iron synthetase or ferrochelatase [44]. The defective enzyme in Wilson's disease might then be a copper synthetase or cuprochelatase. Protein synthesis is required for the elimination of hepatic copper [45, 46], perhaps because of the necessity for production of copper synthetase. The enzyme may add copper in the appropriate form to apoceruloplasmin, the protein portion of ceruloplasmin. This copper incorporation has been demonstrated in vitro but under nonphysiological conditions [47]. Apoceruloplasmin is present in small quantities in normal individuals and in patients with Wilson's disease [48] and has been found in the sera of copper-deficient rats [49]. Copper is not incorporated into ceruloplasmin after apoceruloplasmin has been released into the circulation [50].

Alternatively, a nonenzymatic carrier may be defective or missing. Copper has been shown to pass from albumin into a small-molecular-weight protein in the liver [39]. A copper-containing protein of low molecular weight was previously isolated from liver [51]. Metallothionein, perhaps this same small protein, has been suggested as the site of the defect in Wilson's disease [52]. This small protein may be absent or unable to transport copper within the cell.

We propose that a partially functional form of the "carrier" is present in the Slavic type of Wilson's disease. A slight incorporation of ^{67}Cu into ceruloplasmin has been found, as expected by this hypothesis, in both of two treated patients of the Slavic type. Partial function would result in a slower accumulation of copper in the tissues and a relatively late age of onset of symptoms. Patients of the juvenile type have a more pronounced impairment of carrier function, therefore little capacity for ceruloplasmin production or for biliary copper excretion and an earlier age of onset of symptoms than in the Slavic type. Heterozygotes have a normal concentration of serum ceruloplasmin, suggesting that their one normal gene has a reserve capacity for synthesis. According to our hypothesis, atypical patients have a completely nonfunctional carrier, particularly in its role of incorporation of copper into ceruloplasmin. We predict that their ceruloplasmin concentration would not increase with pregnancy or estrogen therapy. Atypical heterozygotes have a severely limited capacity for ceruloplasmin production and have ceruloplasmin concentrations usually about one-half of the normal mean. Since the function of their normal gene is limited, an inhibitor of the enzyme or hypothetical carrier may be present.

The site of the defect is not necessarily identical in the three types of disease and could involve several mechanisms.

The evidence presented here suggests that Wilson's disease is heterogeneous. Reexamination of data obtained by other investigators is now required to determine the validity of these findings. Recognition of three distinct types of disease may clarify the results of biochemical and metabolic studies.

SUMMARY

Studies have been carried out in 28 families in which Wilson's disease has occurred. Diagnosis of the 44 affected individuals was based on clinical and biochemical criteria and studies with ^{64}Cu and ^{67}Cu. In 81% of our patients, the onset of symptoms occurred before 16 years of age and presenting symptoms were primarily hepatic. Recessive inheritance was confirmed.

In 11 obligate heterozygotes, the concentration of serum ceruloplasmin showed relatively little variation. The serum ceruloplasmin concentration was below normal in three (6.1%) of 47 parents of patients. Ceruloplasmin concentrations were measured in a total of 214 relatives. A screening test for an abnormally low ceruloplasmin concentration was carried out on sera from an additional 167 relatives. Some relatives were identified as heterozygotes by their reduced rate of incorporation of ^{64}Cu or ^{67}Cu into ceruloplasmin.

The data suggest that there are three types of Wilson's disease, probably genetically different. There are two typical types in which heterozygotes have normal concentrations of serum ceruloplasmin: the "juvenile" type usually becomes manifest before 16 years of age, is frequently a hepatic disease, and affects western Europeans and several other ethnic groups; the "Slavic" type has a late age of onset, is predominantly a neurological disease, and occurs mainly among eastern Europeans from the regions of the Slavic languages. The third type of Wilson's disease, which we have called "atypical," is characterized by heterozygotes with a low serum ceruloplasmin concentration. Clinical features are similar to those in the juvenile type. A possible genetic basis for these differences is discussed.

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The National Science Foundation has established a new program to enhance understanding of the human cell. The program will provide preferential support for multidisciplinary research teams investigating problems that require coordinated efforts, and support services that will facilitate research using cultured cells.

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Prospective applicants should direct inquiries to the Cellular Biology Section of the Division of Biological and Medical Sciences, National Science Foundation, Washington, D.C. 20550.