ABSTRACT

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The concentration of ceruloplasmin, the major copper-containing protein in serum, varies widely among individual persons. A study of 309 individuals indicated that the ceruloplasmin concentration changes with age, declining from early childhood to about 12 years of age. Significant familial influences, mainly hereditary, were indicated by family and twin studies.

The gene for recessively-inherited Wilson's disease (hepatolenticular degeneration), is known to accompany a pronounced ceruloplasmin deficiency in affected homozygotes. Ceruloplasmin concentration was determined in 33 parents and 228 other relatives in 25 families of patients with Wilson's disease. In certain 'atypical' kindreds, heterozygotes showed abnormally low ceruloplasmin levels, apparently due to their particular genotype. The infrequent patients with Wilson's disease who have a normal ceruloplasmin level probably carry the same defective gene as that in ceruloplasmin-deficient patients. Studies of rats provided evidence that liver copper concentration influences ceruloplasmin production. A new hypothesis, proposing the absence of a copper chelatase or incorporating enzyme as the primary defect in Wilson's disease, was presented.

Short Title:

CERULOPLASMIN: GENETIC AND ENVIRONMENTAL INFLUENCES

E.D. WILSON COX

GENETIC AND ENVIRONMENTAL INFLUENCES

ON THE

SERUM PROTEIN CERULOPLASMIN

by

E. Diane Wilson Cox

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INTRODUCTION

A protein which formed a cerulean blue solution when purified was isolated from human and pig sera by Holmberg and Laurell in 1948 and was named "coeruloplasmin". Twenty years later, this protein still holds many mysteries.

Impetus for the investigation of ceruloplasmin was provided by the discovery that sera from patients with Wilson's disease have a reduced serum copper oxidase activity due to a relative deficiency of ceruloplasmin (Bearn and Kunkel, 1952; Scheinberg and Gitlin, 1952). Wilson's disease, or hepatolenticular degeneration, is an inherited human disease characterized by increased deposits of copper in many body tissues.

The present study was undertaken to determine what factors influence variation in the concentration of ceruloplasmin in the serum of normal individuals and how such variation might apply in Wilson's disease.

The study is reported in two separate sections. In Section I, present knowledge of ceruloplasmin is reviewed and the author's investigation of some of the factors causing variation in serum ceruloplasmin levels in the normal individual are presented. Section II considers ceruloplasmin in Wilson's disease. New data on the genetic aspects of Wilson's disease is analyzed. Ceruloplasmin levels in relatives of patients with Wilson's disease have been investigated and a new pattern of familial variation has been observed. Qualitative studies have been carried out where applicable. Experimental studies of the effect of penicillamine administration to rats have been carried out to help determine the reason for normal ceruloplasmin levels in certain unusual patients with Wilson's disease.

SECTION I

A STUDY OF SERUM CERULOPLASMIN LEVELS IN NORMAL INDIVIDUALS

REVIEW OF THE LITERATURE

A. Physical and Chemical Characteristics of Ceruloplasmin

Ceruloplasmin is an alpha$_2$-globulin first isolated and purified by Holmberg and Laurell (1948). The name "coeruloplasmin" was suggested by these authors because of the intense blue color of the compound. Almost all, or about 90 to 95 per cent, of the copper in human serum occurs in the form of ceruloplasmin (Holmberg and Laurell, 1947).

Several methods for the large-scale isolation and preparation of ceruloplasmin are available (Broman and Kjellin, 1964; Deutsch, 1960; Deutsch, Kasper, and Walsh, 1962; Morell, Aisen, and Scheinberg, 1962).

The molecular weight of ceruloplasmin has been calculated as 159,000 (Kasper and Deutsch, 1963), a figure somewhat higher than that of 151,000 determined by Dr. K. Pederson on the original purified preparations (Holmberg and Laurell, 1948). The molecule contains eight atoms of copper, or a copper content of 0.32 per cent (Holmberg and Laurell, 1948; Kasper and Deutsch, 1963).

The absorption spectrum of ceruloplasmin shows a broad absorption band with a maximum at 605 to 610 mμ. and a band in the ultraviolet with a maximum at 280 mμ. corresponding to the non-specific protein absorption maximum in this region (Holmberg and Laurell, 1948). The extinction coefficient in a 1 cm. cuvette, $E^{1\%}_{610}$, is 0.68 (Holmberg and Laurell, 1948; Kasper and Deutsch, 1963). The absorption peak at 610 mμ. and the blue color of ceruloplasmin disappear in the presence of reducing agents such as ascorbic acid (Holmberg and Laurell, 1948).

This reversible decolorization is not a reversible oxygenation dependent on the presence of molecular oxygen in the protein molecule (Morell et al., 1964).

Ceruloplasmin shows weak oxidase activity toward a number of polyphenols, particularly to p-phenylenediamine (Holmberg and Laurell, 1951b).

The eight atoms of copper in ceruloplasmin do not have identical properties. Approximately four of the copper atoms are relatively easily exchanged, in vitro, with ionic copper (Scheinberg and Morell, 1957). When the protein is digested with chymotrypsin, four copper atoms are rendered dialyzable (Curzon, 1958). Only about 50 per cent of the copper atoms, those in the cupric (Cu^{2+}) state, can be detected in electron paramagnetic resonance studies (Blumberg et al., 1963; Broman et al., 1962; Kasper, Deutsch and Beinert, 1963). Magnetic susceptibility measurements confirmed that half of the copper is in the cupric state (Ehrenberg et al., 1962). Cupric ions or their specific bonding are responsible for the blue color and the oxidase activity of ceruloplasmin (Broman et al., 1962; Blumberg et al., 1963).

The nature of the copper-protein bond in ceruloplasmin has not been completely determined. Copper is not removed from ceruloplasmin by the chelating agent ethylenediaminetetraacetic acid (EDTA) at pH 5.5 (Levine and Peisach, 1963), but is partially removed at pH 7.4 (Kasper and Deutsch, 1963). When ceruloplasmin is treated with Tris, EDTA, or ascorbic acid at pH 7.4, it is partially converted to a more acidic colorless component with higher anodal mobility on electrophoresis (Kasper and Deutsch, 1963). The colorless derivative contained four atoms of copper per molecule of protein. The molecular weight was

similar to that of native ceruloplasmin, but changes in sedimentation properties indicated an increase in the axial ratio. These results indicate that unfolding of the ceruloplasmin occurs after some of the copper is lost. Kasper and Deutsch have concluded, on the basis of their studies, that copper plays a role in the secondary and tertiary structure of ceruloplasmin. Apoceruloplasmin, ceruloplasmin from which all of the eight copper atoms have been removed, can be prepared using sodium diethyldithiocarbamate in the presence of a reducing agent, followed by dialysis (Morell and Scheinberg, 1958; Morell et al., 1964). These authors have reported that ceruloplasmin with the same properties as that of native ceruloplasmin can be reconstituted from the apoceruloplasmin by adding to it Cu^{2+} in the presence of ascorbic acid at pH 5.4. There are no stable intermediates formed with more than zero or less than eight atoms of copper per molecule (Aisen and Morell, 1965). Apoceruloplasmin has an increased axial ratio compared to that of native ceruloplasmin (Kasper and Deutsch, 1963). The reconstitution of ceruloplasmin from apoceruloplasmin could be due to the refolding of the protein in the presence of Cu^{2+}. While this reaction can proceed in vitro under the given conditions, an enzymatic process could be required under physiological conditions.

Acid-base and spectrophotometric titration studies indicate that histidyl and either lysyl or tyrosyl residues in the protein are concerned with the binding of copper (Kasper and Deutsch, 1963). The results of their spectrophotometric titrations did not appear to implicate tyrosine, although the evidence was equivocal.

Broman (1964) has suggested that the eight atoms of copper in ceruloplasmin may be clustered in a ligand-bound core. The structure of ceruloplasmin has not been elucidated sufficiently, for example by crystallographic techniques, to establish this. In a recent symposium, Blumberg (1966) has discussed information available from physical studies regarding the ligands and positions of the cupric sites in ceruloplasmin. Two of the ligands are weak and may be water molecules; the other ligands are of two types with different relative bonding strengths which produce distortions in the complex. Some speculations about the positions of cuprous and cupric ions in ceruloplasmin have been discussed at the conclusion of the previously-mentioned symposium.

Poulik (1962) converted human ceruloplasmin into subunits which appeared to maintain copper but could not be recombined to restore enzymatic activity. Subunits of ceruloplasmin with a suggested molecular weight in the range of 50,000 were similarly obtained by Kasper and Deutsch (1963). Alkali treatment or succinic anhydride treatment produced discrete subunits representing half-molecules, quarter-molecules, and eighth-molecules (Poillon and Bearn, 1966). Two separate bands of equal intensity appeared on polyacrylamide electrophoresis of the eighth-molecule subunits, suggesting to these authors that ceruloplasmin is composed of two kinds of polypeptide chains, identical in molecular weight but differing in charge.

The carbohydrate portion of human ceruloplasmin has been analysed by Jamieson (1965) and contains per mole: nine moles of sialic acid, 18 moles of N-acetylglucosamine, two moles of fucose, and 36 moles of hexose. Jamieson has suggested that the carbohydrate portion of ceruloplasmin contains nine or ten chains of similar size, but probably of

different composition terminating in sialic acid and joined to the protein moiety via asparaginyl residues. Aspartic and glutamic acids are the most abundant amino acids present in the native protein (Kasper and Deutsch, 1963). Peptide fingerprinting has been carried out after tryptic digestion which left an undigested core of less than ten per cent of the molecule (Holtzman et al., 1967).

Evidence for heterogeneity of ceruloplasmin was presented by Broman (1958), Uriel (1958), and more recently by numerous investigators. Morell and Scheinberg (1960) found four different ceruloplasmins and suggested that the differences were genetic in origin. Differential synthesis of two forms of ceruloplasmin was implicated in Wilson's disease (Richterich et al., 1960). A slower minor electrophoretic component has been found in pooled plasma (Poulik and Bearn, 1962) and in sera from pregnant women (McAlister, Martin and Beditt, 1961) suggesting that a normal minor component may be present in the serum but in a very low concentration. However, it is not clear whether this component is present in vivo or is induced, for example, by aging of the serum (Poulik and Bearn, 1962). Various treatments applied to ceruloplasmin have been shown to alter its physical and chemical properties (Kasper and Deutsch, 1963; Poulik, 1963) as well as its immunological properties (Kasper and Deutsch, 1963). Some of the heterogeneity found therefore appears to be an artifact caused by partial denaturation of the protein. The electrophoretic variant found by McAlister, Martin and Benditt (1961) occurred in a Caucasian and appeared to be genetically determined. No other variants were found in 250 other sera tested. Several genetic variants have been found in Negroes (Shreffler et al., 1967). These authors have not found variants among hundreds of sera from Caucasians suggesting that variants are rare in this racial group.

Reviews of studies on the physical and chemical nature of ceruloplasmin have been presented by Laurell (1960) and Malmström and Neilands (1964).

B. Methods for the Assay of Ceruloplasmin

Immunological Methods:

Immunological methods for the assay of ceruloplasmin have been described (Scheinberg and Gitlin, 1952; Markowitz et al., 1955; Hitzig, 1961). A highly purified preparation of human ceruloplasmin is used to stimulate the production of ceruloplasmin - specific antibody, usually in rabbits. The antibody preparation can be further purified by absorption on sera, from patients with Wilson's disease, which lacks ceruloplasmin.

This method is not used routinely because it is less simple than enzymatic methods.

Colorimetric Methods:

The strong blue color of ceruloplasmin with its peak at 610 mμ. is measured before and after its decolorization. An adaption of the method used by Holmberg and Laurell (1951b) using ascorbic acid as the decolorizing agent has been described by Scheinberg and Morell (1957). Sodium cyanide is a more effective decolorizing agent (Scheinberg et al., 1958).

This method is particularly suitable for the determination of ceruloplasmin in purified preparations and is used to standardize the enzymatic methods.

Enzymatic Methods:

Ceruloplasmin shows weak oxidase activity toward a number of substrates (Holmberg and Laurell, 1951a). The best substrates are p-phenylenediamine (PPD) and its derivative N,N-dimethy-p-phenylenediamine (DPPD). This oxidase activity can be used as the basis

for the quantitative measurement of ceruloplasmin, the only known serum component to show such activity.

The oxidase activity can be measured by determining the uptake of oxygen manometrically (Holmberg and Laurell, 1951a), or more conveniently by measuring colorimetrically the formation of the colored oxidation products. A stable purple product is formed in the final oxidation step with PPD as substrate (Peisach and Levine, 1963).

A method utilizing the oxidation of DPPD was described by Akerfeldt (1957). A lag period was present in the reaction with some sera and was found to correlate with the amount of ascorbic acid in the sera (Akerfeldt, 1957; Aprison and Grosz, 1958). The presence of a long lag period in some sera, accentuated by the use of a short period of measurement of the oxidase activity and a large proportion of serum in the reaction mixture, make this method unsuitable. The cause of the lag period has not been fully elucidated, although ascorbic acid is known to play a role (see discussion by Sass-Kortsak, 1965).

A method described by Broman (1958) is suitable for use with purified ceruloplasmin preparations, for which it was described. The reaction mixture included 4×10^{-5}M ethylenediaminetetraacetic acid (EDTA) to remove contaminating copper ions which can oxidize the substrate, PPD. The short incubation period of ten minutes makes the method unsuitable for use with serum as the lag period

could appreciably affect the results, particularly when the ceruloplasmin level is low.

Suitable methods for measurement of the oxidase activity have been described by Scheinberg and Morell (1957), Houchin (1958), and Ravin (1961). The Houchin method uses a ten-fold dilution of serum and a 15 minute incubation period, so that the lag period is probably insignificant at least with normal sera. The effect of the lag period is further minimized in the Ravin method by greater serum dilution and a one hour incubation period. In both of these methods, the extent of oxidation after an incubation period is measured. The method described by Scheinberg and Morell is not influenced by a lag period because the rate of the reaction is measured. If a lag period is observed, the reaction rate can be measured after it has ended. A revised version of this method is available (Scheinberg, Morell, and Hayes, 1962). Because the reaction can be observed throughout its course, this type of method offers advantages in kinetic studies or in circumstances where the lag period is expected to be prolonged, as in individuals with little serum ceruloplasmin or in inhibition studies.

The advisability of using EDTA in the reaction mixture is open to question. Its use has been suggested to prevent the non-enzymatic oxidation of PPD by copper (Broman, 1958). However, under certain conditions, ceruloplasmin is altered by treatment with EDTA (Kasper and Deutsch, 1963). Levine and Peisach (1963) have shown that Fe^{2+} (ferrous) ions stimulate PPD oxidation at low concentration

and inhibit it at high concentration. The effect of Fe^{2+} ions is less with DPPD as the substrate (Curzon and Cumings, 1966). The addition of EDTA is recommended by these authors (Levine and Peisach, 1963; Curzon and Cumings, 1966) to chelate Fe^{2+} ions which would influence the oxidation rate. However this effect was found with purified preparations of ceruloplasmin and is not a factor in assays of serum since iron is bound to transferrin.

Many other ions have been found to influence the oxidase activity of ceruloplasmin including thiocyanate, chloride, bromide, fluoride, nitrate, sulphate, and acetate, (Holmberg and Laurell, 1951c; Curzon, 1960). Standardized test conditions should avoid effects of these ions.

Rapid Screening Tests:

A rapid screening test, based on the oxidase activity of PPD, has been described for distinguishing ceruloplasmin levels below that of a given standard (Aisen et al., 1960). An automated screening technique is being employed in the 'Genetic Alert' program sponsored by the National Foundation for Neuromuscular Diseases (Scheinberg, 1967, personal communication).

C. Physiological Function of Ceruloplasmin

Oxidation:

Hydroquinone, catechol, pyrogallol, dopa, adrenalin, and ascorbic acid were shown by Holmberg and Laurell (1951b) to act as substates for ceruloplasmin, although the most effective substrate was the non-physiological compound p-phenylenediamine. Soveral physiological substrates: adrenaline, dihydroxphenylalanine, and serotonin are slowly oxidized by ceruloplasmin (Curzon, 1961). None of these weak oxidation reactions are known to be of physiological importance. Indeed Broman (1964) has suggested that ceruloplasmin may be "just an ineffective oxidase in the wrong place, lacking a suitable substrate".

Copper Transport:

About half of the eight atoms of copper per ceruloplasmin molecule were shown to exchange with ^{64}Cu *in vitro* (Scheinberg and Morell, 1957). This finding led these authors to suggest that a reversible release of copper from ceruloplasmin might regulate the absorption of copper from the intestinal tract. However, later studies indicates that there was no exchange of copper from ceruloplasmin *in vivo* (Sternlieb *et al.*, 1961a).

Another transport function for ceruloplasmin has been suggested by Broman (1964). He suggested that ceruloplasmin transports copper into cytochrome oxidase. There is no supporting evidence for this hypothesis to date and the normal functioning of individuals lacking ceruloplasmin and freed from toxic accumulations of copper suggests that there is no concurrent deficiency of cytochrome oxidase.

Copper Excretion:

The incorporation of copper into ceruloplasmin has been suggested as a prerequisite for its excretion via the bile (Gaballah et al., 1965; Osborn and Walsh, 1967). Evidence indicates that this is not true. Very little ceruloplasmin per se is excreted in the bile (Jeunet, Richterich, and Aebi, 1962). The possibility that catabolized ceruloplasmin could provide the copper excreted via the bile was tested by Aisen et al. (1964) by injecting rabbit ceruloplasmin labelled with ^{64}Cu into rabbits. Less than five per cent of the ^{64}Cu was recovered in bile or feces, indicating that the copper of catabolized ceruloplasmin is not preferentially or directly excreted via the bile. Following the oral administration of a dose of copper to humans, there is a rapid increase in the biliary copper excretion within thirty minutes or less (Van [illegible], 1944). In the isolated perfused rat liver, in which the metabolism of ^{64}Cu appears to be the same as in the intact rat, the peak biliary excretion of ^{64}Cu occurs between one and two hours after administration (Owen and Hazelrig, 1966). Since ceruloplasmin has a half life of about five days and its copper cannot be exchanged in vivo until the protein is degraded (Sternlieb et al., 1961a), the biliary excretion of copper could not show such a rapid response if dependent upon ceruloplasmin degradation.

D. Factors Affecting Ceruloplasmin Levels in Normal Individuals

Pregnancy and Estrogenic Hormones:

High levels of copper late in pregnancy were shown to correlate with high oxidase activity toward p-phenylenediamine due to the presence of increased amounts of ceruloplasmin (Holmberg and Laurell, 1951a). By immunological assay, ceruloplasmin levels about twice those in non-pregnant women were observed in women in the last trimester of pregnancy (Scheinberg, Cook, and Murphy, 1954; Markowitz et al., 1955). These high levels contrasted markedly with the low levels found by these authors in cord serum. Many later investigators have confirmed these findings.

The administration of estrogens results in the elevation of ceruloplasmin levels (Russ and Raymunt, 1956). A similar effect was observed in some patients with Wilson's disease treated with estrogens (German and Bearn, 1961). Oral contraceptive preparations containing estrogenic hormones cause an increase in ceruloplasmin levels to about twice the normal levels (Carruthers, Hobbs, and Warren, 1966).

The above results suggest that an increased production of estrogens in pregnancy causes the increase in ceruloplasmin levels.

Sex and Age:

Females have been reported to have higher copper levels than males (Cartwright et al., 1960; Neale and Fischer-Williams, 1958). Ceruloplasmin levels were reported to be higher in females than males, however the sample consisted of only 30 individuals (Cartwright and Wintrobe, 1964).

Levels of serum copper and ceruloplasmin alter appreciably during the early months of life. Newborns have very little ceruloplasmin in their serum (Holmberg and Laurell, 1951; Scheinberg, Cook, and Murphy, 1954; Pojerova and Tovarek, 1960; Richterich, 1961). Pojerova and Tovarek (1960) found that the ceruloplasmin levels had risen from low newborn levels to normal adult levels by one year of age. Levels higher than those of normal adults were found at the age of one year by both immunological (Hitzig, 1961) and enzymatic (Richterich, 1961) assay methods. Ceruloplasmin levels in older children have not been investigated. In studies of whole blood from children from one month to 15 years of age, the highest levels of copper were found during the second year (Sachs, Levine and Fabian, 1936).

E. Ceruloplasmin Levels in Disease States

Hypoceruloplasminemia:

A pronounced deficiency of serum ceruloplasmin is found in Wilson's disease (hepatolenticular degeneration), to be discussed in detail in Section II. This finding has provided stimulation for many of the studies on ceruloplasmin.

Low levels of serum copper and ceruloplasmin are found in the nephrotic syndrome because of loss of the ceruloplasmin in the urine (Markowitz et al., 1955).

Hypoceruloplasminemia is found in kwashiorkor, a form of malnutrition with inadequate protein intake, adequate calorie intake, and multiple vitamin deficiencies (Reiff and Schneiden, 1959). Serum ceruloplasmin levels are also decreased in diseases such as celiac disease and sprue where absorption of copper from the intestinal tract is impaired as shown by studies with ^{64}Cu (Sternlieb and Janowitz, 1964).

Serum ceruloplasmin levels are usually increased in patients with liver disease. However occasional patients with fatal hepatitis, chronic hepatitis, or cirrhosis of the liver with fatty degeneration have been reported with abnormally low ceruloplasmin levels (Walshe and Briggs, 1962; Galt, Stein, and Aronoff, 1966). Studies of mouse liver culture (Hochwald, Thorbecke, and Asofsky, 1961) and isolated perfused rat liver (Owen and Hazelrig, 1966) indicate that ceruloplasmin is synthesized in the liver. When the liver is sufficiently damaged, its synthetic functions fail and ceruloplasmin synthesis is decreased.

Unlike patients with Wilson's disease, these patients with low ceruloplasmin levels secondary to liver disease can incorporate copper into ceruloplasmin (Walshe and Briggs, 1962).

Hyperceruloplasminemia:

Many pathological conditions are associated with an increase in the serum concentration of ceruloplasmin.

The ceruloplasmin and copper levels are increased in acute and chronic infections of many kinds (Brendstrup, 1953; Markowitz et al., 1955). Other diseases that are associated with increased ceruloplasmin levels and correspondingly increased levels of serum copper are rheumatoid arthritis, myocardial infarction, hemochromatosis, leukemias, and various forms of neoplasms (Lahey et al., 1953; Koch, Smith, and McNeely, 1957; Sullivan and Hart, 1960).

Patients with chronic and acute liver disease frequently have high levels of serum copper and ceruloplasmin (Pineda, Ravin, and Rutenberg, 1962; Walshe and Briggs, 1962; Gault, Stein, and Aronoff, 1966). The levels are particularly increased in the presence of obstructive jaundice, probably because the biliary excretion of copper is prevented. An increased level of copper was found in the liver of patients with primary biliary cirrhosis (Hunt et al., 1963).

Controversy has arisen regarding reports of an increase of ceruloplasmin levels in schizophrenia, which was proposed as an aid to diagnosis. However, the increased DPPD oxidase activity found in schizophrenics and other patients with mental illness was explained only partly by

an increase in the levels of ceruloplasmin, and was partly due to reduced levels of ascorbic acid in the serum (Akerfeldt, 1957). While the mean level of ceruloplasmin of a group of patients with schizophrenia may be higher than the normal mean, there is considerable overlap and the ceruloplasmin level has therefore no diagnostic application (Scheinberg et al., 1957; O'Reilly, 1961). The increased oxidase activities can be explained by the use of tests which are affected by reducing substances, such as ascorbic acid, in the serum and by the presence of infections or inflammatory reactions in some patients (Horwitt et al., 1957).

Other references on ceruloplasmin and copper levels in disease can be found in review articles by Sternlieb and Scheinberg (1961) and Sass-Kortsak (1959).

THE PRESENT STUDIES

PART I

Factors Influencing Serum Ceruloplasmin Levels in Normal Individuals

Studies on the effects of pregnancy and hormone administration on the serum ceruloplasmin levels in healthy individuals have been reviewed previously. An increase in the ceruloplasmin level during the early months of life has been found, but factors influencing the levels in normal healthy individuals beyond infancy have not been investigated by others.

Such information is required for assessing abnormal findings in families in which Wilson's disease has occurred. The present study was undertaken to determine the extent of daily variation of the ceruloplasmin level and possible effects of age, sex, body weight, the menstrual cycle, and familial factors. Serum copper levels have also been measured for comparison with the ceruloplasmin levels. Some of the results of these studies have recently been published (Cox, 1966).

No existing assay method for ceruloplasmin was found completely satisfactory for our purposes. An enzymatic method was used because of simplicity and rapidity. Methods which measured the amount of oxidation product formed at the end of a specified incubation period were not considered suitable because of the possible effect of the lag period. The effect could be significant when measuring very low levels of ceruloplasmin or in inhibition studies.

A method in which the rate of the oxidation reaction was measured, using PPD as substrate, was therefore developed. This method is based on that described by Scheinberg and Morell (1957) and later revised (Scheinberg, Morell, and Hayes, 1962). Conditions which increased the enzymatic activity were chosen so that smaller samples of serum could be used.

MATERIALS AND METHODS

The Quantitative Assay of Ceruloplasmin:

The rate of oxidation of paraphenylenediamine dihydrochloride (PPD) was measured in a Beckman DK-2 recording spectrophotometer with a time drive attachment and a temperature-regulated cell holder through which water was circulated from a constant temperature water bath. The water bath was a cylindrical pyrex container, four and one half gallon capacity, controlled by a Bronwill constant temperature circulator. The reaction was carried out at 30.0 ± 0.05°C, as measured in the cell holder. The actual reaction mixture was 0.1°C lower.

All glassware was washed in dilute nitric acid and rinsed thoroughly with deionized distilled water.[1] Deionized distilled water was also used for the preparation of all solutions. P-phenylenediamine dihydrochloride (PPD) was recrystallized as follows (Scheinberg, Morell, and Hayes, 1962): 33 g. of PPD (Fisher certified), 90 ml. concentrated HCl, 60 ml. water, and 2 g. stannous chloride were heated. After adding a further 150 ml. water, heating was continued until as much of the PPD as possible dissolved. Activated charcoal, 3g., was added and the mixture was filtered under pressure through Whatman No. 50 paper with further small additions of hot water. To the filtrate,

[1] Prepared by passing distilled water through cation exchange resin.

chilled in an ice-salt bath, were added 120 ml. concentrated HCl. The filtrate and its precipate were allowed to stand overnight in the refrigerator. The crystals, pure white in color, were filtered off on a Büchner funnel, washed with a few ml. HCl, dried in vacuo over NaOH, and stored in a tightly capped vial. All of the above precautions avoided contamination of the reaction mixture with traces of iron and copper which leads to non-enzymatic oxidation of PPD.

Into a 1 cm. path-length cuvette was placed 2 ml. of acetate buffer (1.0 M, pH 5.2), prewarmed to 30°C. and 1 ml. of a 0.4 per cent solution of recrystallized PPD in the same buffer at room temperature. The PPD solution was freshly prepared for each batch of five to ten determinations. The cuvette was placed in a small container in the water batch for about two minutes while the previous test was being completed. To this reaction mixture was added 0.25 ml. of serum. The cuvette was covered with parafilm and the contents were mixed. The cuvette was left in the cell holder for two minutes for temperature equilibration. The change of optic density (ΔO.D.) was recorded at a wavelength of 530 mμ, against a distilled water blank, for six to eight minutes. The slope of the recorded line was measured to give ΔO.D./minute.

A solution of purified human ceruloplasmin[1] was used to calibrate the method. The ceruloplasmin concentration in this solution was

[1] Prepared at the Connaught Medical Research Laboratories, Toronto, from outdated human plasma collected by the Canadian Red Cross Society from volunteer donors. Preparations were kindly donated to us through the courtesy of Dr. A. M. Fisher.

measured by a spectrophotometric method[1] (Scheinberg et al., 1958) using sodium cyanide as the ceruloplasmin decolorizer. Three or four different small measured aliquots of the ceruloplasmin solution were each added to samples of the same serum, keeping the total volume constant, and the enzyme activity was measured as described above. A straight line was obtained when ΔO.D./minute was plotted against the concentration of added ceruloplasmin. The reciprocal of the slope of this line was used to provide the factor to convert ΔO.D./minute to ceruloplasmin concentration in mg. per 100 ml. (or mg. per cent). This calibration was repeated five times using two different preparations of purified ceruloplasmin and sera from two normal individuals, and two patients with Wilson's disease and a pronounced ceruloplasmin deficiency (two calibrations were carried out on one of the latter sera). A standard serum, stored frozen in suitable aliquots, was tested along with each calibration curve and was measured in duplicate with each batch of determinations. Under the given conditions, using the mean of the five calibrations:

Concentration of ceruloplasmin, in mg./100 ml. = ΔO.D./min. x 830

The calibration factor varies between laboratories and must be determined for each laboratory.

The method can also be carried out at 37°C , a more convenient temperature to maintain in some laboratories. We have found this

[1] Since a 1 cm. path-length cuvette was used, the concentration of ceruloplasmin in mg./100 ml. was found by dividing optical density at 610 mμ. by the extinction coefficient $\left(E^{1\%}_{1\,cm.}\right)$ 0.68.

temperature easier to maintain in our laboratory and now prefer its use. The faster rate of reaction at 37°C with other conditions unchanged allows the use of 0.15 ml. serum. The results of this study, carried out at 30°C, are pertinant at 37°C as only the conversion factor differs. At 37°C:

Concentration of ceruloplasmin, in mg./100 ml = ΔO.D./min. x 730.

Other Methods:

Total copper was determined in most sera, using the method of Eden and Green (1940) as adapted by Sass-Kortsak et al. (1959).

The statistical methods used were described by Steel and Torrie (1960).

Test Subjects:

Venous blood was obtained from 309 unrelated normal Caucasian individuals ranging in age from two to 81 years. These individuals consisted of children attending Toronto nursery, elementary, and secondary schools; university students; hospital and university personnel; and service club members. All were apparently healthy and free from respiratory and other infections.

Blood samples were usually drawn after an overnight fast but in a few cases four or more hours after breakfast. Plastic syringes, disposable needles, and acid-washed collection tubes were used. Serum was separated by centrifugation from the clotted blood.

Individuals were grouped by age and sex into children (males up to and including 14 years, females up to and including 12 years), adolescents (males 15 to 19 years, females 13 to 16 years), and adults.

Age limits selected for adolescent groups were based on the occurrence of physical and physiological changes of puberty (ref. text: Nelson, 1964).

Family Studies:

Blood samples were obtained, as outlined above, from available relatives, usually parents and sibs, of seven test subjects whose serum ceruloplasmin levels were unusually low. These studies included 12 parents, three sibs, four offspring, and nine other relatives of the test subjects.

Blood samples were collected from 19 parents and 26 offspring in ten unrelated normal families.

Twin Studies:

Blood samples were obtained from nine pairs of dizygotic twins (four pairs like-sexed, five pairs unlike-sexed) and nine pairs of monozygotic twins. Zygosity diagnoses were made by Dr. Nancy Simpson of Queen's University, Kingston, Ontario, based on physical characteristics, and analyses of nine blood groups and dermal patterns using the method of Smith and Penrose (1955). Haptoglobin and serum cholinesterase subtypes were also determined for some of the twin pairs. Dermatoglyphic analyses were not available for three pairs of monozygotic twins, but for all monozygotic pairs, the probability of monozygosity as determined from the available data was greater than 0.985. These blood samples, collected for another study, were drawn in plastic syringes and stored in acid-washed vials, but were not centrifuged in acid-washed test tubes. This did not have any detectable effect on the ceruloplasmin levels. Serum copper levels were not measured.

RESULTS

Assessment of the Method for Quantitative Assay of Ceruloplasmin:

A buffer of 1.0 M acetate, pH 5.2, produced a greater rate of oxidation than did a 0.5 M buffer, pH 5.2, over a range of substrate concentrations, as shown in Fig. 1. The 1.0 M acetate buffer was therefore used for the assay, resulting in a concentration of 0.33 M acetate in the final reaction mixture, exclusive of serum. The pH remained constant throughout the reaction period when 1.0 M buffer was used.

The substrate concentration used in the assay allowed optimal oxidation. The velocity of reaction is shown in Fig. 1. for several concentrations of substrate (PPD). The substrate concentration used was 6.8×10^{-3} M under the conditions of the experiment, or 7.4×10^{-3} M in the reaction mixture exclusive of serum. A concentration of PPD of 2.0×10^{-2} M did not produce an increase in the rate of oxidation; this highly acidic PPD solution depleted the buffering capacity of the reaction mixture, causing a lowering of the pH.

The pH optimum under the conditions described lies between 5.0 and 5.3, as shown in Fig. 2.

When the serum was diluted with physiological saline at intervals down to one in eight, a linear relation was obtained between the concentration of ceruloplasmin, as determined by the amount of sera used, and the ΔO.D./minute. The results are shown in Fig. 3.

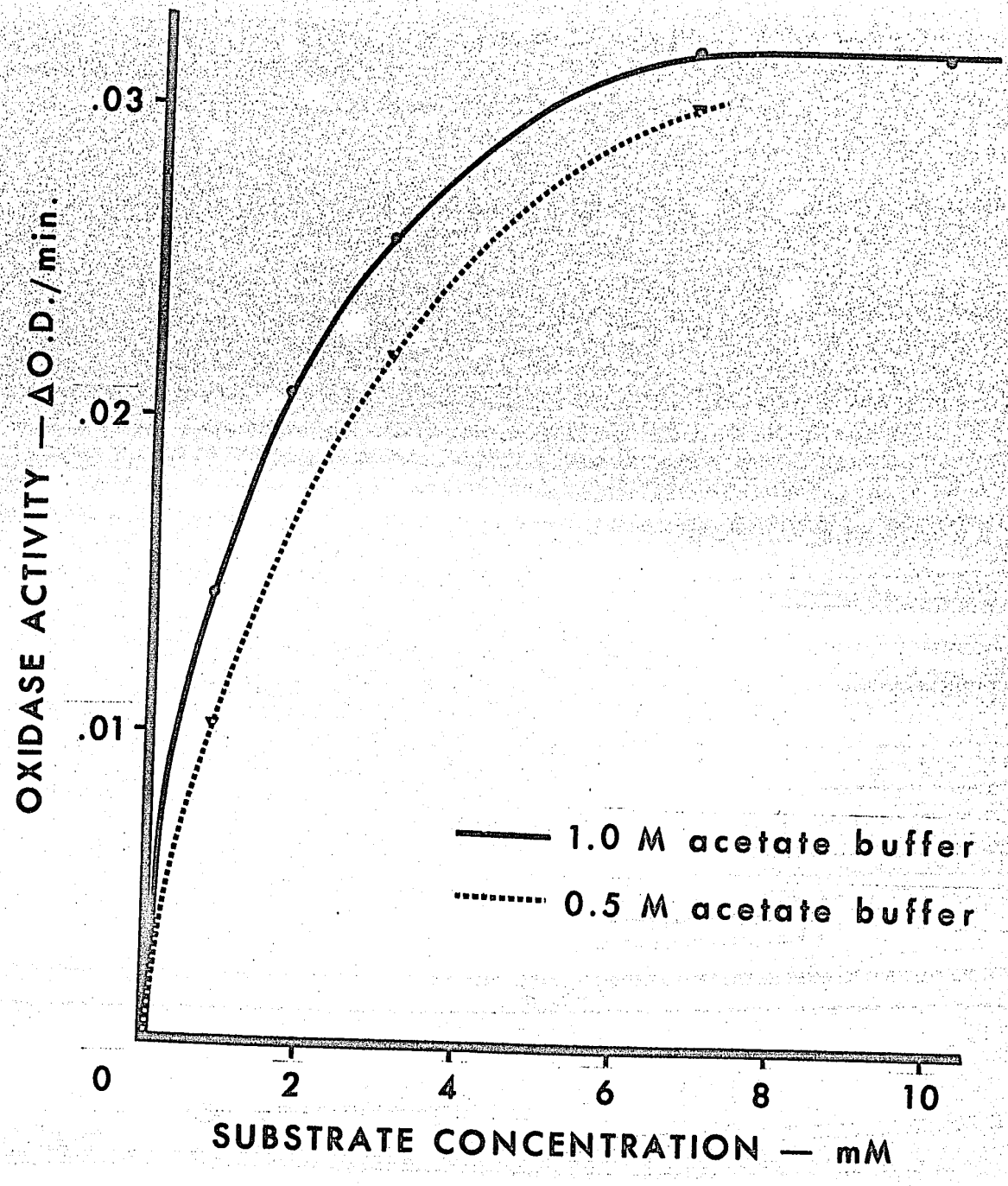

Fig. 1. Rate of reaction plotted against substrate (PPD) concentration. Reaction was carried out in acetate buffer at pH 5.2 at acetate concentrations of 0.5 M and 1.0 M; 0.25 ml. human serum; 30° C.

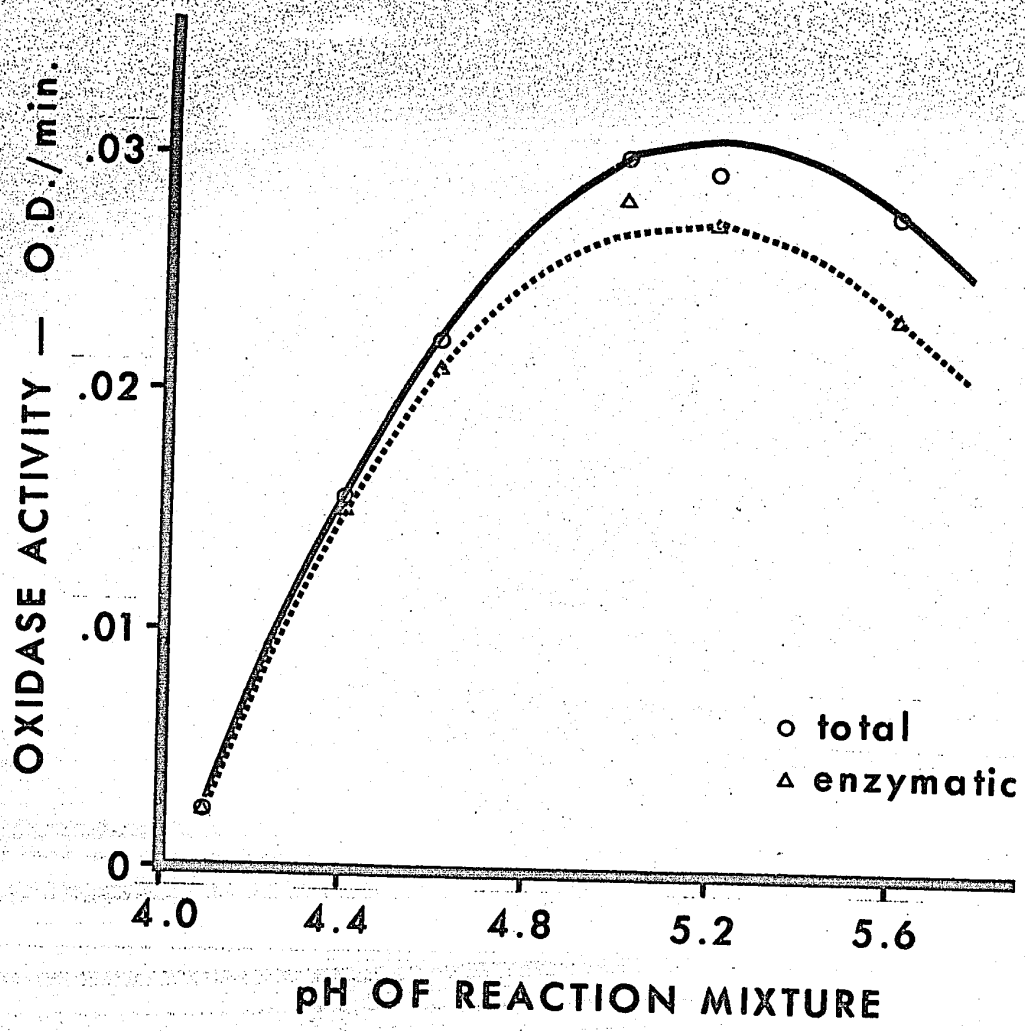

Fig. 2. Rate of reaction plotted against pH of acetate buffer. Acetate concentration was 1.0 M; 0.25 ml. human serum; 30° C.

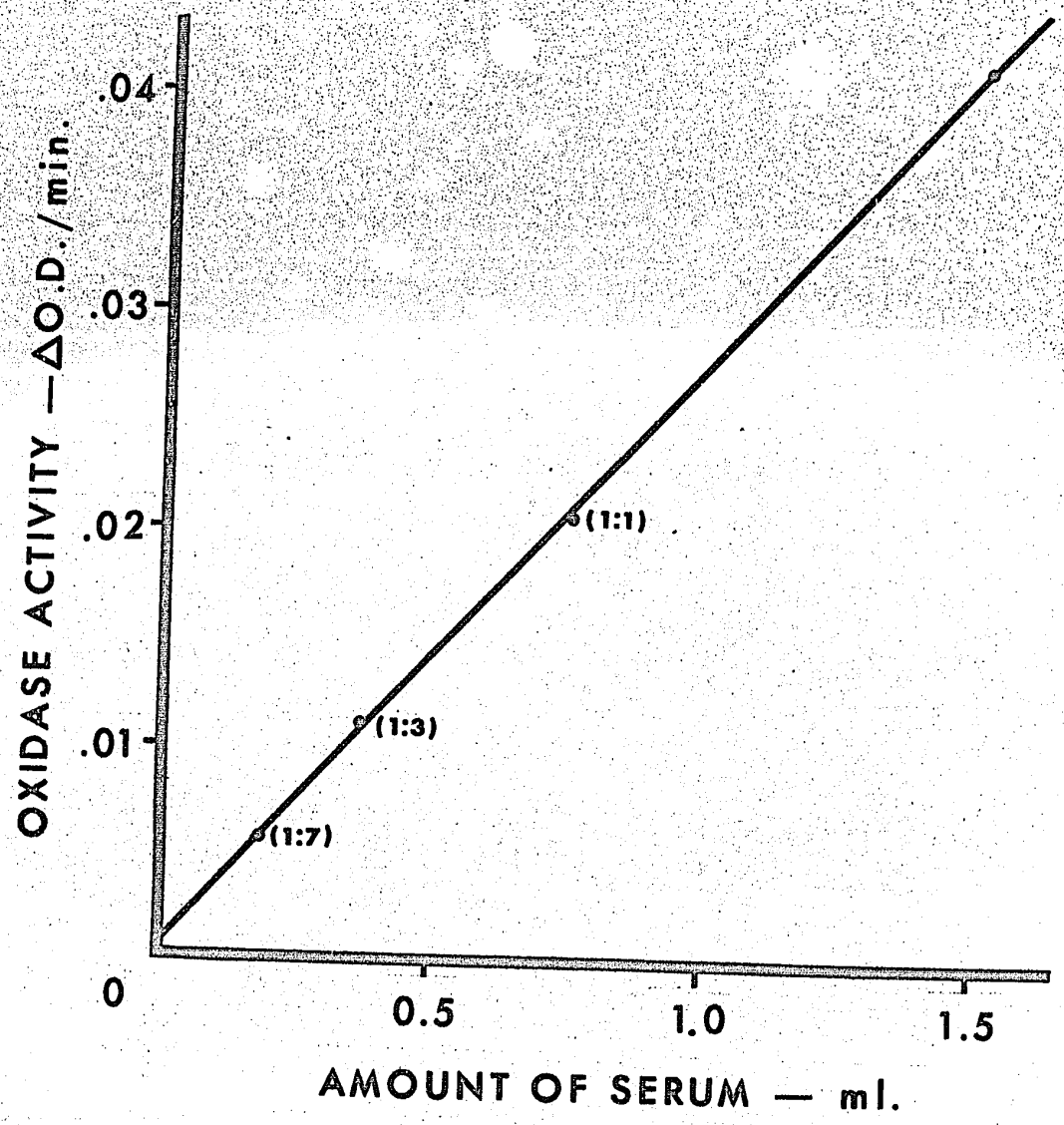

Fig. 3. Rate of oxidase reaction in human serum plotted against amount of serum used. Dilution of serum used for each point is indicated in parentheses. Acetate buffer, 1.0 M, pH 5.2; 30° C.

Lipemic serum could be used provided that the rate of reaction was linear. This was tested by measuring the level of ceruloplasmin in serum obtained from the same individual before and one hour after a fatty meal; the levels were the same. Severely lipemic serum produced very irregular changes in optical density and the rate of reaction could not be measured.

A small aliquot of a hemoglobin solution was added to serum to produce a solution containing 75 mg./100 ml. hemoglobin. This vivid red mixture showed a three per cent increase in oxidation activity over that of the serum alone. Results of the assay were therefore altered very little by a degree of hemolysis more extreme than would be encountered in serum samples.

The lag period cannot cause erroneous results in this method. With normal sera, the lag period, during which time ΔO.D. is zero, is brief and is completed well before the completion of the interval allowed for temperature equilibration. However, with sera having a low ceruloplasmin level, as in Wilson's disease, and particularly in such patients on penicillamine treatment, the lag period may last ten minutes or longer. The rate of the reaction in such cases is not recorded until the end of the lag period.

The method is highly reproducible. The mean difference between duplicate determinations on the same day was 0.09 mg./100 ml. ceruloplasmin, based on 30 sets of duplicates. The difference is not significant from zero ($t = 1.1$, d.f. $= 29$, $P > 0.2$). Because of the close agreement, duplicate determinations were made only when the first appeared unusual. In all such cases, duplicates were almost identical.

Differences between measurements on the same sample carried out on different days were also nonsignificant. Analysis of variance of measurements of sera from ten randomly chosen individuals, tested in duplicate on two successive days and stored at 4°C between tests, showed that the effect of day of measurement within individuals was not significant ($F = 2.3$; d.f. = 10, 20; $P > 0.05$) but variation between individuals was highly significant ($F = 196.2$; d.f. = 9, 10; $P < 0.005$).

Effects of Freezing and Storage of Sera:

Sera could be stored under refrigeration at approximately 4°C for at least a week with no apparent alteration of activity. In the present study, the sera were frozen immediately after preparation and were stored for later testing, usually within several weeks. Sera from eight individuals were tested on the day of collection (test 1), on the following day (test 2), and again after 15 to 21 months of frozen storage at -15 to -20°C (test 3). Analyses of differences between paired results showed no significance for tests 1 and 2 ($t = 1.52$, d.f. = 7, $P > 0.1$), tests 1 and 3 ($t = 0.8$, d.f. = 5, $P > 0.4$), or tests 2 and 3 ($t = 2.21$, d.f. = 5, $P > 0.05$). Two sera were omitted from test 3 because of heavy turbidity. Turbidity has not developed in samples stored for about a year or less.

Daily Variation Within Individuals:

From three individuals, a blood sample was drawn on each of six days one month or more apart. The sera were frozen as obtained and all were measured in one batch to avoid possible variations due to the method. The variation of the results for each individual was

small (mean ± S.D.: 27.5 ± 1.3, 36.1 ± 1.6, 31.8 ± 1.1 mg./100 ml.). Analysis of variance showed no significant effect of day of sampling ($F < 1$; d.f. = 5, 10; $P > 0.2$) but highly significant variation between individuals ($F = 30.4$; d.f. = 2, 10; $P < 0.005$).

Effects of Age and Sex:

The means and linear regressions of ceruloplasmin level on age for adults and adolescents are shown in Table 1. None of the regressions are significant at the one per cent level, although a suggestion of age effect (five per cent level of significance) was found for both groups of males. The positive regression for adult males appears to be due to slighly lower levels among the younger male adults. Between the four groups, comparisons of means were made as follows:

female adolescents vs adults: $t = 1.35$, d.f. = 93, $P > 0.10$

male adolescents vs adults: $t = 4.63$, d.f. = 95, $P < 0.001$

male adolescents vs female adolescents: $t = 3.84$, d.f. = 48, $P < 0.001$

male adults vs female adults: $t = 1.20$, d.f. = 140, $P > 0.20$

Adolescent females and adult males and females were therefore all combined, but adolescent males kept separate. Means and confidence limits for the population are shown in Table 2.

Adolescent male levels can be adjusted to adult values as follows:

$$\text{Adjusted level} = \text{observed level} \times \frac{\text{mean of all adults}}{\text{mean of adolescent males}}$$

$$= \text{observed level} \times 1.17$$

The values for adolescent males appear to cover a relatively narrow range. However, the variances are homogeneous ($F = 1.97$; d.f. = 67, 28; $P > 0.025$). Based on our present sample, the given conversion therefore appears to be appropriate.

TABLE 1

CERULOPLASMIN LEVELS OF ADULTS AND ADOLESCENTS BY SEX

Group	Age (years)	Sex	No.	Ceruloplasmin (mg./100 ml.) mean ± S.D.	Regression coefficient $b \pm s_b$	t	P
Adolescents	15-19	M	29	25.8 ± 3.2	-0.98 ± 0.44	2.20	<0.05
Adolescents	13-16	F	21	29.4 ± 3.3	-0.16 ± 0.71	0.23	>0.50
Adults	>19	M	68	30.1 ± 4.5	0.09 ± 0.04	2.24	<0.05
Adults	>16	F	74	31.1 ± 5.7	0.07 ± 0.05	1.40	>0.1

TABLE 2

MEANS AND CONFIDENCE LIMITS FOR CERULOPLASMIN LEVELS

Group (age in years)	No.	Ceruloplasmin (mg./100 ml.) mean ± S.D.	Confidence limits 95% (mean ± $t_{.05}$ S.D.)	99% (mean ± $t_{.01}$ S.D.)
Males (15-19)	29	25.8 ± 3.2	19.1, 32.4	16.8, 34.7
Adults (males >19) (females >12)	163	30.4 ± 5.0	20.7, 40.2	17.6, 43.3

TABLE 3

REGRESSION OF CERULOPLASMIN LEVEL ON AGE IN CHILDREN

(MALES 2 to 15 YEARS, FEMALES 2 to 12 YEARS)

	No.	Mean age (years)	Ceruloplasmin (mg./100 ml.) mean	$s_{y.x}$	Regression coefficient b ± s_b	t	p
Males	63	9.1	34.2	6.34	-1.14 ± 0.23	4.92	<0.001
Females	54	7.7	36.3	5.48	-1.18 ± 0.24	4.82	<0.001
All children	117	8.4	35.2	5.91	-1.17 ± 0.16	7.16	<0.001

Among both male and female children, there is a significant regression of ceruloplasmin level on age, as shown in Table 3. The two samples have a common variance ($F = 1.3$; d.f. = 62, 53; $P > 0.1$). The slopes of the two regressions are the same ($t = 0.10$, d.f. = 113, $P > 0.5$). Analysis of covariance indicated that there is no difference in elevation of the regressions ($F < 1$; d.f. = 1, 114; $P > 0.2$). The samples could therefore be combined, to provide the results shown for all children in Table 3. This regression is also significant at the 0.1 per cent level. The individual levels and the regression line are shown in Fig. 4.

At the age of 12 years, children have approximately the same mean ceruloplasmin level as adults, although in males levels appear to show a further decrease. Children's values can therefore be adjusted to age 12, that is to adult values, as follows:

Adjusted level = observed level - 1.17 x (12 - age in years)

The histogram in Fig. 5 shows the levels of all 309 individuals in this study. Levels for children have been adjusted to 12 years; levels for adolescent males have been adjusted to adult values. The expected frequencies for a normal distribution are shown. There is some suggestion that the observed distribution differs from the normal curve, using a chi-square test and combining the first two and last three observed intervals ($\chi^2 = 21.6$, d.f. = 12, $P < 0.02$). The difference is due mainly to an excess of observed individuals in the highest interval. The high values may be due to the response of ceruloplasmin to acute and chronic infection which could be present but undiagnosed, or to familial factors.

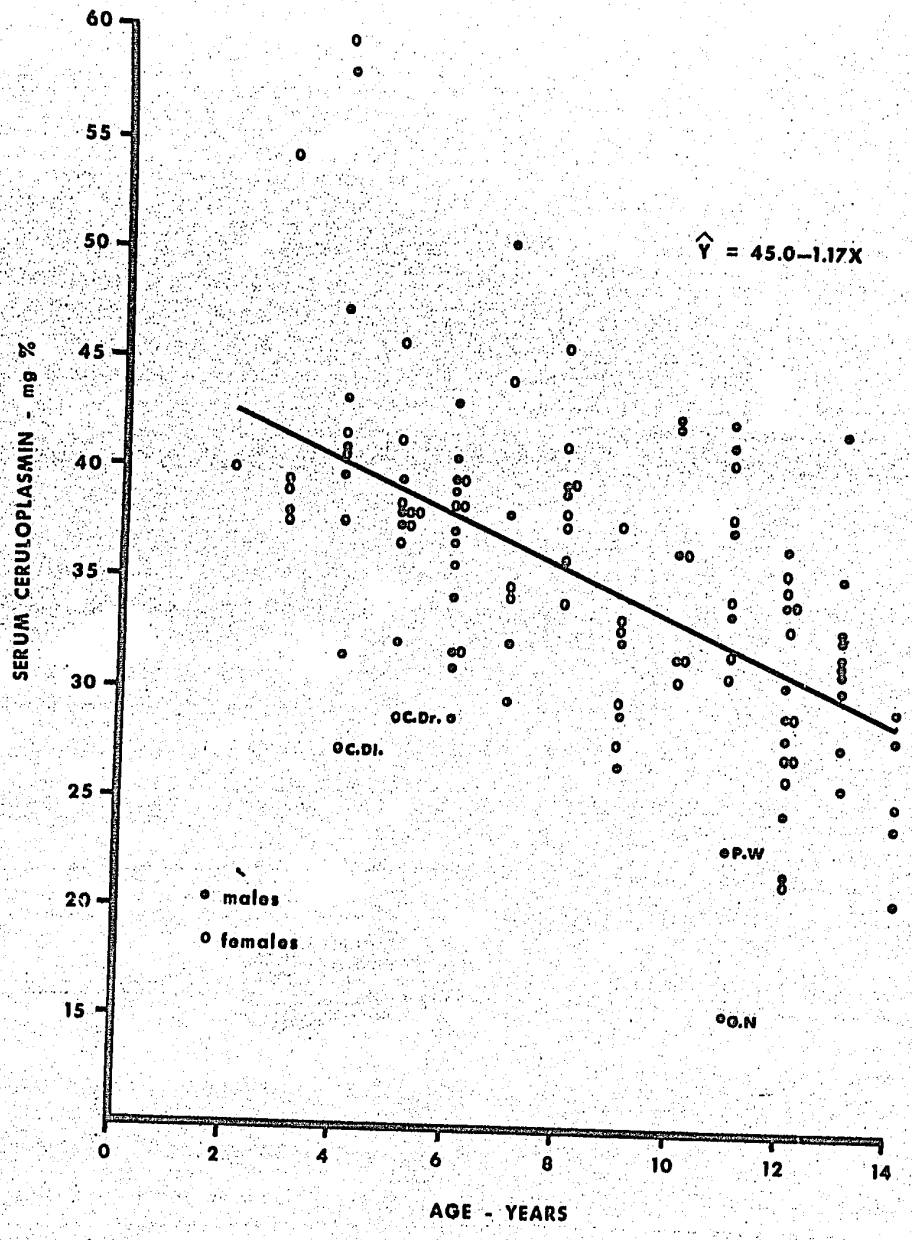

Fig. 4. Serum ceruloplasmin levels of 117 normal children plotted against age. The regression line and its equation are shown.

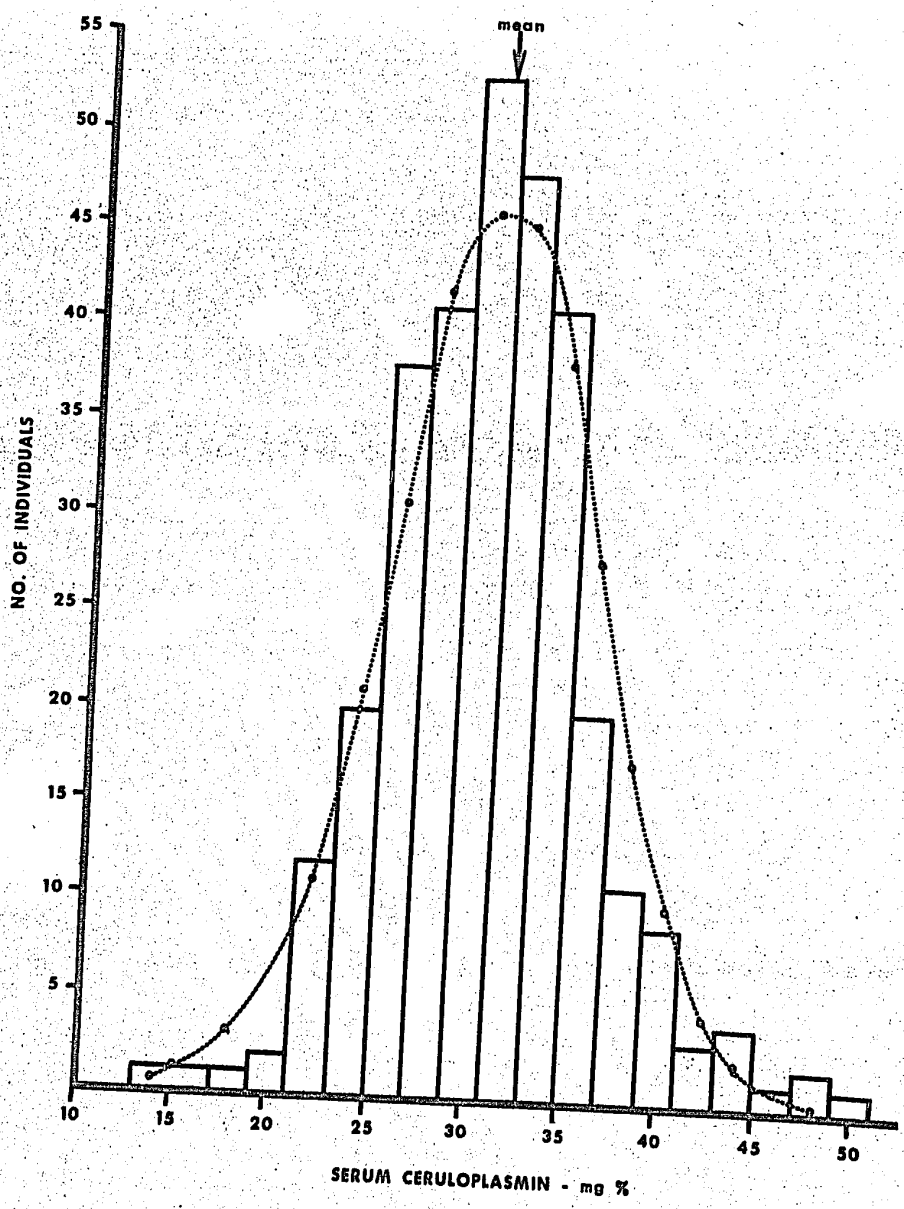

Fig. 5. Distribution of serum ceruloplasmin levels of 309 normal individuals. Levels of children and adolescent males have been adjusted to adult levels, as described in the text. The dotted curve shows expected values, assuming a normal distribution.

Serum copper was measured in most of the samples and the findings were consistent with those of ceruloplasmin levels. There were no significant differences between mean copper levels of 54 male and 50 female adults (105.4 ± 18.6 µg./100 ml. and 112.8 ± 23.5 µg./100 ml., respectively; $t = 1.79$, d.f. = 102, $P > 0.05$) and 21 female adolescents (103.7 ± 16.0 µg./100 ml.), consequently these were combined. The combined mean ± 1 S.D. was 108.1 ± 20.6 µg./100 ml. (95 per cent confidence limits: 67 to 149 µg./100 ml.). Copper levels of male adolescents differed from those of male adults ($t = 2.49$, d.f. = 74, $P < 0.02$), although the difference was not as marked as for ceruloplasmin levels. The mean ± 1 S.D. for male adolescents was 96.5 ± 17.3 µg./100 ml. (95 per cent confidence limits: 61 to 132 µg./100 ml.). There was no significant difference between male and female adolescents, however females were combined with adults to be consistent with the way in which ceruloplasmin levels were treated. Both male and female children showed a significant regression of copper level on age. Analysis of covariance showed that the regressions were the same. The slope of the regression line from the combined data (62 males, 50 females) is -3.05 ± 0.44 µg./100 ml. and differs significantly from zero ($t = 6.99$, d.f. = 110, $P < 0.001$). The individual levels and the regression line are shown in Fig. 6. Children's copper levels are approximately the same as those of adults at the age of 12 years and can be adjusted for comparison as follows:

Adjusted level = observed level - 3.05 x (12 - age in years)

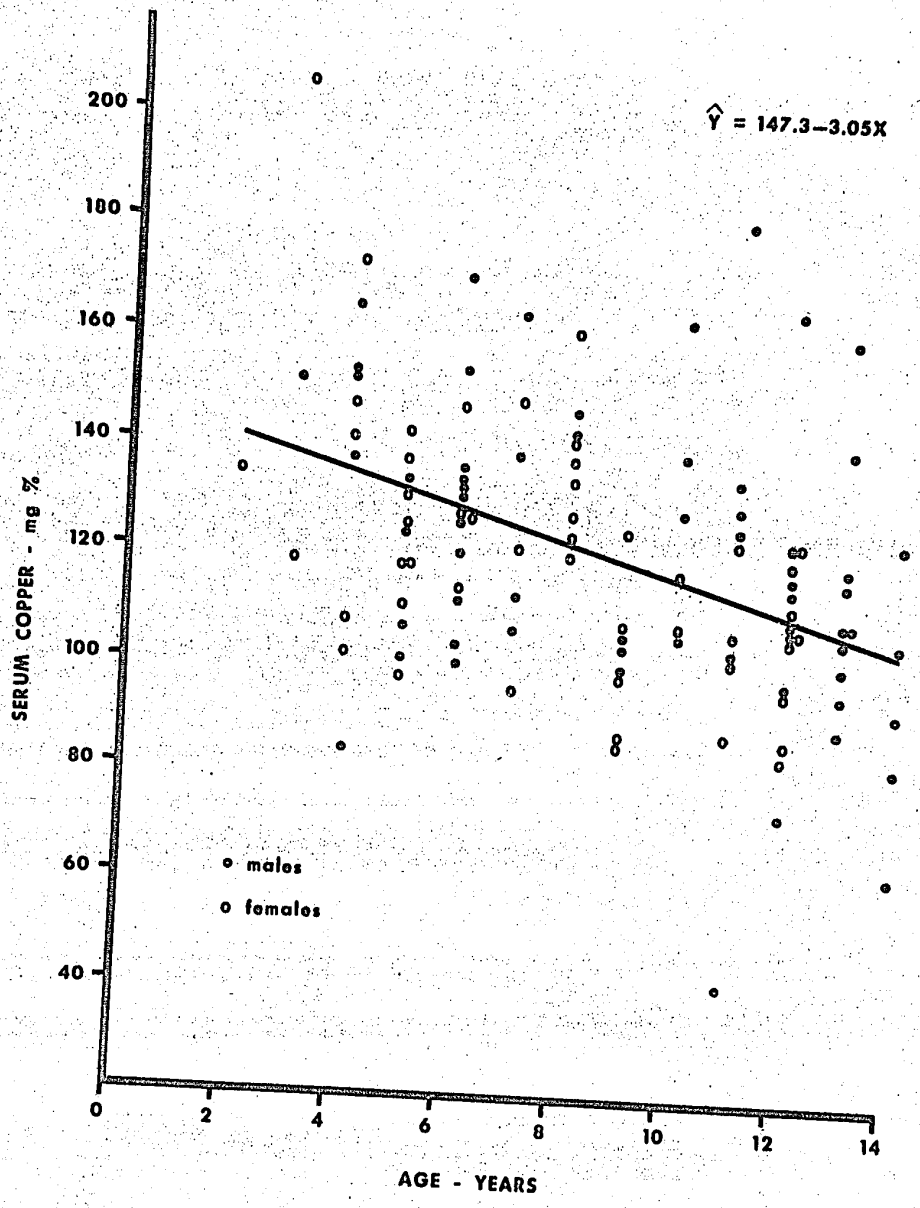

Fig. 6. Serum copper levels of 112 normal children plotted against age. The regression line and its equation are shown.

Effect of the Menstrual Cycle:

Pregnancy and estrogen administration are known to alter copper metabolism and in particular to lead to an increase in the level of ceruloplasmin, as previously reviewed. The question of possible effects of hormonal influences during the menstrual cycle therefore arose.

No consistent variation of ceruloplasmin level was observed in two normal women tested at weekly intervals over a period of 10 and 14 weeks, respectively. The menstrual cycle of 42 women was divided into four periods: days 1 to 7, 8 to 14, 15 to 21, and 22 and over. Numbers of women in each group were 6, 13, 9 and 14 respectively. Analysis of variance comparing variation among time periods to that within time periods showed no significant differences ($F < 1$; d.f. = 3, 38; $P > 0.2$).

Effect of Body Weight:

The effect of body weight was tested in 40 adult males. Results were grouped into seven weight categories of ten-pound intervals. Analysis of variance comparing variation among weight intervals to that within weight intervals showed no significant difference ($F < 1$; d.f. = 6, 33; $P > 0.2$).

Family Studies:

Parents and sibs, or offspring, of seven individuals with low levels of ceruloplasmin were tested. Five of these individuals in the normal series had ceruloplasmin levels which were somewhat low but above the 99 per cent lower confidence limit. These

individuals are C. Di., C. Dr., and P.W. shown in Fig. 4; G.M., a 22-year old male with a ceruloplasmin level of 22.5 mg./100 ml.; and D.W., a 15 - year old male with a ceruloplasmin level of 21.5 mg./100 ml. All of these had ceruloplasmin levels above the 95 per cent lower confidence limit except for C.Di., whose level was between the 95 and 99 per cent lower confidence limits. The ceruloplasmin levels of ten parents and three sibs of these individuals are shown in Table 4. All levels are normal except for the father of D.W. whose level lies just above the lower 99 per cent confidence limit. In these families, there does not appear to be a single gene exerting a pronounced effect on the ceruloplasmin level.

G.N., an 11-year old male, and N.R., a 46-year old female (see Fig. 7) had ceruloplasmin levels below the 99 per cent confidence limits. N.R. had a normal copper level (75 μg./100 ml.); that of G.N. was low (42 μg./100 ml.). Both were clinically normal, had no Kayser-Fleischer rings by slit lamp examination, and had normal urinary copper excretion and liver function tests, making a diagnosis of Wilson's disease virtually untenable. Their pedigrees are shown in Fig. 7. Ceruloplasmin levels in these two families were either normal or considerably below the established normal limits. All individuals were in good health. In these two families there appears to be a single gene exerting a pronounced effect on the ceruloplasmin level. In five individuals with low ceruloplasmin levels in family N., the copper levels were correspondingly low. In family R., the serum copper level of N.R. was higher, on two occasions, than would be expected from the level of ceruloplasmin present. It was calculated that from 21 to 25 per cent

TABLE 4

CERULOPLASMIN LEVELS IN RELATIVES OF INDIVIDUALS WITH LOW CERULOPLASMIN LEVELS

Proband	Ceruloplasmin levels (mg./100 mls.)		
	Father	Mother	Sib[b]
C.Dr.	26.4	28.7	32.9
C.Di.	35.2	31.2	46.7 (36.7)
G.M.	29.5	58.4	
D.W.	17.8	31.5	
P.W.	33.4	21.3	25.2

[a] Refers to normal individual with low ceruloplasmin through whom family was ascertained.

[b] Adjusted for age effects in parentheses.

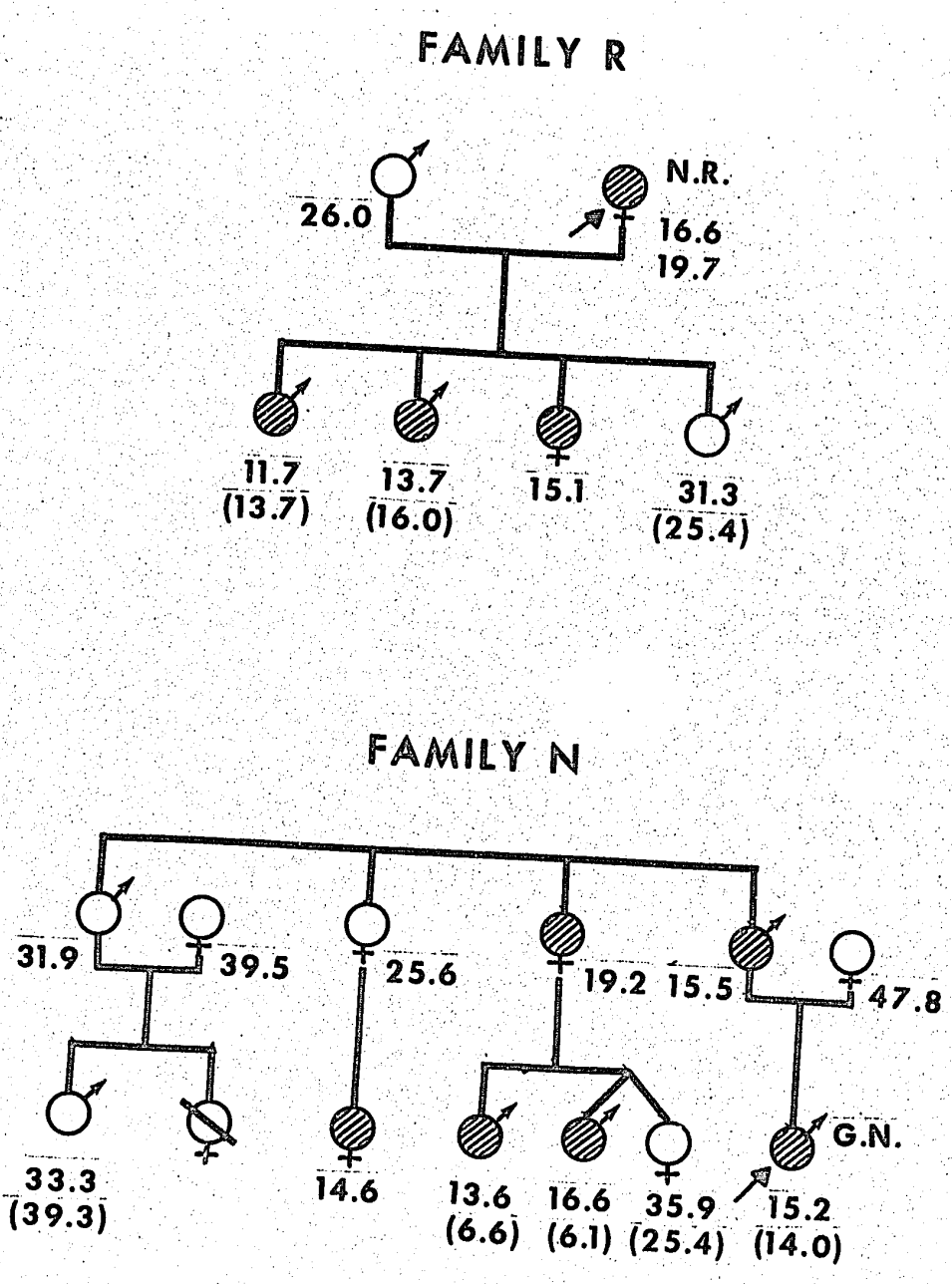

Fig. 7. Pedigrees of families in which abnormally low serum ceruloplasmin levels occur, showing those individuals tested. Figures are ceruloplasmin levels in mg./100 ml. Age-adjusted values, when required, are shown in parentheses. Shaded symbols represent individuals with a low ceruloplasmin level.

of the copper was present in the serum not bound to ceruloplasmin. Such values are infrequently found in normal individuals and may be due to contamination of the serum sample with extraneous copper. It could also be explained by the presence of a normal amount of ceruloplasmin with an abnormally low oxidase activity or by a real increase in the amount of non-ceruloplasmin bound copper in the serum. Family R became inaccessible for further study after our early findings. The mobility of the ceruloplasmin of the father of G.N., N.R., and the two sons of N.R. were all identical to that of normal ceruloplasmin by vertical starch gel electrophoresis in borate buffer of pH 9.5. (Details of this method are presented in Section II). The uptake of copper-64 into ceruloplasmin as measured in heterozygotes for Wilson's disease (described in Section II) was unique. The uptake during the first 24 hours of the study was normal, then became reduced, as in typical heterozygotes for Wilson's disease, during the remainder of the study. His response is shown in Fig. 17.

Less specific familial influences also appear to influence the ceruloplasmin level. Ceruloplasmin levels were measured in sera from 45 parents and offspring of ten normal families. Variation between families was significantly greater than variation within families ($F = 3.1$; d.f. = 9, 35; $P < 0.01$).

Twin Studies:

The serum ceruloplasmin levels of twins were evaluated to determine if the familial influences were due to hereditary or non-hereditory factors. Analysis of variance and intraclass correlation (Steel and

Torrie, 1960), as applied to twin data by Kempthorne and Osborne (1961), were used for the analyses.

The ceruloplasmin levels of children and adolescents were adjusted to equivalent adult values as described previously. The age distribution of the MZ and DZ twin pairs was similar; seven pairs of each type were between eight and 19 years of age. Four pairs of unliked-sexed DZ twins were in the adolescent age group, in which the level of only the male twin required age adjustment.

The values determined for monozygotic (MZ), all dizygotic (DZ), and same-sexed (SS) DZ twin pairs were as follows:

	B	W	P	σ_x^2	r_1
MZ (9 pairs)	33.32	5.90	<0.01	19.61	0.70
DZ (9 pairs)	71.67	110.86	n.s.	91.27	-0.22
DZ - SS (4 pairs)	99.96	31.28	n.s.	65.62	0.52

The symbols used above are as follows: B = mean square between pairs; W = mean square within pairs; P = the probability that F differs from zero, where F, as determined from the analysis of variance, $= \frac{B}{W}$; σ_x^2 = variance for individuals; r_1 = intraclass correlation. Kempthorne and Osborne (1961) pointed out that when a trait is influenced mainly by hereditary factors, the variance for individuals (σ_x^2) should be the same for the two types of twins but the intraclass correlation should be greater for MZ pairs than for DZ pairs.

In these data, the individual variation and that between pairs is less for MZ than for DZ pairs. This could suggest that the sampling has not been random: either the range of values covered by the MZ pairs is less than in the normal population, the range of values

covered by the DZ pairs is greater than in the normal population, or there is an unusually large spread of values within the DZ pairs. An examination of individual ceruloplasmin levels shows an exceptionally large difference within three pairs of twins, all of them unlike-sexed. The ages of these twin pairs were 14, 16, and 51 years. In all cases, the female of the pair had a much higher ceruloplasmin level than did the male. Correction was made for the sex difference which exists during the adolescent years, as determined in this study. The females were not known to be pregnant, taking hormone treatment, or having severe infection. The reason for this discrepancy is, therefore, unknown. The same-sexed DZ twins were analyzed separately and also show a larger individual variation, but the number studied is too small to draw valid conclusions.

The analysis of variance for MZ twins indicated a significant component of variance due to differences between twins when compared to that within twins ($F = 5.64$; d.f. = 8,9; $P < 0.01$). The intraclass correlation is greater for the MZ twins than for either group of DZ twins, suggesting that hereditary rather than environmental factors predominate in influencing the serum ceruloplasmin level.

DISCUSSION

The method for the quantitative assay of ceruloplasmin presented here was high precision and reproducibility. The buffer system and substrate concentration used permit maximum enzyme activity. Measurement of the rate of enzyme activity provides one major advantage over colorimetric methods in which a single measurement is made

after an incubation period: effects of the lag period are avoided by measuring the rate of reaction. The other type of colorimetric method may provide erroneous results under circumstances in which the lag period is long, for example when the ceruloplasmin level is very low.

Moderate hemolysis and lipemia have little effect upon the method described. Sera should not be used, however, if turbidity has developed after prolonged storage since protein denaturation may have occured.

The ceruloplasmin level of individuals remained relatively constant over a period of time but differences in levels between individuals were marked. The mean ceruloplasmin level in 163 adults (females over 12 years, males over 19 years) was 30.4 ± 5.0 mg./100 ml. with 95 per cent confidence limits of 20.7 to 40.2 mg./100 ml. This result was similar to the value of 32.3 ± 4.9 mg./100 ml. obtained from 100 blood donors of unspecified ages using another enzymatic method (Ravin, 1961). The mean copper level of 125 adults in the present series was 108.1 ± 20.6 µg./100 ml. This is close to the values found in two other series of normal adults: 114 ± 14 µg./100 ml. in 205 adults (Cartwright et al., 1960) and 104 ± 14 µg./100 ml. in 106 adults (Neale and Fischer-Williams, 1958). Copper and ceruloplasmin levels in the present series agreed well with each other, with copper in ceruloplasmin accounting for about 96 per cent of the serum copper, as expected.

Body weight in males and the menstrual cycle in females did not significantly affect the ceruloplasmin level. No significant effect

of sex was found for ceruloplasmin or copper levels in children or adults. Ceruloplasmin levels were reported by Cartwright and Wintrobe (1964) to be higher in females than males although the sample size of 30 was much smaller than in the present series. The mean serum copper level was slightly higher in females than in males but the difference was not significant in the present study. A significant difference has been reported by Cartwright et al. (1960) and Neale and Fischer-Williams (1958). Male adolescents had significantly lower ceruloplasmin levels than those of female adolescents, but the difference was not significant for copper levels.

A significant alteration of ceruloplasmin with age was found. Normal levels in young children were considerably higher than those in adults, and there was a constant decline with age, reaching adult levels by the age of 12 years. The rate of decline was the same for both male and female children. Males, however, appeared to have a further decline in ceruloplasmin levels, such that levels in adolescent males were lower than in adolescent females and in adults. Serum copper levels of the same samples showed similar age variations. Previous studies, as reviewed from the literature, have demonstrated low levels of ceruloplasmin in newborns, increasing levels during the first year of life, and levels equal to or higher than adult levels by the age of one year. When these data are combined with those of the present investigation, the overall picture of the ceruloplasmin level in serum is as follows: low levels in the newborn are followed by a gradual rise during the early months of life, probably indicating the maturation of an enzyme system; adult levels are

reached in the latter half of the first year and the levels continue to rise to a peak at two or three years of age; a gradual decline follows, with adult levels reached by about 12 years of age.

These findings are of practical importance, particularly in studies of relatives of patients with Wilson's disease. The age of a child must be considered in assessing the ceruloplasmin or copper levels. The use of adult values for children leads to the underdiagnosis of hypoceruloplasminemia or hypocupremia. The correction factors are particularly useful when comparisons of individuals of different ages within families are desired, for example in families in which Wilson's disease has occurred. The corrections may not apply to patients with Wilson's disease who usually have a pronounced deficiency of ceruloplasmin, except possibly in advanced stages of the disease. There is no evidence that the already defective mechanism for ceruloplasmin production in such patients is influenced by the factors that are altered by age in normal individuals.

Ceruloplasmin and copper levels of adolescent males (defined as 15 to 19 years of age) are lower than those of adults. The observed values can be converted to those of adults by the factor given previously, although this assumes homogeneity of variance between the adolescent male and adult groups. This assumption is apparently valid. As an alternative to conversion, the appropriate confidence limits found for the individuals in this group can be used. Males

13 and 14 years of age could have been included in the adolescent group as the group boundaries are arbitrary.

If other laboratory methods for the determination of ceruloplasmin or copper levels are used, the given correction factors should still be applicable even though the normal mean and confidence limits may differ.

Studies in normal families indicated that serum ceruloplasmin levels are influenced by familial factors. In two families ascertained through a study of normal individuals, low ceruloplasmin levels showed a regular pattern of inheritance. The pedigrees were consistent with the affected individuals being heterozygotes for a gene which produces a low enzymatic activity of ceruloplasmin. There was one exception to this pattern in family N. This may indicate that a single dominant gene is involved which lacks complete penetrance. On the other hand, the gene for Wilson's disease may be present in these families and may be responsible, as will be presented in Section II, for the low ceruloplasmin levels. The results of ^{64}Cu studies carried out on one individual in family N were unusual and it was not possible to draw definite conclusions regarding the presence or absence of the gene for Wilson's disease. The two families may have decreased ceruloplasmin levels for different reasons, with the Wilson's disease gene implicated in one family, but not the other. The studies of heterozygotes of the Wilson's disease gene presented in Section II of this thesis indicate that about 6.1 per cent of heterozytoes have ceruloplasmin levels

below the 99 per cent confidence limits of the normal population. This figure is 10.0 per cent among heterozygotes of western European origin. The normal series presented here is almost entirely of western European origin, reflecting the population origin of the city area from which the sample was selected. If the frequency of heterozygotes is as high as one in 265, (Section II) then in a population of western European origin the expected frequency of heterozygotes for the Wilson's disease gene having a low level of ceruloplasmin would be about one in 3,000. Inclusion of two such individuals in a sample of 309 would be rather unlikely, however we may have, by chance, done so. With the data at present available it is possible to conclude only that low ceruloplasmin levels appear to be inherited in occasional families and their relation to the gene for Wilson's disease is not known.

The results of the twin studies indicated that ceruloplasmin levels are influenced by genetic factors. This interpretation must be accepted with reservation because of the unexplained increased individual variation for DZ compared with MZ twins. A similar conclusion was reached by considering only DZ twins of the same sex, which may be a more valid comparison, however, the number of such twin pairs was small.

SECTION II

STUDIES ON WILSON'S DISEASE

PART 2

A Screening Test for the Detection of Low Levels of Ceruloplasmin

Since a study of the ceruloplasmin levels of relatives of patients with Wilson's disease would involve a great many tests, a rapid screening method appeared to offer a feasible substitute for the usual assay.

A spot screening test, based on the oxidase activity of PPD, was reported to distinguish ceruloplasmin levels below that of a given standard (Aisen et al., 1960). In our experience, the paper test strips were found inconvenient to prepare and not suitable for storage, and test results were frequently difficult to interpret. A commercially available kit, prepared for the detection of patients with Wilson's disease, utilizes paper discs and an unspecified color-producing reaction. The standard serum in this kit is suitable for use with sera from adults, but our findings of a change of ceruloplasmin level with age made this standard unsuitable for the testing of children. The kit would detect the severely deficient ceruloplasmin levels of children with Wilson's disease, but would not discriminate children with less-pronounced decreases below the normal level for their age.

The screening test devised can handle large numbers of sera at one time, is low in cost, and can be adapted to test sera from children of any age.

MATERIALS AND METHODS

A standard serum (or plasma) containing a known amount of ceruloplasmin was prepared by mixing, in suitable proportions, serum from a normal adult and serum deficient in ceruloplasmin. The latter was obtained either from the umbilical cord of a newborn or from a patient with Wilson's disease. The ceruloplasmin levels of the sera used for the standard and for the final standard mixture were determined by the assay method described in Part 1. A suitable standard serum for adults contains 20.7 mg./100 ml. ceruloplasmin. This is the lower 95 per cent confidence limit found in the normal population sample tested in Part 1. Suitable standard sera for children are as follows:

ages 2 to 3 years - 30 mg./100 ml.
ages 4 to 6 years - 27 mg./100 ml.
ages 7 to 9 years - 30 mg./100 ml.
ages 10 years & up - adult standard

A heparinized capillary tube (hematocrit tube, 1.3-1.5 mm. diameter) was filled with blood from a finger puncture. One end of the tube was plugged with plasticine and the plasma was separated by centrifugation or gravity. The cell-containing portion of the tube was broken off and discarded. Into each well of a microtiter 'U' plate (Canlab. No. 21828/4) was placed a thick filter paper disc for antibiotic assay (Fisher No. 9-897), (or 1.3 cm. diameter disc cut from Whatman No. 3 MM. filter paper). The microtiter plate was marked off into squares of nine wells each. The centre well of each group was used for the standard. The centre of each disc was touched with a capillary tube of standard, or plasma to be tested, allowing the absorption of about one inch of the capillary tube

contents into the disc. The amount of plasma is not critical as it determines, within limits, only the size of spot produced. On each disc was dropped two drops of a 0.3 per cent solution of PPD dihydrochloride (Fisher certified[1]) in acetate buffer (pH 5.2, 1.0 M), prepared immediately before testing. The plate was covered with plastic film (saran wrap) and was placed in a shallow water bath (rectangular pyrex dish) at 35 to 40°C for ten minutes, or until the standard discs were pale blue. The plate was then uncovered and read against a white background. A test disc as pale or paler than the standard indicated a positive test result, that is a low level of ceruloplasmin. A disc darker than the standard indicated a normal level ceruloplasmin. The appearance of the discs is shown in Fig. 8.

It is convenient to test 48 samples at one time. The microtiter plate contains 80 wells, however we used six groups of nine wells each at one time. The use of one control in each group of nine compensates for differences in reaction times.

[1]Recrystallization is not required when the PPD crystals are white and their solution is clear. If either are colored, PPD can be crystallized as described previously in Part 1.

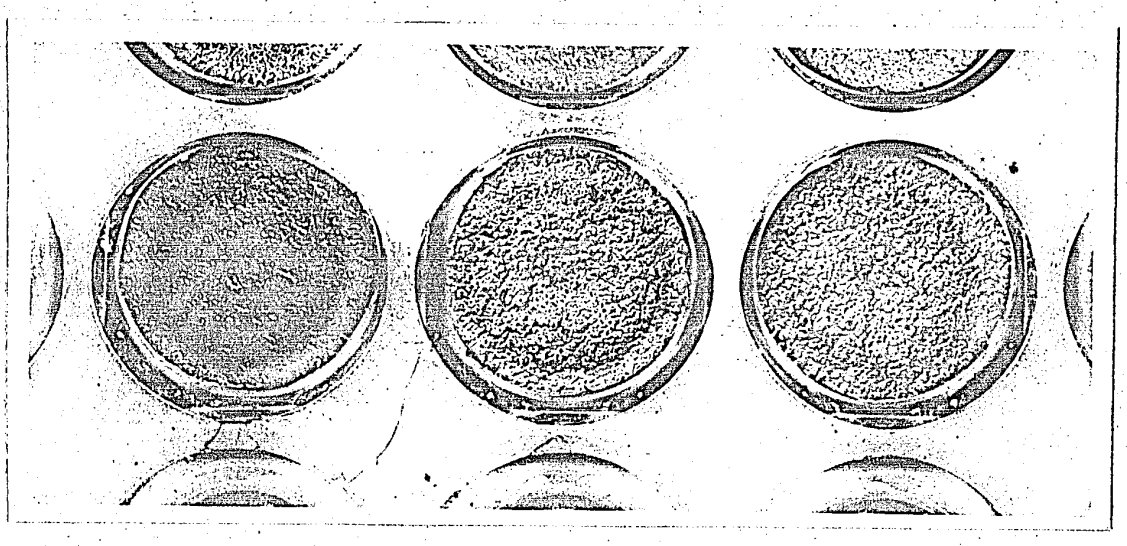

Fig. 8. Appearance of test discs, used in screening test, after development. From left to right, ceruloplasmin levels of sera (by qualitative assay) are: 29.4 mg./100 ml.(negative); 18.9 mg./100 ml. (standard); 7.2 mg./100 ml. (positive).

The screening test was evaluated on 50 sera, having a broad range of ceruloplasmin levels. These sera were obtained from normal individuals and from patients with Wilson's disease and their relatives. The standard serum in this evaluation contained 18.9 mg./100 ml. ceruloplasmin. Two investigators examined the test discs independently, without knowledge of the source of the sera, and recorded their conclusions. The amount of ceruloplasmin was measured in these sera by the assay method described previously in Part 1 and the results of the two tests were compared.

RESULTS

The results of the evaluation of the screening test are shown in Table 5. A test result was classified as uncertain when the two investigators disagreed or when one or both investigations considered a result open to question. In practice, such results should be considered positive and investigated further.

The screening test was found effective in detecting ceruloplasmin levels below that of the standard; in all ten cases the pertinent sera were classified as positive in the screening test. Sera with ceruloplasmin levels up to 2 mg./100 ml. above the standard registered as false positives, as did occasionally those with levels up to 5 mg./100 ml. above the standard. The screening test therefore sometimes falsely registered sera with normal ceruloplasmin levels but did not fail to detect sera with low ceruloplasmin levels.

TABLE 5

RESULTS OF SCREENING TEST COMPARED TO QUANTITATIVE ASSAY OF CERULOPLASMIN

Ceruloplasmin concentration[a] (mg./100 ml.)	Number tested	Results of Screening		
		Negative	Positive	Uncertain
40.0 - 50.0	6	6		
30.0 - 40.0	19	19		
25.0 - 30.0	9	9		
23.0 - 24.9	2	1		1
21.0 - 22.9	2	2		
19.0 - 20.9	2		1	1
Standard (18.9)				
17.0 - 18.9	1		1	
15.0 - 16.9	3		3	
less than 15.0	6		6	

[a]Measured by quantitative determination of oxidase activity

The test results were apparently not altered after blood samples had spent two days in transit. This was confirmed by measuring the ceruloplasmin level in fresh serum and serum left unseparated from the red blood cells for two days at room temperature. Freezing was avoided during transit. The tubes were well sealed and were placed in a corrugated cardboard holder. This holder was then placed in a small mailing container.

DISCUSSION

This screening test is rapid and requires relatively simple materials. The test is reliable for detecting ceruloplasmin levels equal to or below a given standard. There were no false negative results in 50 tests. False positives are likely to occur when the ceruloplasmin level is up to 2 mg./100 ml. above the standard, however our previous studies have shown that a very small percentage of the levels of the normal population lie in this range.

This screening test has been tested for large-scale use on 336 patients in a psychiatric hospital to detect possible patients with Wilson's disease (Cox, 1967). In this study, one false positive result was encountered among 336 samples tested. The test was found convenient and rapid for the screening of large numbers of samples.

The screening test is useful for the detection of individuals with Wilson's disease. An abnormally low level of ceruloplasmin is characteristic of the majority of patients with Wilson's disease.

In one series of 111 patients, 96.4 per cent of patients had ceruloplasmin levels below 20 mg./100 ml. (Sternlieb and Scheinberg, 1963). Probably about one in 20 patients would, therefore, not be detected by the screening test. This test could be suitably applied to the following individuals who could be considered suspects for having Wilson's disease: individuals with liver disease, extrapyramidal disfunction or psychiatric disorders, and children with unexplainable behaviour problems. Such screening could facilitate early diagnosis and consequently more successful treatment for patients with Wilson's disease.

While this method has been particularly convenient for large-scale testing, it can also be used for testing small numbers of samples. The standard sera can be stored frozen in small aliquots to be used and refrozen repeatedly unless turbidity develops. The test is suitable for use in hospital laboratories where a minimum amount of equipment is desired for screening of the ceruloplasmin level. The more extensive testing, which must be carried out on individuals showing a positive test result, could then be referred to centres equipped for such investigation.

This test was developed particularly for studies of relatives of patients with Wilson's disease. By using the appropriate standard sera, ceruloplasmin levels below the normal population limits can be detected in children or adults. Relatives with a low level of ceruloplasmin must then be investigated further to determine if they are heterozygotes or asymptomatic homozygotes for the Wilson's disease gene. The simplicity of the test allows its use on field

trips where there may be no laboratory facilities available. Because the test is not affected by transit of samples in the mail, individuals can conveniently be tested who might otherwise be inaccessible. These features allow the convenient study of large kindreds in which Wilson's disease has occurred.

SECTION II

STUDIES ON WILSON'S DISEASE

REVIEW OF THE LITERATURE

A. Clinical Features of Wilson's Disease

Kinnier Wilson (1912), in his classical monograph, first recognized the combination of neurological disease and cirrhosis of the liver as a disease entity which he called progressive lenticular degeneration. He described in detail the clinical and pathological features of four of his own patients and six from the literature. Diagnosis of the disease has frequently been difficult because many patients show features deviating from Wilson's classical form of the disease.

Patients generally fall into two broad categories. In one, the symptoms involve primarily the nervous system; in the other, primarily the liver.

Neurological Symptoms:

The neurological symptoms arise mainly from disorganization of the lenticular region of the brain. Tremor like that seen in Parkinson's disease, rigidity, dysarthria, and dysphagia appear. Spastic rigidity usually predominates in the neurological picture in younger patients, whereas progressively worsening tremor is common when the disease appears later in life. Denny-Brown (1964) has suggested that the two types of symptoms have two different causes, with tremor arising from the toxic effect of copper, and rigidity or dystonia from the effect of a second hepatic factor. Liver damage may be apparent in these forms from abnormalities in liver function tests or may be diagnosed only at biopsy.

Hepatic Symptoms:

With continued refinement of biochemical tests for Wilson's disease, the hepatic form of the disease is recognized as more common than was previously believed. The early hepatic symptoms resemble those of acute or chronic hepatitis. Post-necrotic cirrhosis follows. This type is sometimes referred to as the "abdominal form" of Wilson's disease.

Age of Onset:

The age of onset is generally between adolescence and the twenties. The age of onset in three large series of patients ranged from nine to 31 years, mean of 18.1 years, for 33 patients (Cartwright et al., 1960); nine to 36 years, mean of 23.2 years, for 32 patients (Bearn, 1960); and four to 15 years for 19 Japanese patients, 12 of whom were less than nine years of age (Arima and Kurumada, 1962a). The latter series suggests an earlier onset in the Japanese. In general, the hepatic form of the disease predominates in younger patients and is rapidly progressive; the neurological form predominates in older patients and the disease may worsen slowly, if untreated, over a period of years.

Corneal Pigmentation:

The only important clinical feature not described in Kinnier Wilson's thorough account of 1912 was the Kayser-Fleischer ring. These corneal rings had been previously described independently by Kayser and Fleischer, and were recognized by Fleischer (1912) as characteristic of

Wilson's type of pseudosclerosis. The rings are caused by the deposition of golden or greenish copper-containing pigments in the outer edge of the cornea. A review of studies of the ring and an excellent colour photograph have been published by Sternlieb (1966). The rings, which are sometimes visible only by slit-lamp examination, are almost always present in symptomatic Wilson's disease and are absolute diagnostic criteria. However, they may be lacking in young patients with the hepatic form of the disease and their absence does not exclude the diagnosis.

Other Clinical Features:

The kidney is also frequently involved in Wilson's disease, resulting in generalized aminoaciduria and other signs of abnormal renal tubular function. These are usually not clinically obvious, although they have been known to cause bone lesions.

Psychiatric disturbances have been reported in many cases of Wilson's disease. In one series of 33 cases, 15 per cent had psychiatric disturbances as their first deviation from good health and in another 25 per cent, such disturbances developed with other clinical symptoms (Sternlieb and Scheinberg, 1964). Beard (1959), in a review of cases of Wilson's disease with psychiatric disturbances, has pointed out that the psychiatric abnormalities present have often been mistaken for specific psychiatric entities, for example schizophrenia.

Bone lesions and hemolytic crises infrequently accompany the disease.

Reviews of clinical features of Wilson's disease, including numerous references have been prepared by: Bearn (1957), Boudin and Pepin (1959), Sass-Kortsak (1965), and Scheinberg and Sternlieb (1965).

B. Biochemical Abnormalities in Wilson's Disease

In his 1912 monograph, Wilson concluded that the disease was acquired and was caused by a toxin, not microbial but possibly chemical in nature. Now, over 50 years later, evidence indicates that the disease is in fact produced by the toxic action of copper. However, the toxicity is not acquired, but results from an inborn error of metabolism.

Copper is an element essential to life and is present in abundance in the human diet. Copper deficiency is unknown in man. The normal metabolism of copper allows retention of the trace amounts required by the body and elimination of excess amounts which would otherwise prove toxic.

Numerous references to studies on normal metabolism of copper and on the biochemical aspects of Wilson's disease can be found in reviews by Cumings (1959); Peisach, Aisen, and Blumberg (1966); Sass-Kortsak (1965); Scheinberg and Sternlieb (1965); and Walshe (1967). The main biochemical features are outlined below.

Tissue Copper:

Metabolic intoxication in Wilson's disease had been suggested in the German literature soon after the description of the disease. In 1945, Glazebrook investigated a patient with Wilson's disease and found, at autopsy, a high copper content in liver and brain (Glazebrook, 1945). More detailed studies followed, and have established the presence of abnormally high copper concentrations in the liver, brain, kidney, and cornea.

Tissue copper levels in patients have been listed by Cumings (1959). Copper concentration apparently increases as the disease progresses. In one study, liver copper levels varied from 4 to 40 times the normal mean levels, and brain copper levels varied from 7 to 17 times the normal mean level (Butt et al., 1958). It is of interest that these authors found the silver content of liver and brain to be increased by 12 and 4 times, respectively. The increase in silver content has been mentioned in earlier German literature and has apparently never been adequately explained.

An increased copper content of the liver may be useful as a diagnostic feature in asymptomatic Wilson's disease (Sternlieb and Scheinberg, 1963), although these authors would now accept 250 µg./g. dry liver rather than 100 µg./g. dry liver as the upper limit for normal copper content.

Elevated tissue copper levels presented by Butt et al. (1958) in "juvenile cirrhosis" are misleading because several of the patients were infants, in which copper levels are normally high, and at least "some", if not all of the other patients were later reported to have Wilson's disease (Butt and Nusbaum, 1962).

Urine Copper:

Mandelbrote et al. (1948) observed an increased amount of urinary copper in a patient with the disease. Porter (1949) soon confirmed this in two more patients. Many other investigators have similarly found increased levels.

Patients with symptomatic Wilson's disease usually excrete more than 100 ug. copper per 24 hours in the urine compared with less than 50 ug.

per 24 hours in normal individuals. The level of urinary copper can vary considerably. Levels arenot usually increased before clinical symptoms appear.

Bearn and Kunkel (1954a) found high urinary copper levels in cirrhosis due to biliary obstruction. Urinary copper levels may also be increased in nephrosis (Cartwright, Gubler, and Wintrobe, 1954) due to loss of ceruloplasmin in the urine. Both these conditions are clinically distinguishable from Wilson's disease.

Biliary Copper:

The copper content in bile has been reported normal in four patients with Wilson's disease (Denny-Brown and Porter 1951; Cartwright et al., 1954). The significance of this is discussed in the section on the dynamic aspects of copper metabolism.

Serum Copper:

Total serum copper levels were found to be below normal levels in most patients with Wilson's disease (Bearn and Kunkel, 1952; Cartwright et al., 1954), an observation since confirmed in many patients. In normal humans, about 95 per cent of the serum copper is firmly bound in the form of ceruloplasmin (Holmberg and Laurell, 1947; 1948). The finding of low serum copper values in patients prompted an examination of ceruloplasmin levels, as discussed below. In patients with Wilson's disease, the direct-reacting copper fraction, which is not bound to ceruloplasmin, is increased (Cartwright et al., 1954).

A low total serum copper level is not a reliable feature of Wilson's disease. A moderately low ceruloplasmin level combined with an increased direct-reacting copper fraction can produce a normal serum copper level in a patient.

Ceruloplasmin:

Reduced levels of serum oxidase activity, as well as total serum copper, were found in patients with Wilson's disease by Bearn and Kunkel (1952). Serum oxidase activity had previously been shown to be almost entirely due to ceruloplasmin (Holmberg and Laurell, 1947; 1948). Scheinberg and Gitlin (1952) found a deficiency of ceruloplasmin in patients by spectrophotemetric and immunologic methods. The majority of patients have a pronounced deficiency of ceruloplasmin, for example, in one series of 111 patients, 81.1 per cent had levels of less than one half of the lower normal limit of about 20 mg./100 ml. (Sternlieb and Scheinberg, 1963). A decreased ceruloplasmin level provides one of the most reliable diagnostic tests for the disease.

The ceruloplasmin levels of patients do extend over a range from complete deficiency to normal levels (Markowitz et al., 1955; Sternlieb and Scheinberg, 1963). In these two series, four (2.9 per cent) of a total of 139 patients have levels above their normal lower limits. More than a dozen patients with normal ceruloplasmin have been reported in the literature (reviewed by Kurtze, 1962; Scheinberg and Sternlieb, 1963). The presence of a normal ceruloplasmin level does, therefore, not exclude the diagnosis of Wilson's disease. The levels, even when normal, tend to be in the lower region of normal. No values have been reported above the normal mean of about 30 to 32 mg./100 ml.

Other Copper Proteins:

Many proteins can form complexes with copper. A true copper protein, however, has copper as an integral part of its molecule and in a definite proportion. Some of these copper-proteins have enzymatic functions. Copper and other metallo-proteins are discussed in reviews by Vallee (1955) and Malmström and Neilands (1964).

There is no evidence that patients with Wilson's disease have abnormalities of any other copper proteins. Tyrosinase must not be markedly decreased, as in albinism, because patients have normal pigmentation. Chromatographically defined copper proteins of the brain are the same in normal individuals and in patients although, in the latter, copper is also complexed to a variety of proteins which are not normally associated with copper (Porter, 1963; 1964). Excess copper in the liver of patients is bound to a protein in the mitochondrial fraction, which shows some properties similar to the mitochondrocuprein of neonatal liver (Porter, 1963). The mean level of copper in erythrocytes, most of which is bound in erythrocuprein (Markowitz et al., 1959), was found to be similar in patients with Wilson's disease and in normal controls (Cartwright et al., 1954).

C. Copper Metabolism in Wilson's Disease

The use of radioactive isotopes of copper has helped elucidate the metabolic pathway of copper, not only in Wilson's disease, but in the normal individual. Copper-64 (^{64}Cu), with a half-life of only 12.8 hours, has been used almost exclusively in metabolic studies to date because of its commercial avilability. The longer-lived copper-67 (^{67}Cu), with a half-life of 58.5 hours, is not available commercially.

Copper Absorption:

Orally ingested ^{64}Cu appears rapidly in the blood, indicating rapid absorption from the intestinal tract. Comparisons of the response curve, following an oral dose of ^{64}Cu, have shown an increased peak of ^{64}Cu and decreased rate of disappearance of ^{64}Cu from plasma or serum in patients with Wilson's disease when compared with control subjects (Earl, Moulton, and Silverstone, 1954; Bearn and Kunkel, 1955; Bush et al., 1955; Jensen and Kamin, 1957). The higher peak of radioactivity has been interpreted to be a result of increased intestinal absorption in the patient with Wilson's disease. However, an equally valid explanation would be delayed clearance of ^{64}Cu from the plasma. In fact, when ^{64}Cu is administered intravenously, patients show a delayed fall in the ^{64}Cu level from their plasma in comparison with normal controls (Earl, Moulton, and Silverstone, 1954; Bearn and Kunkel, 1955; Bush et al., 1955). Delayed clearance of ^{64}Cu from the serum, perhaps because of failure of an excretory pathway, is thus a feasible explanation for the results.

Copper Incorporation into Ceruloplasmin:

In the normal individual, the fall in the level of plasma ^{64}Cu after its oral or intravenous administration is followed by a secondary rise. Bearn and Kunkel (1954b) demonstrated that ^{64}Cu was immediately bound to albumin after its entry into plasma and that the secondary rise in plasma ^{64}Cu was due to its incorporation into ceruloplasmin. These authors found that patients with Wilson's disease didnot show this incorporation of ^{64}Cu into the ceruloplasmin fraction. The failure of patients with Wilson's disease to incorporate ^{64}Cu into ceruloplasmin is now well documented (Bush et al., 1961; Sternlieb, Morell, and Scheinberg, 1961a) and has also been observed in a patient with a normal level of ceruloplasmin (Sass-Kortsak et al., 1959).

Ceruloplasmin - bound copper, although exchanged in vitro (Scheinberg and Morell, 1957), is not exchanged in vivo (Sternlieb et al., 1961a). Ceruloplasmin, therefore, cannot function in the transport of copper. Copper is apparently transported to other body tissues from the small albumin-bound fraction, which contains only about five to ten per cent of the total serum copper. A small third fraction in equilibrium with albumin-bound copper is bound to amino acids and may be important in the transport of copper through membranes (Neumann and Sass-Kortsak, 1963; 1967).

Copper Uptake into the Liver:

As the albumin-bound fraction of ^{64}Cu disappears from the blood, ^{64}Cu appears in the liver, an observation first made by Bush et al. (1955). The uptake of ^{64}Cu into the liver, the main organ in which

concentration takes place, was found to be slower in patients with Wilson's disease than in control subjects (Sass-Kortsak et al., 1962). A study of eight patients indicated that their livers took up a smaller fraction of the administered dose than did the normal liver (Osborn and Walshe, 1961; Osborn, Roberts, and Walshe, 1963). In the normal individual, the ^{64}Cu activity in the liver reaches a peak four to ten hours after administration then gradually declines, while in the patient the activity rises more slowly and continues to rise apparently because there is no discharge of the ^{64}Cu from the liver (Osborn and Walshe, 1961; Sass-Kortsak et al., 1962; Osborn, Roberts, and Walshe, 1963). This reduced ability of the liver to take up radioactive copper appears to be a consequence of the disease process. Aspin and Sass-Kortsak (1966), using a tracer dose of ^{64}Cu, found that a patient with clinically mild disease had a rapid uptake of ^{64}Cu into the liver during the first ten hours after intravenous administration of ^{64}Cu, similar to the uptake in unaffected individuals. The patient showed a further slow accumulation of activity in the liver instead of the expected decrease. The initial uptake appeared to be more rapid than in the unaffected individuals, but could rather indicate a decreased excretion from the liver since the response curve is the net affect of ^{64}Cu input and excretion. After a re-examination of their data, Osborn and Walshe (1967) found that the impairment of copper concentration in the liver is seldom found in asymptomatic patients but is pronounced in severely affected patients. After several years of treatment with penicillamine, the liver regains some of its copper-concentrating function (Osborn and Walsh, 1967). The improvement could be due to removal of the heavy copper deposits from the liver or to repair of previously damaged liver.

The former explanation is more likely since the uptake of ^{64}Cu into the liver was normal in five out of six patients with liver damage not due to Wilson's disease (Osborn, Roberts, and Walsh, 1963).

Biliary Excretion of Copper:

Copper has been shown to be excreted mainly via the bile in dogs (Mahoney et al., 1955) and humans (Bush et al., 1955). In humans given oral or intravenous copper, the amount of copper in the bile increases rapidly, and shows a considerable increase after 30 minutes (van Ravesteyn, 1944). Further evidence that biliary copper excretion responds quickly to increased copper intake is provided by studies in mice, in which the ^{64}Cu content of feces increases with the administered dose (Gitlin, Hughes, and Janeway, 1960) and in rat liver, in which recovery of ^{64}Cu in bile increases with the administered dose (Owen and Hazelrig, 1966). With the high concentrations of copper in the liver of patients with Wilson's disease, an increased concentration of copper would be expected in the bile. Scheuer and Barka (1964) have shown that rats whose livers have been loaded with copper prior to the administration of ^{64}Cu have an increased excretion of ^{64}Cu in the bile. The ratio between bile and liver activity was similar in copper-loaded and control rats. The uptake of ^{64}Cu into the liver was increased in the copper-loaded rats. This is similar to the finding in early Wilson's disease (Aspin and Sass-Kortsak, 1966). This evidence suggests that the low net uptake of ^{64}Cu found in symptomatic patients is a result of saturation of the normal copper binding sites in the liver.

Several studies have indicated a decreased fecal excretion of ^{64}Cu in patients with Wilson's disease (Matthews, 1954; Bush et al., 1955; Bearn and Kunkel, 1955; Aspin and Sass-Kortsak, 1966). Since most of this copper originates from the bile (Mahoney et al., 1955), these data combined with those outlined above provides strong evidence that biliary excretion of copper is impaired in Wilson's disease.

Urinary Excretion of Copper:

The urinary excretion of ^{64}Cu is higher in patients than in controls (Earl, Moulton, and Silverstone, 1954; Bearn and Kunkel, 1955), even in early stages of the disease (Aspin and Sass-Kortsak, 1966). However the total of urinary and fecal copper excretion is nevertheless considerably reduced.

Uptake of Copper into Erythrocytes:

The initial uptake of ^{64}Cu into erythrocytes was found to be greater in one patient than in his normal brother and the secondary rise lower in the patient (Neumann and Silverberg, 1967). They suggested that the higher initial rise was due to the longer half-life of circulating albumin-bound ^{64}Cu. This is probably a consequence of its slower removal from the circulation via the liver. The small reduction in secondary rise may be a chance deviation. Studies on other patients have shown a normal uptake of ^{64}Cu into the erythrocytes (Bush et al., 1955; Jensen and Kamin, 1957).

Summary of Biochemical and Metabolic Abnormalities:

The main biochemical and metabolic abnormalities in untreated patients with Wilson's disease are as follows:

1. increased copper content in liver and brain
2. increased urinary excretion of copper
3. decreased total serum copper
4. decreased serum ceruloplasmin
5. decreased rate of incorporation of ^{64}Cu into ceruloplasmin
6. reduced uptake of ^{64}Cu into liver (in advanced disease)
7. probably reduced biliary excretion of copper

D. Genetic Aspects of Wilson's Disease

Racial Distribution:

Patients with Wilson's disease have been reported from many countries, and in individuals of European, Chinese, Japanese, Indian, Eskimo, Indonesian, and Negro stock (references in Cumings, 1959; Scheinberg and Sternlieb, 1965). The occurrence of the gene for Wilson's disease in African negroes is uncertain since in reported cases of American negroes, one had white ancestors (Herz and Drew, 1950) and pedigrees of three other were not given (Bearn, 1960; Holtzman et al., 1967). In the series of 32 patients compiled by Bearn (1960), 14 were Jews from Eastern Europe, eight from the Mediterranean, and ten from various other origins. This particular distribution is of course influenced by the racial distribution of the inhabitants of New York City from where this sample was drawn.

Clinical Type of Disease:

Jewish patients from Eastern Europe appear to have predominantly neurological symptoms with a later age of onset and more slowly progressive character than is the tendency in other patients (Bearn, 1960). The hepatic form of the disease, with early onset and rapid deterioration is more prevalent among Oriental patients (Arima and Kurumada, 1962a; Tu, 1963). These clinical differences may be influenced by genetic factors. However, the copper-rich diet of Oriental peoples may well be a contributing factor to the earlier onset of the disease (Tu, 1963).

Although it has recently been suggested that the hepatic form of the disease is a separate genetic entity (Levi, Sherlock, and Scheuer, 1967), present evidence does not favor this interpretation. In many reported cases, the combination of symptoms makes classification of the disease into either a hepatic or neurological form impossible. A brief period of hepatic disease may be followed many years later by typical neurological symptoms. While the disease is frequently similar within a sibship, in other sibships the clinical manifestations are different (Bearn, 1960; Walshe, 1962).

Mode of Inheritance:

Hall (1921) was the first to suggest that Wilson's disease is inherited. He suggested recessive inheritance was a possibility but favored a two-gene hypothesis in which the disease occurred in the presence of a gene spread generally in the population, plus a recessive gene within the family. Evidence presented by Kehrer (1930), Stadler (1939), and André and van Bogaert (1950) suggested recessive inheritance. Matthews, Milne, and Bell (1952) analyzed family data, mainly from the three previously stated authors, and concluded that the disease resulted from a recessive hereditary factor, in spite of a significant increase in the number of affected sibs over that expected. Correction had been made for the exclusion of carrier parent pairs who have only normal children, however, bias towards selection for families with more than one affected sib was probable.

Early studies were hampered by lack of knowledge of biochemical abnormalities of the disease. Usually sibs under a given age were excluded because it was impossible to determine if they had the disease. Bearn (1953, 1960), from an analysis of his own data rather than reports from the literature, confirmed that the ratio of affected sibs of probands was that expected for recessive inheritance. However, those unaffected sibs under the age of 30 years or those who died before 30 years were excluded because biochemical tests were not carried out on all sibs.

Analysis of pedigrees of 21 Polish families (Wald, 1962), 18 Japanese families (Arima and Kurumada, 1962b), and nine Chinese families (Tu, 1963) support recessive inheritance.

The recent suggestion that the hepatic form of Wilson's disease is not inherited as a simple recessive trait (Levi, Sherlock, and Scheuer, 1967) is based on the erroneous use of data from only five families in which no correction has been made for heterozygous parent pairs who have, by chance, had no affected children.

The consanguinity rate is increased among the parents, as expected for a rare recessive gene. Bearn (1960) found a first cousin consanguinity rate of 36.7 per cent among 30 families in his series. The rate was particularly high among Eastern European Jews. A first cousin consanguinity rate of 40.3 per cent was found among parents of patients in Japan, compared with a rate of four to six per cent in the general population (Arima and Kurumada, 1962b). On the other hand, Walshe (1967) has found a first cousin consanguinity rate of only 9.3 per cent among 43 families in England, where the

consanguinity rate of a "general hospital population" has been estimated at 0.61 per cent (Bell, 1940). Dahlberg has demonstrated that the consanguinity rate among parents heterozygous for a recessive gene decreases as the frequency of the gene increases, (Stern, 1960, p. 376), suggesting that the gene frequency is rather high among the population of England.

Gene Frequency:

Bearn (1953) estimated a gene frequency of from one in one thousand to one in two thousand, with a corresponding disease incidence of from one in four million to one in one million. This estimate was derived from Dahlberg's formula (see Stern, 1960), assuming panmixia, and using the approximation $q = \frac{a\ (1 - x)}{16}$, where q = gene frequency, a = frequency of cousin marriages in the general population, and x = observed incidence of cousin marriages in the genetic material to be analyzed. Figures of from 0.5 to 1.0 per cent were used as the consanguinity rate in the population, both of which are probably too high. Many more cases were present in Bearn's series from New York than would be expected from the estimates. Scheinberg (1967) has suggested a prevalence of about one in one hundred thousand for Wilson's disease in the United States. The difference between prevalence and incidence of diseases has been discussed (Myrianthopoulos, 1961). Since the prevalence of Wilson's disease is expectedly lower than the incidence at birth because early death can occur, Bearn's estimates appear to be inadequate. Bearn has concluded that his data were derived from a non-homogeneous population of genetic isolates with a higher gene

frequency and higher consanguinity rate than in the general population. Actually, a higher consanguinity rate alone could account for the observed findings.

The gene frequency has been reported to be ten times higher in Japan than the estimate derived from Bearn's study. Using Dahlberg's formula, Arima and Kurumada (1962b) have estimated a gene frequency of one in 135 to 90, with a corresponding disease incidence of one in 73 thousand to 33 thousand. These figures were calculated on the basis of an incidence of consanguinity in the general population of Japan of four and six per cent, respectively. There are insufficient data to estimate the gene frequency among the Chinese. However, no consanguinity was found in nine families with Wilson's disease. This may be due only to the lower rate of consanguinity among the Chinese compared with that of the Japanese (Tu, 1963) and not to a different gene frequency.

The gene frequency in England can be estimated by Dahlberg's formula to be one in 274, with a disease incidence of one in 75 thousand. These figures are based on a consanguinity rate of 0.6 per cent in the general population (Bell, 1940) and 9.3 per cent in parents of patients with Wilson's disease (Walshe, 1967). The gene frequency in the English population is very similar to the lowest estimate for Japan. Probably the gene frequency is similar in Caucasian and Oriental races. Bearn's very different estimates may reflect the particular genetic constitution of the New York City population from which he has ascertained his patients, as described previously.

His material is in fact quite unique in having a high proportion of Eastern European Jews with a late, predominantly neurological onset. There is little genetic information on the patients described by Walshe (1967). The source population may be more homogeneous than that of New York City.

Detection of Heterozygotes:

Heterozygotes can be helpful in the identification of the basic defect of a recessively inherited condition.

Individuals heterozygous for the Wilson's disease gene, that is parents of patients, occasionally have a ceruloplasmin level below the normal range (Cartwright et al., 1960; Sass-Kortsak et al., 1961; and Sternlieb, et al., 1961b). In fact, some heterozygotes have ceruloplasmin levels as low as those typically found in patients, yet do not have any clinical signs of the disease. Sternlieb, Morell, and Scheinberg (1961) have estimated, from reports in the literature, that about 20 per cent of heterozygotes have decreased ceruloplasmin levels. In the largest single series presented (Sternlieb et al., 1961b), two of 19 parents of patients had ceruloplasmin levels below those usually found in normal individuals: 19.2 and 9.5 mg./100 ml. respectively.

A more frequently found defect in heterozygotes is a decreased rate of uptake of ^{64}Cu into ceruloplasmin after an oral dose of ^{64}Cu (Sass-Kortsak et al., 1961; Sternlieb et al., 1961b). The response curves frequency lie between those of individuals homozygous for the normal alleles and those homozygous for the recessive alleles. Almost all heterozygotes may show reduced incorporation when suitable

techniques are used, including the intravenous rather than oral administration of ^{64}Cu (Taux et al., 1966; Sass-Kortsak, 1966).

Heterozygotes do not have clinical symptoms of Wilson's disease or hypercupuria. They can usually be differentiated from asymptomatic patients by their lower hepatic copper concentration (Sternlieb and Scheinberg, 1963) and by their response to ^{64}Cu. In rare cases, the distinction between heterozygote and affected homozygote cannot be made despite all investigations.

E. Chelation Therapy for Wilson's Disease

Clinical Aspects:

Effective treatment of Wilson's disease requires the elimination of the excess stores of copper from the body and the prevention of its reaccumulation.

Cumings (1948) suggested that BAL (2, 3-dimercaptopropanol) might be used to reduce the copper content of the tissues in Wilson's disease and at about the same time Mandelbrote and co-workers (1948) reported that BAL injections were followed by increased urinary excretion of copper. Many patients were treated with BAL with varying degrees of success. The necessity for intramuscular injections plus frequent side effects did not make BAL suitable treatment for prolonged therapy.

In 1956, Walshe showed that penicillamine (β, β-dimethylcysteine) increased the urinary excretion of copper in Wilson's disease and suggested it as a convenient form of therapy (Walshe, 1956). Penicillamine can be taken orally, is relatively non-toxic, and is effective in the maintenance of a negative copper balance. Many patients are now being treated on continuous penicillamine therapy usually with good results (Walshe, 1960; Sternlieb and Scheinberg, 1964), provided that treatment is carried on long enough at an adequate dosage, usually of about 1 g. penicillamine daily. Clinical improvement may not be apparent for several months but usually occurs, provided the disease is not in terminal stages before treatment is started. With early diagnosis, the prognosis in Wilson's disease appears to be extremely good and the disease is no longer "progressive and invariably fatal" as described by Wilson in 1912.

The L-isomer of penicillamine produces toxic reactions apparently because it is a pyridoxine (Vitamin B_6) antagonist (Kuchinskas and du Vigneaud, 1957). Studies in rats (Heddle, McHenry, and Beaton, 1962) and man (Jaffe, Altman, and Merryman, 1964) provided biochemical evidence of pyridoxine deficiency following the administration of D,L-penicillamine. The D-isomer of penicillamine is preferred for treatment in Wilson's disease. A disturbance of tryptophan metabolism was produced, with high doses of D-penicillamine, in rats (Asatoor, 1964) and in man (Jaffe, Altman, and Merryman, 1964) although six of the nine patients in the latter study were not normal in that they had rheumatoid arthritis. One patient with Wilson's disease among 19 on D-penicillamine treatment showed evidence of pyridoxine deficiency as indicated by increased xanthurenic acid excretion after a tryptophan load (Gibbs and Walshe, 1966). The deficient individual was an adolescent boy taking a daily dose of 1800 mg. penicillamine, or 47 mg./kg. body weight. These authors suggested that the administration of pyridoxine to patients on penicillamine therapy is probably unnecessary unless the daily dose exceeds 40 mg./kg. or during active growth periods.

Another copper-chelating agent, sodium diethyldithiocarbamate has been claimed to be particularly effective in mobilizing copper from the brain although it is less effective in promoting the urinary excretion of copper (Sunderman, White, and Sunderman, 1963). This form of treatment has not yet been adequately evaluated.

In addition to continuous treatment with a chelating agent, patients usually have a diet which excludes copper-rich foods such as oysters, liver, nuts, and chocolate.

A comprehensive outline of treatment has been presented by Sternlieb and Scheinberg (1964).

The Effects of Penicillamine Upon Copper and Ceruloplasmin Levels: Direct evidence from liver biopsies indicates that penicillamine reduces the copper content of the liver (Sternlieb and Scheinberg, 1964) as would be expected from the clinical improvement shown. Penicillamine probably competes for copper normally bound to albumin thus making the copper available for filtration at the glomerulus (Walshe, 1963b; Osborn and Walshe, 1964). Within the first six hours after the administration of penicillamine to normal individuals, the plasma copper level fell, then rose to normal levels, probably due to its replacement from tissue copper stores (Walshe, 1964). The copper-protein bond in liver is fairly resistant to the direct action of penicillamine (Freyer and Walshe, 1963). These data suggest that penicillamine may deplete albumin-bound copper, which is then made up from tissue deposits, rather than by direct removal of copper from the tissues.

A pronounced decrease in the serum copper concentration is produced in patients with Wilson's disease after continuous penicillamine treatment; this is sometimes evident after only four months of treatment (Sternlieb and Scheinberg, 1964; Walshe, 1964). In four patients with schizoprenia and presumably with normal copper metabolism, serum copper levels showed only a slight decrease after one year of penicillamine treatment (Walshe, 1964).

The level of serum ceruloplasmin usually falls in patients with Wilson's disease after penicillamine therapy. After penicillamine therapy, in 17 patients with measurable ceruloplasmin levels prior to therapy, Sternlieb and Scheinberg (1964) found that the ceruloplasmin levels of eight patients were no longer measurable, six showed no change, and three showed a slight increase, one of the latter during an infection. In another series, in five out of six patients who had ceruloplasmin levels over 5 mg./100 ml. before any penicillamine therapy was started, the ceruloplasmin level decreased considerably after four to 12 months of treatment; the level was unchanged in one patient after six months of treatment (Walshe, 1964). At least three patients have been reported in which the ceruloplasmin level was normal or nearly so prior to treatment and fell progressively to negligible amounts after 18, eight and six months respectively (Rosenoer, 1961; Walshe, 1964; Holtzman, Elliott, and Heller, 1966). On the other hand, the ceruloplasmin levels were unchanged in four schizophrenics after taking 1200 mg. penicillamine each per day for one year (Walshe, 1964).

F. Pathogenesis of Wilson's Disease

There is ample evidence, as reviewed previously, that copper is present in excessive amounts in certain organs of individuals with Wilson's disease. When the copper deposits are removed by effective chelation therapy, as outlined, the symptoms of the disease regress. Wilson's disease is apparently synomymous with copper intoxication.

Experimental studies on the toxicity of copper have been reviewed by Scheinberg and Sternlieb (1965) and Walshe (1967). Evidence has been presented that, in the cell, copper exerts a toxic effect on the mitochondria (Vogel and Kemper, 1963). In other studies, copper was shown to inhibit ATPase from the microsomal fraction of pigeon and rat brain homogenates (Peters, Shorthouse, and Walshe, 1966; Peters and Walshe, 1966). These authors therefore suggested that copper exerts its toxic effects by inhibiting ATPase and consequently the membrane transport of ions. The unanswered question now is which of the above two mechanisms is more important, or indeed, whether either one is the primary mechanism.

The primary defect causing the copper intoxication is not yet known. A genetically determined defect in protein metabolism was proposed as the primary defect of Wilson's disease, with disordered copper metabolism as a secondary effect (Uzman, Iber, and Chalmers, 1956). These authors suggested that abnormal proteolytic activity in the tissues led to the formation of protein or polypeptide residues with a high affinity for copper. Aminoaciduria and peptiduria, consequences of proteolytic activity, were considered to be primary biochemical manifestations of the disease. There is little support

for this hypothesis. Abnormal copper-binding proteins or polypeptides have not been isolated from the liver (Porter, 1963; Morell, Shapiro, and Scheinberg, 1961). The delayed uptake of copper into the liver in advanced stages of Wilson's disease (Osborn, Roberts, and Walshe, 1963) does not support the presence of abnormal substances with a high copper affinity. Furthermore, aminoaciduria is generally found as a late symptom, while excess copper deposits are found even in completely asymptomatic patients. Only in rare families is aminoaciduria found regularly in unaffected relatives (Uzman and Hood, 1952; Soothill et al., 1961).

The more favoured hypothesis is that the basic defect lies in some aspect of copper metabolism. The ceruloplasmin deficiency, when first recognized, was proposed as the cause of Wilson's disease (Scheinberg and Gitlin, 1952). Richterich et al. (1960) proposed that this deficiency arises because of lack of an enzyme converting ceruloplasmin from a precursor to final form.

A number of hypotheses, involving various aspects of the transfer of copper, have been proposed. A deficiency of an enzyme which concentrates copper in the liver was suggested by Walshe (1963a). Broman (1964) has proposed that the transfer of copper into ceruloplasmin is blocked and that ceruloplasmin is the only form in which copper is made available for the formation of cytochrome oxidase. Other hypotheses have included an unspecified block in the intracellular transport of copper (Sass-Kortsak, 1965); the lack of enzymatic incorporation of copper into ceruloplasmin which is required for the biliary excretion of copper (Gaballah et al., 1965), and impaired transport of copper across cell membranes (Neumann and Silverberg, 1966).

THE PRESENT STUDIES

PART I

Genetic Studies of Wilson's Disease and Studies of Ceruloplasmin in Relatives of Patients

Genetic studies have been carried out by Bearn on a group of patients in New York City (Bearn, 1953, 1960). The studies reported here were carried out on a different group of patients and it was of interest to determine if any of the genetic features differed in the two groups.

The heterozygotes occurring in the families investigated were of particular interest. Some heterozygotes for the Wilson's disease gene have reduced serum levels of ceruloplasmin. These have generally been regarded as an extreme expression of the heterozygous Wilson's disease gene which, in the homozygous condition, causes a pronounced reduction of the ceruloplasmin level. Arima and Kurumada (1962a) repeated measurements of ceruloplasmin levels, usually on one occasion, in ten parents of patients with Wilson's disease. In seven of these parents, the levels were normal on one determination and below normal at another, but effects of variation due to technical factors could not be evaluated. Part of the present study was undertaken to determine how much variation in the serum ceruloplasmin level occurs in a heterozygote for the Wilson's disease gene and what factors cause low levels in certain heterozygotes.

Walshe and Carpenter (1965) have reported a statistically significant difference in the inhibitory effects of sodium azide and of iproniazid on serum from Wilson's disease patients compared with normal individuals. We have carried out further inhibition studies on sera from heterozygotes, patients and normal individuals.

MATERIALS AND METHODS

Probands:

Many of the patients included in this study were admitted to either The Hospital for Sick Children, Toronto, or The Montreal Children's Hospital. Others were referred to these hospitals for special study or were ascertained from records of other hospitals. All diagnosed patients known in the cities of Montreal and Toronto and surrounding areas up to mid-1967 have been included, with the exception of one adult patient in Montreal who could who could not be included. A total of 33 proven cases from 25 families have been studied. The place of residence of these patients is as follows: Toronto and vicinity - five, other Ontario centres - nine, Montreal - four, Nova Scotia - one, Newfoundland - one, the United States - five.

All patients were diagnosed definitely as cases of Wilson's disease by biochemical tests, tests of liver and renal function, clinical features generally including Kayser-Fleischer rings, and frequently metabolic studies with ^{64}Cu. Clinical diagnoses were confirmed by Dr. Andrew Sass-Kortsak, at the Hospital for Sick Children, or Dr. Mervin Silverberg at The Montreal Children's Hospital.

Relatives of Patients:

Extensive family pedigrees were obtained from each of the 25 families. In almost all families, the pedigree was traced back to ancestry in Europe or Asia.

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. In addition, some had liver function tests, measurement of urinary copper excretion and studies with ^{64}Cu. The latter tests were always carried out when there was some question regarding diagnosis.

Eleven genetically proven heterozygotes (ten parents of patients with Wilson's disease and the monozygotic twin of a parent) were studied over periods of six to seven months to determine the variation of their serum ceruloplasmin levels. Blood was obtained by venepuncture at approximately monthly intervals for a total of six or seven determinations. Serum copper was also assayed in these samples. The serum samples were frozen as obtained and stored at $-4^{o}C$. Determinations of serum ceruloplasmin and copper were made on all samples for each individual at the same time, at the end of the test period, to eliminate variation due to technical error.

In 13 of the families, as many as possible of the living aunts, uncles, grandparents, and cousins had a venous blood sample taken for ceruloplasmin assay, or a finger capillary blood sample taken for screening for ceruloplasmin deficiency.

Biochemical Techniques:

The method used for the quantitative assay of ceruloplasmin and the screening method for the detection of low ceruloplasmin levels were described in Section I of this thesis.

Total serum copper was measured by the modified method of Eden and and Green (1940) as for studies in Section I.

Starch gel electrophoresis was carried out on some of the sera using vertical electrophoresis (Smithies, 1959). The electrophoretic conditions were those described by Shreffler et al. (1967) to obtain optimal resolution of electrophoretic variants in sera from certain Negroes. The gel buffer was 0.016 M boric acid, 0.010 M sodium hydroxide, pH 9.5. The cell buffer was 0.21 M boric acid, 0.085 M sodium hydroxide, pH 9.0. Electrophoresis was carried out at 4°C for 22 hours at 180 v (6v/cm.). Serum samples were diluted one to two with water when the ceruloplasmin concentration was normal or one to one when the ceruloplasmin concentration was low. The gel was sliced in half horizontally. One half was stained for ceruloplasmin with o-dianisidine by a method slightly modified from that of Owen and Smith (1961). The staining solution consisted of 20 ml. 0.5 per cent o-dianisidine stock solution in 0.05 N HCl, 30 ml. ethanol, and 10 ml. 0.2 M acetate buffer of pH 5.5, made up to a total volume of 100 ml. solution. The ceruloplasmin appears as a yellowish-brown band after about one and one-half hours at 37°C. This same half of the gel was usually stained for haptoglobin. The ceruloplasmin stain was poured off after staining was completed and

to the gel was added 20 ml. 0.5 per cent o-dianisidine stock solution made up in 0.05 N HCl, 30 ml. ethanol, 10 ml. 0.1 M acetate buffer of pH 5.7, and 1 ml. 10 per cent hydrogen peroxide, made up to a total volume of 100 ml. solution. This solution stains the hemoglobin-haptoglobin bands in five to ten minutes at room temperature. The gel was washed for about ten minutes in water. The ceruloplasmin and haptoglobin bands were examined over an X-ray illuminator and were classified. The gel was then photographed, using transmitted light, with a Polaroid camera using 146-L film. The gels must be photographed because the ceruloplasmin-stained areas broaden and diffuse in a period of hours. The other half of each gel was stained with Amido-Black 10B to localize all serum proteins.

Inhibition studies were carried out on sera from some of the patients with Wilson's disease and heterozygotes to determine possible differences in response from that of sera from normal individuals. Sodium azide and iproniazid[1] were the inhibitors used. The method used was basically the same as that described for ceruloplasmin determination, with the following change: the appropriate concentration of inhibitor in 0.5 ml. buffer plus 1.5 ml. buffer was used, instead of 2.0 ml. buffer only. Concentrations of inhibitor were selected such that a range of inhibition from almost zero to almost total was included. Reagents were added in the order buffer, buffer plus inhibitor, substrate (PPD), serum. Reactions were carried out at 37°C using 0.15 ml. aliquots of serum.

[1] Kindly provided by Frank W. Horner, Ltd., Montreal, Canada.

Studies with Copper - 64:

The fate of a dose of ^{64}Cu was studied in some of the parents and other relatives. The investigations were directed by Drs. Andrew Sass-Kortsak and Norman Aspin at the Hospital for Sick Children. The techniques previously described for the oral administration (Sass-Kortsak et al., 1961) and intravenous administration of ^{64}Cu (Aspin and Sass-Kortsak, 1966) were used.

RESULTS

Features of the Probands and Their Affected Sibs:

The 25 probands had a total of 14 affected sibs. Included among the 39 patients are six asymptomatic patients and five sibs who probably had Wilson's disease, in retrospect, but in whom no firm diagnosis was ever made. Four sibs were excluded; one died in infancy, one died at six years of nephrosis, one died accidentally at 12 years, and one 31 year-old brother refused to be tested.

In the appendix are listed all patients included in the study with the following information: sex of patient and of age of onset of disease, racial origin and consanguinity of the parents, and the number of normal and affected sibs.

The first symptoms of Wilson's disease were primarily neurological in 13 patients and hepatic in 20 patients. All five of the patients with unconfirmed diagnoses died of hepatic failure following brief illnesses. Their symptoms were so typical of the course of the hepatic form of the disease that they can be included with reasonable certainty.

There were 23 male and 16 female patients.

The age of onset of the disease was 16 years or less for 27 of the 33 clinically affected patients.

The age of onset of the 33 clinically affected patients is plotted against the geographical origin of their parents in Fig. 9. Three patients, distinctively marked, are shown twice because their parents have different geographical origins. Sibs are indicated. Twenty-six patients from 18 families have one or both parents originating in western Europe (Great Britain, France and Germany). Eight patients from eight families have one or both parents originating in central-eastern Europe (Poland, Czechoslovakia, western Russia). In many families, several generations had been born in Canada or the U.S.A. and geographical origin refers to the country of their first foreign-born ancestors.

The age of onset tended to be earlier in patients of western European origin that in patients of central-eastern European origin, although the numbers involved are too small for statistically valid comparisons.

The distribution of ceruloplasmin levels in the patients is shown in Table 6. These levels, with one exception, were measured prior to the commencement of penicillamine therapy. Three patients have normal or nearly normal levels: one Jewish male, S.C., had a level of 18.0 mg./100 ml. at the earliest available determination four years after treatment with penicillamine was started and 21.4 mg./100 ml.

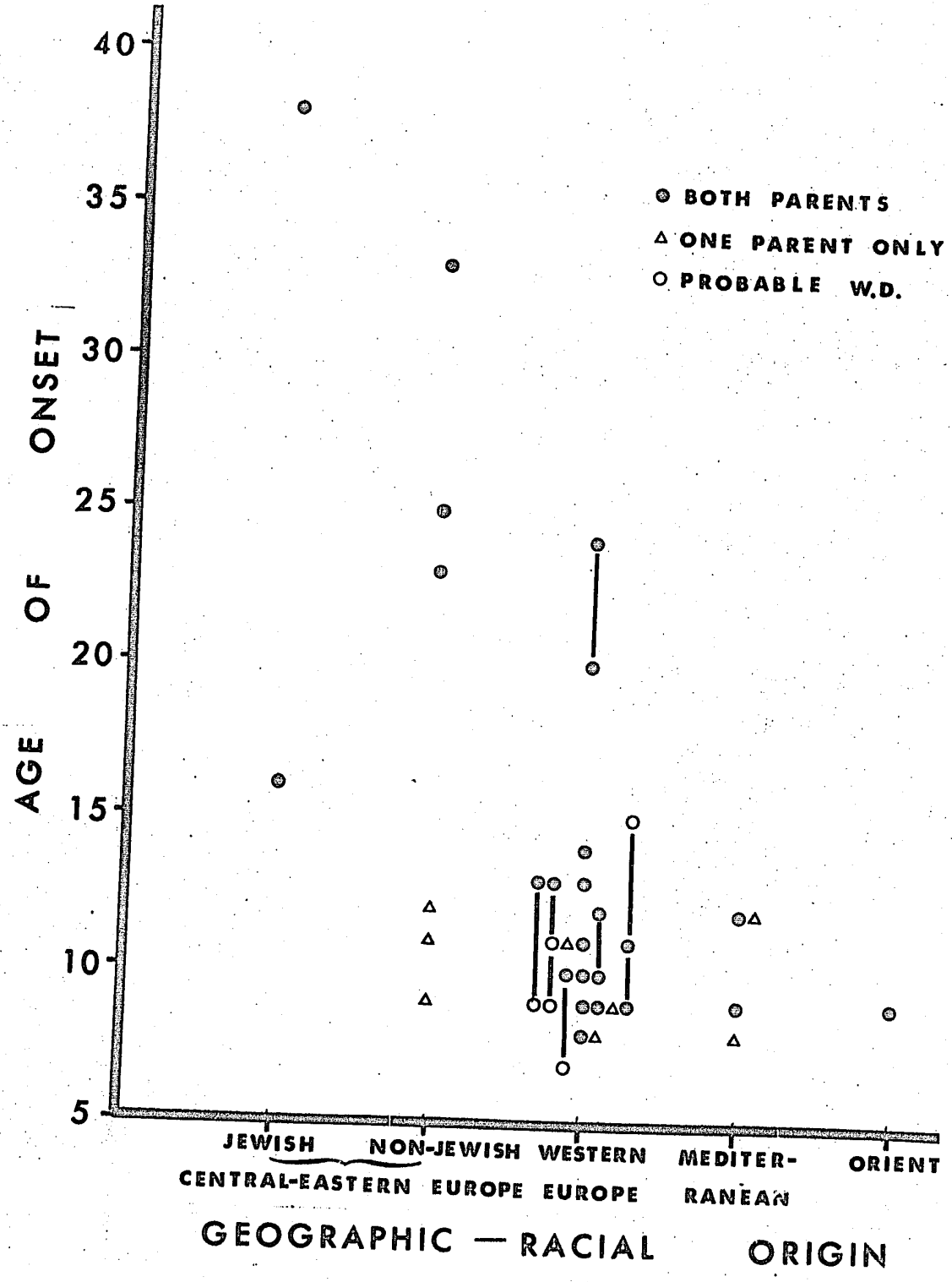

Fig. 9. Age of onset of Wilson's disease plotted against geographic-racial origin of patient. Six asymptomatic patients are not included. Sibs are joined by vertical lines.

TABLE 6

DISTRIBUTION OF CERULOPLASMIN LEVELS IN PATIENTS WITH WILSON'S DISEASE

Ceruloplasmin level (mg./100 ml.)	No. patients observed
0 - 4.9	20
5.0 - 9.9	3
10.0 - 14.9	4
15.0 - 29.9	0
>20	3
unknown	9

after a further four years of treatment. He was reported to have a 'low normal' level prior to treatment (Dauphinee, 1965; personal communication). The two remaining patients were sibs, with ceruloplasmin levels in the low normal region. They have been reported in detail previously (Sass-Kortsak et al., 1959). Both S.C. and a patient with a low ceruloplasmin level (J.O.) had ceruloplasmin which showed the usual mobility on starch gel electrophoresis.

Mode of Inheritance:

Ascertainment is probably fairly complete for the large urban centres. Among cases referred from outlying areas, there may be a tendency to refer patients after at least one sib has been diagnosed or has died from the disease.

Assuming complete ascertainment, the 'a priori method' can be used to calculate the true proportion of affected children, correcting for the exclusion from our sample of families of two heterozygous parents in which, by chance, no children have been affected (Li, 1961, p.61). If we assume recessive inheritance, the probability that a child of two heterozygous parents is affected is 1/4. The probability that a sibship of size s will not contain any affected offspring is $(3/4)^s$. Since a sibship is ascertained only when at least one offspring is affected, we can identify only $1 - (3/4)^s$ families of size s.

Since the total number of offspring observed, t_s, represents $1 - (3/4)^s$ of the theoretical total, c, then:

$$c = \frac{t_s}{1 - (3/4)^s}$$

The theoretical total number of children for each sibship is shown in Table 7. The corrected proportion of affected offspring is therefore:

$$b = \frac{\Sigma r_s}{\Sigma c_s} = \frac{39}{152.7} = 0.255$$

The variance, calculated according to the method outlined by Li (1961) is:

$$V(b) = \frac{1}{163.57} = .0061$$

The difference between the observed proportion, 0.255, and the expected proportion, 0.250, is much less than two standard deviations and is clearly not significant.

If ascertainment is considered to be incomplete and the data collection is by single selection, in which only one affected individual per family is ascertained, then the following method of analysis is appropriate (Neel and Schull, 1954, p. 223):

$$p = \frac{R-N}{T-N} = \frac{39 - 25}{105 - 25} = 0.175$$

where p = correct estimate of recessive proportion, R = total number of affected individuals recorded, N = total number of sibships, and T = total number of children.

TABLE 7

ANALYSIS OF 25 SIBSHIPS WITH AT LEAST ONE CASE OF WILSON'S DISEASE BY 'A PRIORI' METHOD

Family size s	No. of families n_s	No. of offspring $sn_s = t_s$	Theoretical total offspring $c_s = \frac{t_s}{1 - (3/4)^s}$	No. of affected observed
2	5	10	22.857	5
3	9	27	46.697	12
4	5	20	29.252	10
5	2	10	13.110	4
7	1	7	8.078	1
10	2	20	21.190	4
11	1	11	11.483	3
Total		105	152.667	39

The variance of this estimate is as follows:

$$\sigma^2 = \frac{(T-R)(N-R)}{(T-N)^3} = 0.0018$$

The standard error of the estimate is 0.0425. The estimated proportion p is not significantly different from 0.25 (z = 0.176). Recessive inheritance is supported also by this method of analysis.

Gene Frequency:

The frequency of consanguineous marriages (first cousin) among the parents is three in 25, that is 0.12 in this series. One pair of parents were second cousins and one pair third cousins. There were no consanguineous marriages among the five pairs of parents of central-eastern European origin.

The frequency (q) of the recessive gene for Wilson's disease can be calculated from Dahlberg's formula (Stern, 1960, p.375):

$$q = \frac{c(1-k)}{16k - 15c - ck}$$

where c = frequency of first cousin marriages in the general population and k = observed incidence of cousin marriages among parents of the patients with Wilson's disease. This formula assumes random mating. The value to use for c poses some difficulty. An incidence of consanguinity of 0.4 per cent was found among parents of children in a general hospital population in England, however this may be an overestimate if illnesses requiring hospitalization are increased by consanguineous marriages. An incidence of 0.05 per cent was found in a large city in the U.S.A. The frequency of

consanguineous marriages in the whole of North America perhaps lies between these values, but probably closer to the lower figure. The consanguinity frequency was probably higher in the population of small European towns where four of the pairs of parents originated, and in an isolated area of Canada where one parent pair originated. However, since the majority of parents were born and married on this continent, the overall consanguinity rate would be similar to that of the general North American population. The incidence of cousin marriages in the population from which our sample was drawn probably lies between 0.2 and 0.6 per cent. The calculations of gene frequency (q) incidence of affected individuals (q^2) and incidence of heterozygotes (2pq) are shown in Table 8 for values of c of 0.2 and 0.6.

The present data suggest that the gene frequency is of the order of 1.9×10^{-3} to 9.3×10^{-4} with a corresponding disease incidence of one in 280,000 to one in 1,100,000. The frequency of heterozygotes would then be one in 265 to one in 537.

The corresponding estimates calculated from the data of Bearn (1953), Arima and Kurumada (1962b), and Walshe (1967) are shown in Table 8 for comparison. These will be discussed later.

Variability of Ceruloplasmin and Copper Levels in Heterozygotes: The ceruloplasmin levels were found to be remarkably constant in most heterozygotes over the period of the study in the 11 heterozygotes (ten parents, one identical twin of a parent). The individual determinations, means and standard deviations are shown

TABLE 8

ESTIMATIONS OF THE INCIDENCE OF THE RECESSIVE GENE, AFFECTED INDIVIDUALS, AND HETEROZYGOTES FROM THE PRESENT DATA AND FROM THE LITERATURE

Reference	Population consang. c	Parental consang. k	Gene frequency q	Disease incidence $q^2 \times 10^{-6}$	Heterozygote incidence 2 pq
Present Study	0.004	0.12	1.9×10^{-3}	3.6	1 per 265
	0.002	0.12	9.3×10^{-4}	0.87	1 per 537
Bearn (1953)	0.01	0.375	1.1×10^{-3}	1.1	1 per 467
Bearn (1953)	0.005	0.375	5.3×10^{-4}	0.28	1 per 945
Arima and Kurumada (1962b)	0.06	0.403	6.6×10^{-3}	68.0[a]	1 per 77[b]
	0.04	0.403	4.1×10^{-3}	27.0[a]	1 per 122[b]
Walshe (1967)	0.004	0.093	2.5×10^{-3}	6.4	1 per 197

[a] Disease incidence = $Fq + (1-F)q^2$

[b] Heterozygote incidence = $2(1-F)\,pq$

where F = coefficient of inbreeding = 0.004

for each heterozygote in Fig. 10. The mean standard deviation of the ceruloplasmin levels of the 11 heterozygotes was 2.1 ± 0.8 mg./100 ml. (coefficient of variation 8.3 per cent).

The 95 per cent lower confidence limit of 20.7 mg./100 ml. and the 99 per cent lower confidence limit of 17.6 mg./100 ml. for the normal population studied in Section I are shown in Fig. 10. If the 95 per cent limit is chosen to define the lower acceptable limit of normality, three of the ten parents and the identical twin of one of these parents had an abnormally low mean ceruloplasmin level. Two of the three parents and the identical twin had low levels at each occasion of testing, with mean levels of 13.3, 16.4, and 15.1 mg./100 ml., all below the 99 per cent lower confidence limit. The levels of the third parent fluctuated around the lower 95 per cent limit with a mean of 20.4 mg./100 ml. The ceruloplasmin levels of the remaining seven parents were within the normal range on all six or seven test occasions. It, therefore, appears that the ceruloplasmin level, whether normal or low, is usually characteristic for any given heterozygote. A heterozygote with a level close to the lower limit of normal on a single determination is difficult to classify.

The serum copper levels 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. (coefficient of variation 9.2 per cent). The serum copper level was less effective in distinguishing the heterozygote, with only one parent having a

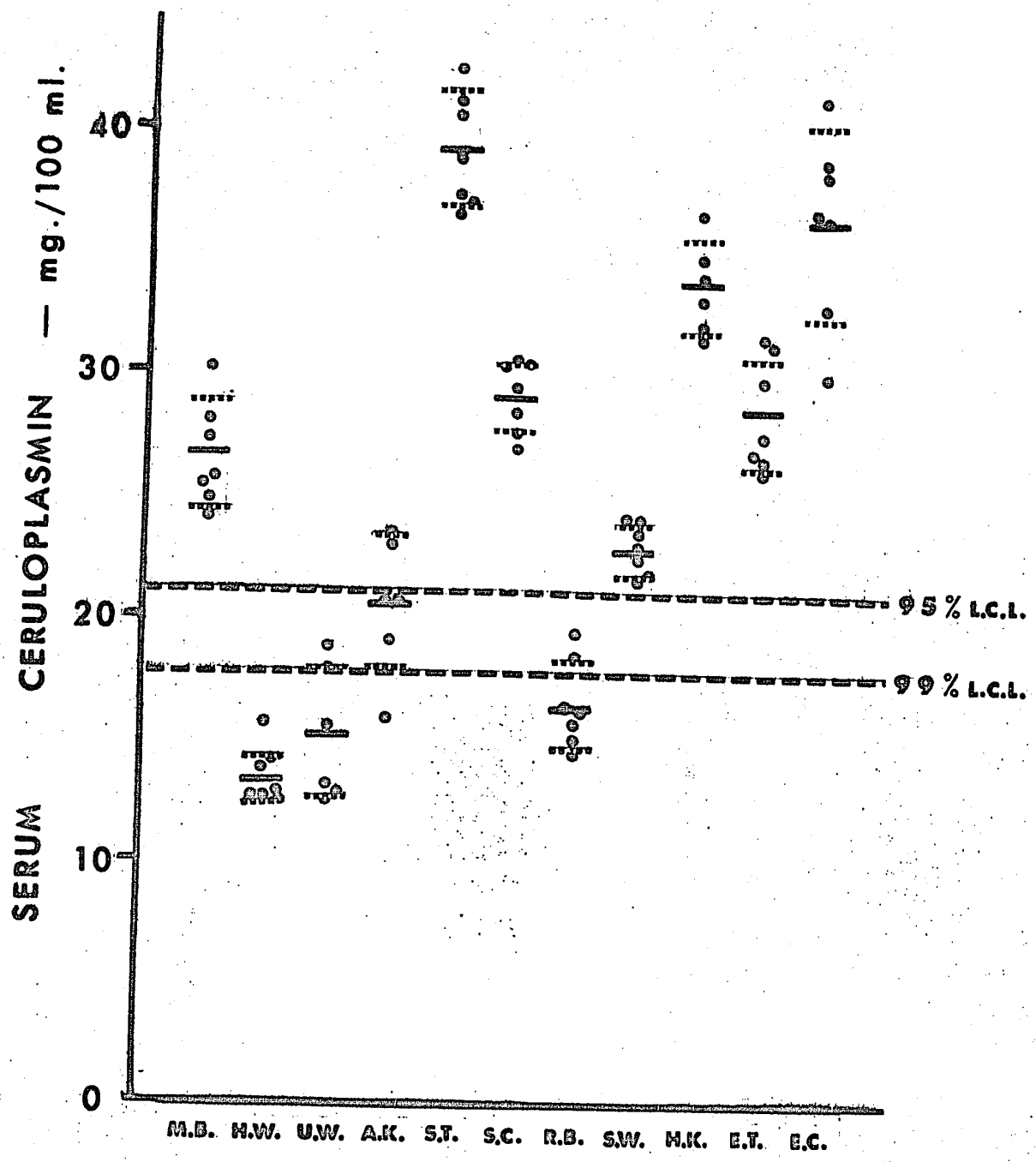

Fig. 10. Serial determinations of serum ceruloplasmin levels in heterozygotes (10 parents, 1 monozygotic twin of a parent). Solid lines - mean; dotted lines - 1 S.D. above and below mean; continuous dashed lines - lower confidence limits (L.C.L.) for normal population.

mean copper level below the normal limit (normal mean 108 µg./100 ml., 95 per cent confidence limits 67 to 149 µg./100 ml. as shown in Section I).

It should be noted that mild respiratory infections, which occured at some time during the test period in most of the heterozygotes, had no apparent effect on ceruloplasmin or copper levels. None of the female heterozygotes were pregnant or taking oral contraceptives at the time of the study.

In addition to the presumably heterozygous parents studied over several months, the ceruloplasmin levels in 23 other parents of patients with Wilson's disease were determined, usually on at least two occasions. Of the total 33 parents studied (excluding the identical twin), all had normal ceruloplasmin levels except for the three with low levels as mentioned previously. These parents include 17 fathers and 16 mothers of 18 different families. In this sample, 9.1 per cent of the parents had ceruloplasmin levels below the 95 per cent confidence limits, 6.1 per cent were below the 99 per cent limits. Of the 33 parents, one pair were first cousins and one pair were second cousins; the parents with low levels were from three different unrelated parent pairs. If the related parents are counted only once, then two of 31 or 6.5 per cent of heterozygotes have ceruloplasmin levels below the 99 per cent confidence limits of the normal population. The incidence of such heterozygotes among all heterozygotes of western European origin is 10.0 per cent.

In a series of 19 parents of patients, Sternlieb et al. (1961) found two with ceruloplasmin levels, apparently determined on one occasion, below the normal limit. These two parents had levels of 19.2 and 9.5 mg./100 ml., respectively, and the normal lower limit was considered to be 20 mg./100 ml. The assay method was basically similar to that used in the present series. When these two series are combined, four of a total of 52 presumed heterozygotes, or 7.7 per cent had ceruloplasmin levels below the normal limit, assuming the limit of the latter series to be at the 99 per cent level, and omitting our borderline parent. This figure cannot be corrected for the occurrence of related heterozygotes as these data are not given by Sternlieb et al. (1961).

The mean ceruloplasmin level of the 33 parents was 30.3 ± 6.6 mg./100 ml. When the two parents with abnormally low levels were omitted, the mean level was 31.3 ± 5.4 mg./100 ml. These values differ from the mean of 30.7 mg./100 ml. for the normal population by less than one standard deviation and are clearly not significantly different.

Ceruloplasmin Levels in Other Relatives:

More extensive testing of ceruloplasmin levels was carried out in 13 kindreds. Only those kindreds in which five or more relatives, including parents and sibs, were tested have been included. These relatives were usually tested on only one occasion, unless the level was abnormal on the first testing.

The ceruloplasmin level was measured in a total of 112 other relatives from 11 kindreds including 35 unaffected sibs, five offspring (presumably heterozygotes), 25 aunts and uncles, six grandparents, 36 first cousins, and five nieces and nephews. Children less than three years of age, pregnant women, and women on oral contraceptives were not included in these numbers.

The distribution of ceruloplasmin levels of these relatives and the 24 parents from the same kindreds is shown in Fig. 11b. The corresponding distribution of a normal population is shown in Fig. 11a. The distribution shown in Fig. 11a was obtained from the study of 309 normal individuals reported in Section 1 (see Fig. 5). The scale for the number of individuals was adjusted in Fig. 11a so that the heights of the histogram are equivalent to those of the sample of 136 individuals in Fig. 11b making direct comparison possible. The ceruloplasmin levels of females less than 12 years of age and males less than 15 years of age were adjusted to the equivalent of 12 years as outlined in Section I. The 95 per cent confidence limits for males 15 to 19 years of age are 19.1 and 32.4 mg./100 ml. (99 per cent limits: 16.8 and 34.7 mg./100 ml.). The age adjustment factor for male adolescents was applied only for use on the histogram, but in practice this may result in broader confidence interval than actually exists in this group. Relatives who are probably heterozygous for the Wilson's disease gene either from pedigree studies (that is, parents or offspring of a patient) or by studies of the rate of incorporation of ^{64}Cu into

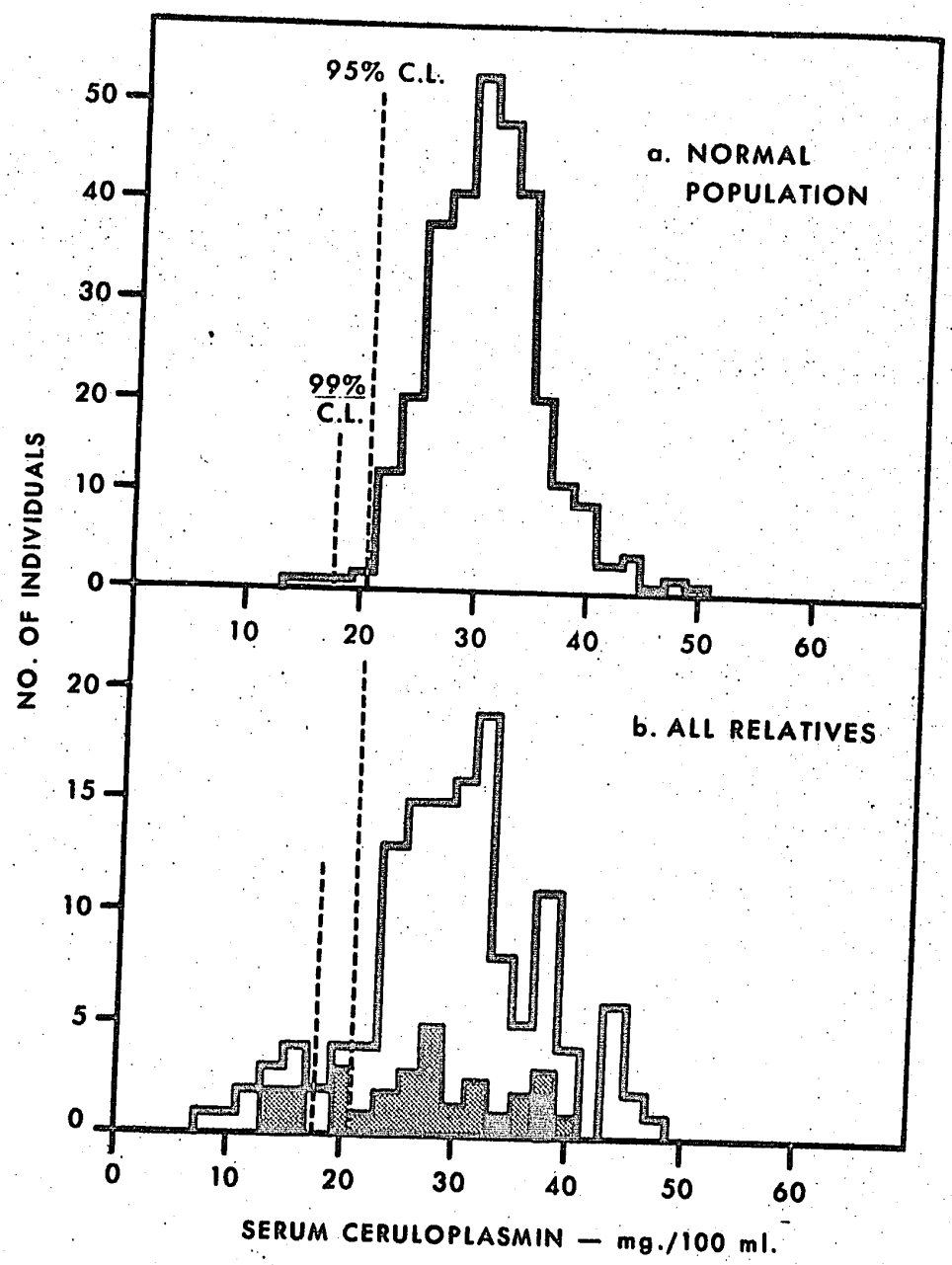

Fig. 11a. Distribution of serum ceruloplasmin levels in normal population (309 individuals). Scale is adjusted so that 11a and 11b are directly comparable. 95 and 99 per cent lower confidence limits are shown.
Fig. 11b. Distribution of serum ceruloplasmin levels in 136 relatives of patients with Wilson's disease.
Shaded squares - heterozygotes; solid squares - relatives of patients with normal ceruloplasmin levels.

ceruloplasmin are indicated. Three of these presumed heterozygotes were relatives (offspring) of one of the probands with a near normal level of ceruloplasmin and are indicated on the histogram. Fig. 11 shows that there is a pronounced excess of relatives with low ceruloplasmin levels over the number expected in a sample from the normal population. The 95 per cent lower confidence limit (20.7 mg./100 ml.) and 99 per cent lower confidence limit (17.6 mg./100 ml.) are shown.

An additional 116 relatives were tested by the screening test for the detection of reduced ceruloplasmin levels as described in Part 2 of Section I. The relatives included 15 aunts and uncles, two grandparents, 97 first cousins, one nephew, and one first cousin once removed. A standard of 20.7 mg./100 ml. was used for adults and appropriately higher standards were used for children (refer to Section I). Children less than three years of age, pregnant women, and women on oral contraceptives, were again excluded from the results since no reliable normal figures were available. However no relatives in these categories had decreased ceruloplasmin levels. Of the 116 relatives tested, one had a positive test result and the remainder were negative. The one positive result was that of the first cousin once removed, a three and one half year old male, in kindred B as shown in Fig. 12. A quantitative assay of ceruloplasmin from a blood sample obtained by venepuncture confirmed a low level: 11.9 mg./100 ml. as assayed, 2.0 mg./ml. after age correction. This age correction may not be valid in such a case where production of ceruloplasmin is probably already very low and may not respond to the factors which normally cause the age effect.

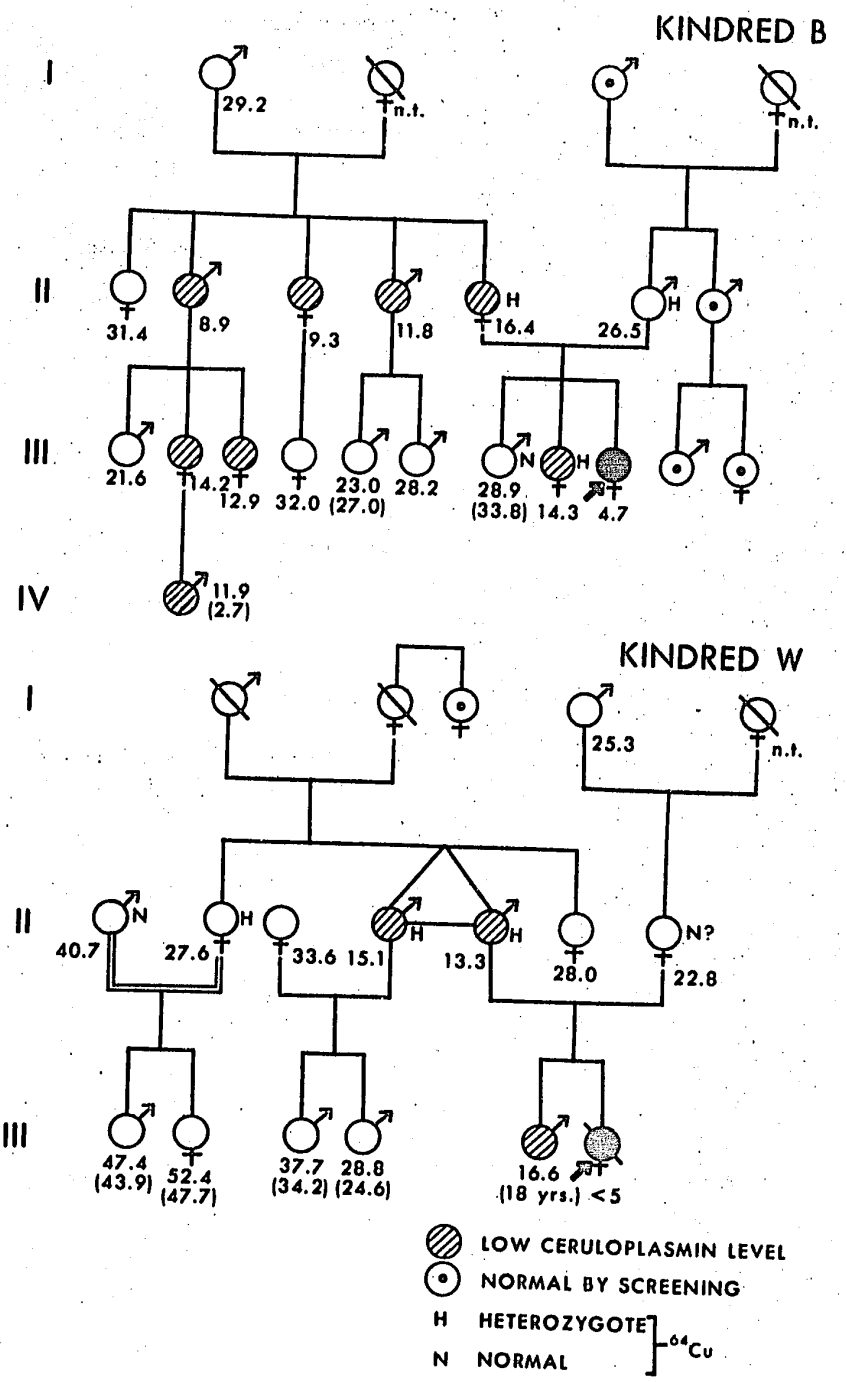

Fig. 12. Pedigrees of atypical kindreds B and W, showing only those individuals tested. Figures are ceruloplasmin levels in mg./100 ml.; age adjusted values, when required, are shown in parentheses. Shaded symbols represent individuals with a low ceruloplasmin. n.t. - not tested.

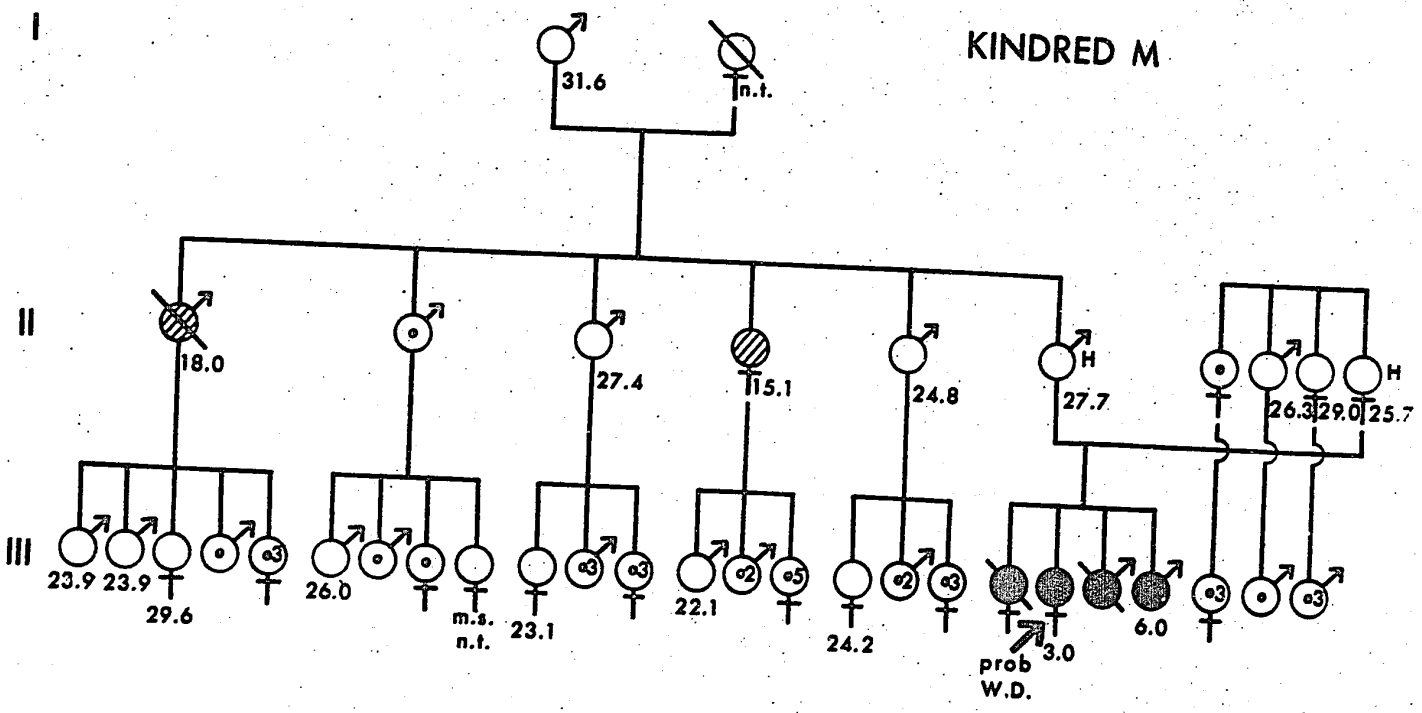

Fig. 13. Pedigree of atypical kindred M. For explanation of symbols, see Fig. 12.
m.s. - multiple sclerosis.

The 13 kindreds whose members had ceruloplasmin levels determined by quantitative assay or by screening were not known to be interrelated. Seventeen relatives with levels below 20.7 mg./100 ml. were found in seven families. All of these relatives were clinically normal. Studies of five sibs, three parents, and one uncle with low ceruloplasmin levels indicated a normal urinary excretion of copper, normal liver function tests, and no abnormal findings on physical examination. The two cousins and first cousin once removed in kindred B (Fig. 12) had normal liver function tests and were normal upon physical examination. The young first cousin once removed will be examined in more detail at a later date but on the basis of his pedigree, plus the extreme deficiency of ceruloplasmin found in the proband in this family, it is unlikely that he has Wilson's disease. The results of studies of the incorporation of ^{64}Cu on these relatives will be discussed subsequently.

Eleven of the relatives with low ceruloplasmin levels cluster in the three kindreds shown in Figs. 12 and 13. All relatives tested are shown and their ceruloplasmin levels are indicated. The distribution of ceruloplasmin levels of all relatives tested is shown in Fig. 14. The levels of relatives in the three 'atypical' kindreds have been separated from those of the relatives in the ten 'typical kindreds. The distribution of ceruloplasmin levels are distinctly bimodol in the atypical kindreds, with one mode lying below the normal limit. Five relatives with low levels of ceruloplasmin

were found in four of the typical kindreds and include one offspring, one parent (previously mentioned as borderline), and three sibs of patients from two kindreds. Both parent pairs of the three sibs had normal ceruloplasmin levels on at least two occasions. The ceruloplasmin levels of these five relatives in the typical families are, even after age correction, above the 99 per cent lower confidence limit for the normal population. On the other hand, ten of 11 relatives from the atypical kindreds have levels below the 99 per cent confidence limit, the exception being an uncle in kindred M who died before he could be retested. In Fig. 15, only adult relatives have been included. The bimodality of the distribution of ceruloplasmin levels in the atypical kindreds is again clearly shown. The borderline levels in the typical families are now almost entirely eliminated. While the formula for age correction is valid on the average, each specific individual may deviate from the mean so that children with borderline corrected levels are difficult to classify with certainty.

Individuals known to be heterozygotes, either from pedigree analysis or studies of the incorporation of ^{64}Cu into ceruloplasmin, to be discussed subsequently, are indicated in Figs. 14 and 15. The ceruloplasmin levels of heterozygotes in the typical kindreds cover a wide range not apparently different from that of the normal population.

Metabolism of Copper-64 in Relatives of Patients:

In two of the atypical kindreds (B and W as shown in Fig. 12), 12 relatives were studied to determine the rate of incorporation of

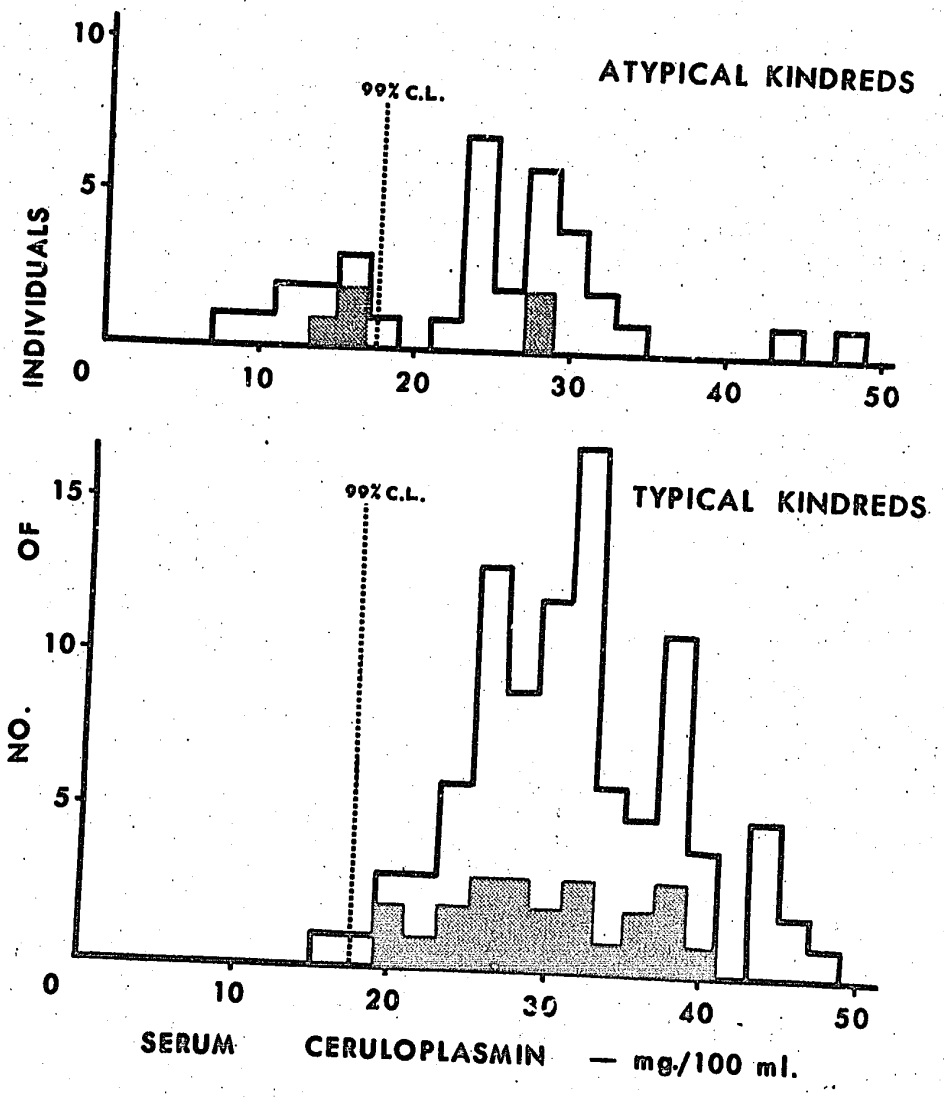

Fig. 14. Distribution of serum ceruloplasmin levels in all relatives of patients with Wilson's disease in atypical and typical kindreds. Shaded areas - heterozygotes, genetically or by ^{64}Cu studies.

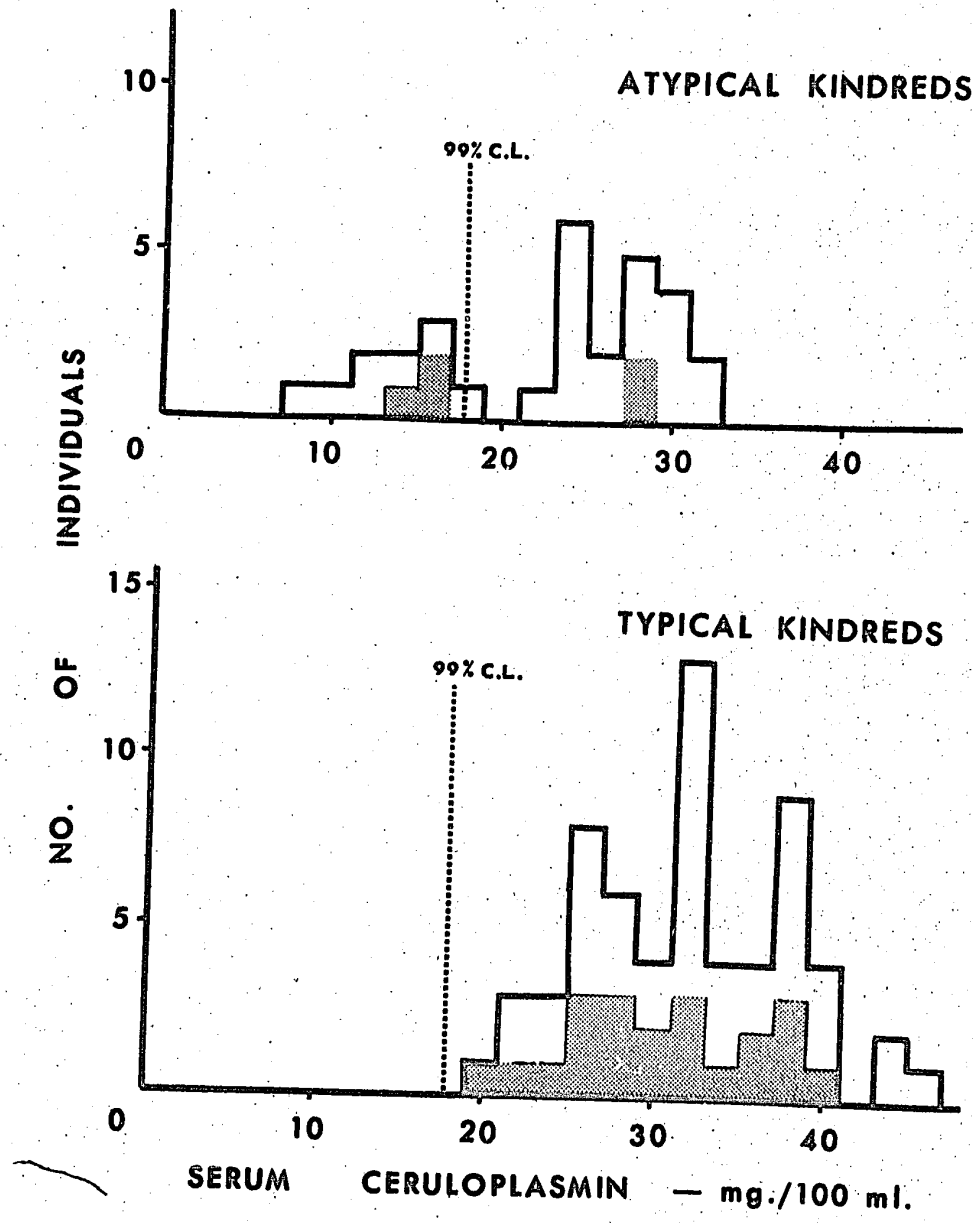

Fig. 15. Distribution of serum ceruloplasmin levels in adult relatives of patients with Wilson's disease in atypical and typical kindreds. Shaded areas - heterozygotes, genetically or by ^{64}Cu studies.

^{64}Cu into ceruloplasmin after the administration of an oral dose of 0.5 or 1.0 mc. ^{64}Cu. The results of these studies have been published (Sass-Kortsak et al., 1961) and are presented in greater detail in Fig. 16. The raw data for these graphs were kindly provided by Dr. Andrew Sass-Kortsak. A high rate of incorporation was shown by the mother S.W. (II-7 in Fig. 12) and a maternal great-uncle (II-1) in kindred W, and sib R.B (III-7) in kindred B. Both parents in kindred B (II-5 and II-6) had a lower rate of incorporation of ^{64}Cu into ceruloplasmin, presumably indicating their heterozygous state. Definitely reduced rates of incorporation of ^{64}Cu were shown by the father H.W. (II-5), sib D.W. (III-5), aunt M.K. (II-2), and uncle U.W. (II-4) in kindred W, and sib B.B. (III-8) in kindred B, all apparently heterozygous for the gene for Wilson's disease.

The ratio, R, of the fraction of the administered ^{64}Cu in the serum 48 hours after administration compared with the fraction of administered ^{64}Cu in the serum after one or two hours (using the higher value) was calculated for each individual in kindred B to be as follows: father (II-5) 0.278, mother (II-6) 0.290, sib R.B. (III-7) 0.730, sib B.B. (III-8) 0.191. If a heterozygote usually has an R value of less than 0.559 and a normal homozygote usually has an R value greater than 1.253 (Sternlieb et al., 1961), then the classification of heterzygous individuals in kindred B remains the same as by graphic analysis, but sib R.B. now falls into the overlap region where classification is uncertain. In kindred W, R values could not be calculated for the aunt (II-2) and

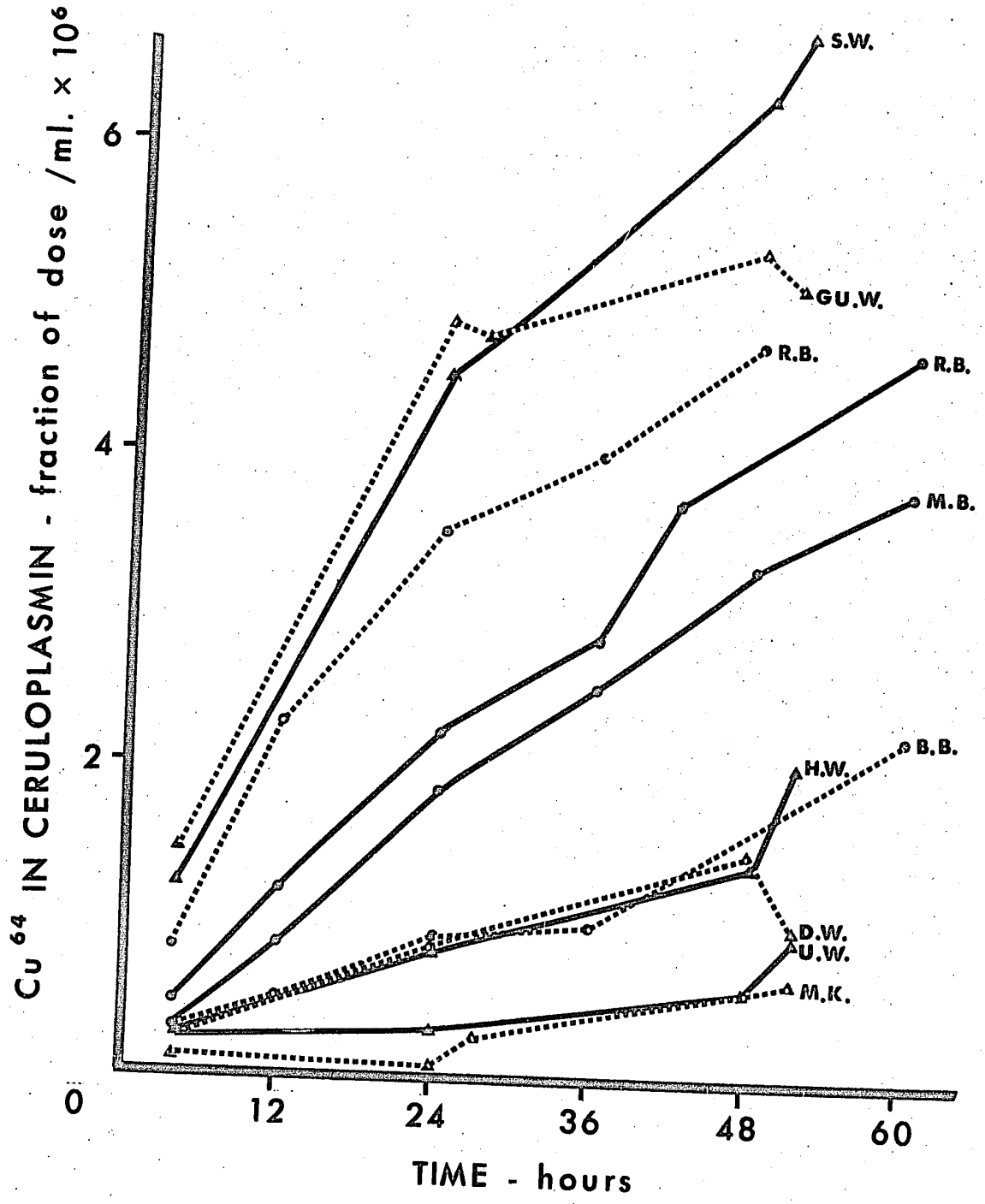

Fig. 16. Incorporation of ^{64}Cu into ceruloplasmin in relatives of patients with Wilson's disease following oral administration of ^{64}Cu. Solid lines - presumed heterozygotes (parents, one monozygotic twin of parent); dotted lines - other relatives, as described in text. ⊙ - relatives from kindred B; △ - relatives from kindred W.

great-uncle (II-1) because serum ^{64}Cu levels were not measured until four hours after ^{64}Cu administration. For the other members of this kindred, only two hour measurements were available and these do not necessarily represent the peak of the one and two hour measurements. However, using the fraction of administered ^{64}Cu in the serum at two hours, the R values are as follows: father (11-5) 0.330, mother (II-7) 1.40, uncle (II-4) 0.216, sib D.W. (III-5) 0.343. As by graphic analysis, the mother cannot be distinguished from normal homozygotes and the other individuals are probably heterozygous for the Wilson's disease gene.

A number of other relatives were studied, after the intravenous administration of from 0.15 to 0.73 mc. of ^{64}Cu, to determine the following aspects of metabolism of ^{64}Cu: rate of disappearance from and reappearance in plasma, rate of incorporation into ceruloplasmin, rate of uptake into the liver, and excretion in urine and feces. The rate of uptake of ^{64}Cu into ceruloplasmin is the most useful criterion, as previously reviewed, for distinguishing heterozygotes from normal homozygotes. Seven patients with Wilson's disease, nineteen relatives of patients, and two normal controls were studied. High specific activity ^{64}Cu, allowing the use of tracer doses of copper, was used for all tests except for those of the two parents from atypical kindred M. The relatives consisted of the two parents from atypical kindred M, three parents and four sibs from typical kindreds included in the present study, and three parents and seven sibs from kindreds not classified and not included because of insufficient family data. The raw data on the incorporation of ^{64}Cu into ceruloplasmin

provided by Drs. A. Sass-Kortsak and N. Aspin, was analyzed graphically as shown in Fig. 17. The bands indicate the ranges of values found at each time of measurement. None of the seven patients showed measurable incorporation of ^{64}Cu into ceruloplasmin. The two normal controls showed high rates of incorporation. Seven of the heterozygous parents (six of western European origin and one of central-eastern European origin) showed rates of incorporation of ^{64}Cu into ceruloplasmin well below the normal rates, as indicated in Fig. 17. One parent (J.Or.), of Italian origin, showed a somewhat higher rate of incorporation as plotted separately in Fig. 17. The father of the patients in atypical kindred M showed one of the lowest rates of ^{64}Cu incorporation. All of these parents had normal serum ceruloplasmin levels. The sibs could be divided into two groups, one having a low rate of ^{64}Cu incorporation similar to that of the heterozygous parents, and the other showing a high rate of ^{64}Cu incorporation similar to that of the normal controls. The six sibs with a low rate of incorporation, as shown in Fig. 17, are probably heterozygous for the Wilson's disease gene. Two of these sibs had ceruloplasmin levels, after correction for age effects, between the 95 and 99 per cent lower confidence limits of the normal population; the other four sibs had normal ceruloplasmin levels. The five sibs with high rates of incorporation are probably homozygous normal individuals. Four of these sibs were included with the normal controls in Fig. 17; the rate of ^{64}Cu incorporation of one sib (J. D.) was exceptionally high and is shown separately. All of the sibs in the latter group had normal serum ceruloplasmin levels,

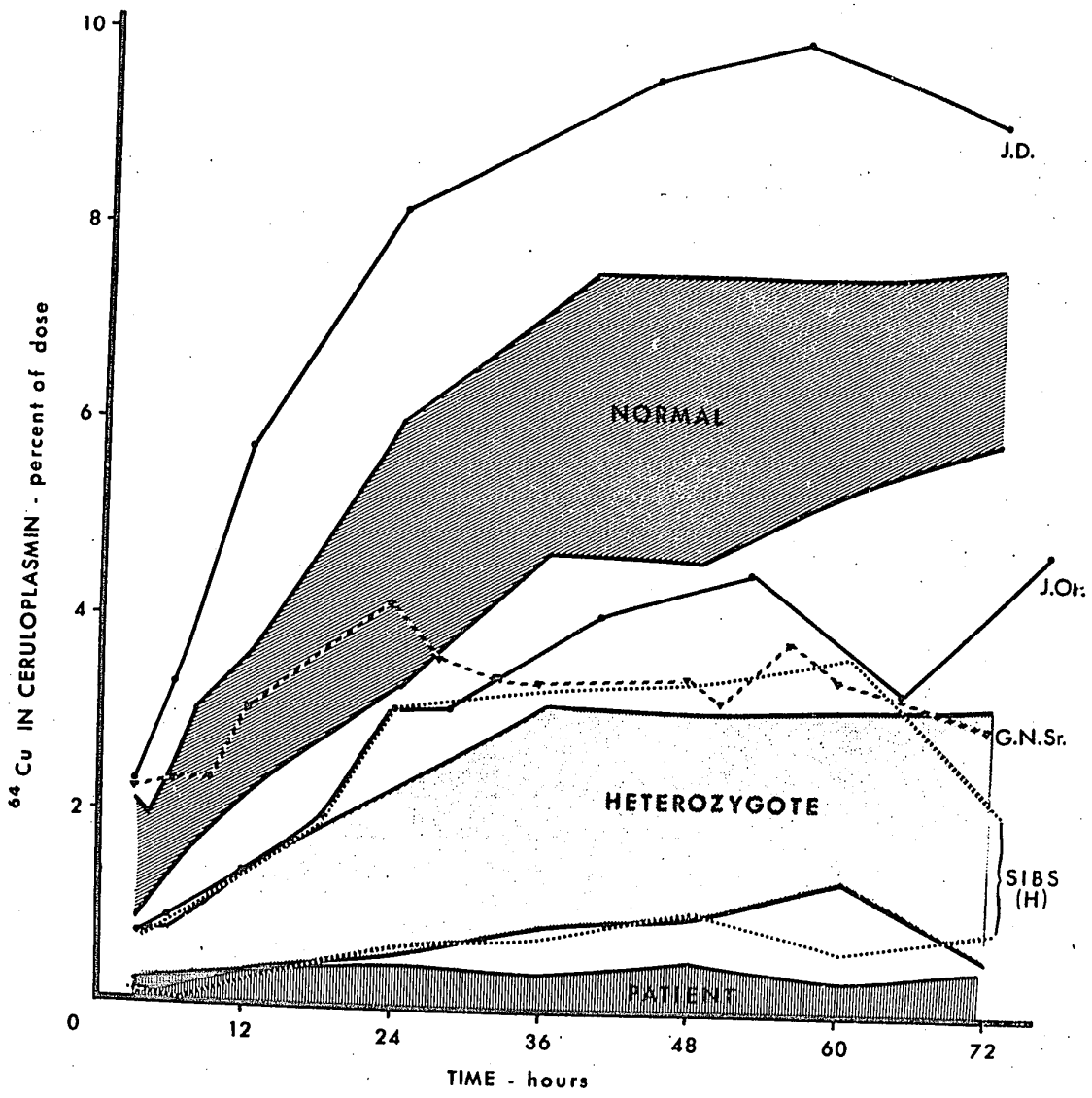

Fig. 17. Incorporation of ^{64}Cu into ceruloplasmin in relatives of patients with Wilson's disease following intravenous administration of ^{64}Cu.

with the exception of sib J.D. who had an abnormally high serum ceruloplasmin level.

The usual pattern of ^{64}Cu incorporation shown by an individual from family N (G.N.Sr.), ascertained from the normal population, is also shown in Fig. 17 and has been discussed in Section I.

Starch Gel Electrophoresis of Sera of Presumed Heterozygotes:

Sera from individuals with low serum levels of ceruloplasmin from the three atypical kindreds were studied by starch gel electrophoresis. All relatives tested had ceruloplasmin which migrated with the same mobility as that of normal ceruloplasmin. The following relatives were tested: II-1 and II-5 in kindred B; II-4 in kindred M; II-2, II-5, and III-2 in kindred W as shown in Figs. 2 and 13.

Individuals in families R and N, ascertained from the normal population, but perhaps heterozygotes for the Wilson's disease gene, also had ceruloplasmin showing normal electrophoretic mobility. Those tested were I-1, and II-1, and II-2 in family R and 1-5 in family N as shown in Fig. 4.

Inhibition Studies on Sera of Presumed Heterozygotes:

Sodium azide: Concentrations of sodium azide of 0, 10^{-5} M, 10^{-4}M, and 10^{-3} M, were used to cover the range from no inhibition to almost complete inhibition. A response curve obtained with normal serum is shown in Fig. 18. The values for ΔO.D. were corrected for non-enzymatic oxidation as this becomes an appreciable part of the oxidation when inhibition is high. The non-enzymatic oxidation

was determined by assaying the oxidase activity in the reagent mixture exclusive of serum in the presence of each of the concentrations of inhibitors used. The ΔO.D. due to non-enzymatic oxidation was low (0.0010) and was the same for all concentrations of inhibitor.

Sera from three patients with Wilson's disease, three probable heterozygotes with low ceruloplasmin levels, and five normal individuals showed a similar response to the inhibiting effect of sodium azide. The results are shown in Table 9.

Iproniazid: Concentrations of iproniazid of 0, 10^{-5}M, 10^{-4}M, and 10^{-3}M were used to cover the full range of inhibition. Correction was made for non-enzymatic oxidation as above.

Sera from three patients with Wilson's disease, three probable heterozygotes with low ceruloplasmin levels, and four normal individuals showed a similar response to the inhibiting effect of iproniazid. The results are shown in Table 9.

The lag period in the above studies was usually less than two minutes so that by the time recording of the optical density was started, the lag period had ended. However, when the lag period was timed, it was observed that the lag period was noticeably longer at higher inhibitor concentrations (10^{-3}M). The lag period was particularly long (one and one half to two minutes) for the serum from patients with Wilson's disease in which the oxidase activity was already low.

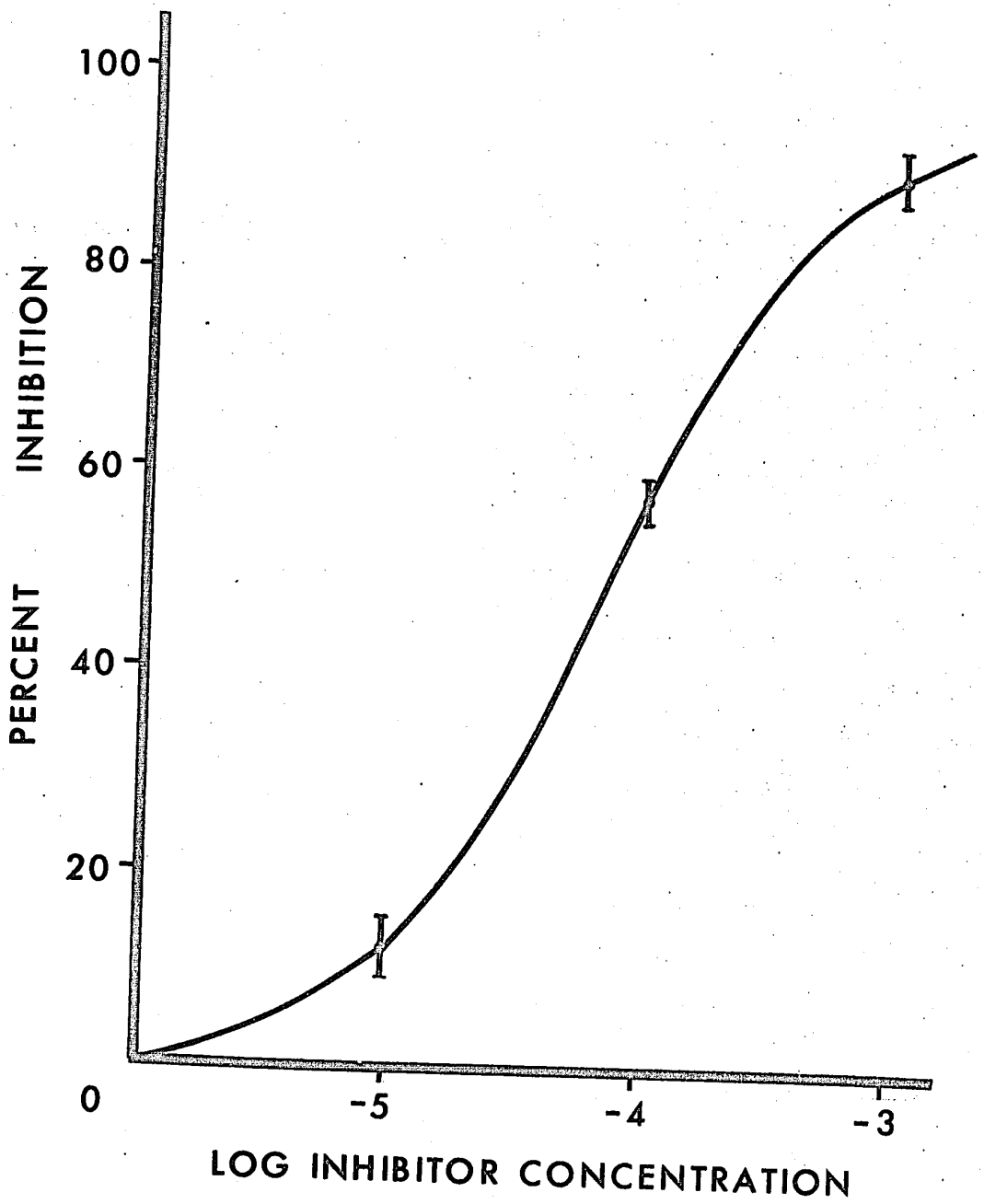

Fig. 18. Inhibition of ceruloplasmin oxidase activity, in human sera, with sodium azide. At each concentration indicated, the mean $\pm$ 1 S.D. obtained with sera from five normal individuals is shown. Acetate buffer, 1.0 M, pH 5.2; 37° C.

TABLE 9

PERCENTAGE INHIBITION OF OXIDASE ACTIVITY

IN HUMAN SERA

Subject	Sodium azide concentration			Iproniazid concentration		
	10^{-5}M	10^{-4}M	10^{-3}M	10^{-4}M	10^{-3}M	10^{-2}M
W.D. Patients:						
S.C.	13.8	59.7	90.4	7.7	47.1	81.0
E.T.	-	57.2	90.2	0	26.3	100.0
F.B.	-	59.2	82.8	-	-	-
M.T.	-	-	-	8.8	52.7	100.0
Heterozygotes:						
R.B.	6.4	51.2	84.9	10.3	51.0	82.8
J.K.	13.5	59.9	94.8	-	-	-
M.K.	-	-	-	0	47.2	91.9
G.N.Sr.	17.9	56.3	90.0	4.6	42.9	88.8
N.R.	-	-	-	5.5	46.2	87.6
Normal (5):						
mean	12.4	47.0	89.8	8.3	47.6	90.3
range	9.4-16.7	54.7-59.2	87.1-92.6	6.0-12.7	42.0-52.6	86.3-94.2

DISCUSSION

Clinical Data and Genetic Parameters:

The majority of patients in this series had an early age of onset of Wilson's disease and are of Western European origin. This series is very different from that of Bearn (1960), which consisted primarily of Jewish patients of eastern European origin. The earlier age of onset in the present series may be partly due to bias from referrals predominantly of children from outside centres since the study was centred in two children's hospitals. An early age of onset also appears to be common in a series from England (Walshe, 1967) in which 75 per cent of a total of 20 symptomatic patients seen prior to treatment were 16 years of age or less at the time of onset. These patients were not asertained primarily through children's hospitals. In the present series, there are three families in which both parents trace their origins to central-eastern Europe and are not aware of any Jewish ancestry. However, they are similar to the Jewish patients from the same area in Bearn's series and in this series in having a later age of onset and their disease primarily of a neurological type. The central-eastern European patients, whether of Jewish or non-Jewish origin, appear to form a clinically different and probably homogeneous group with respect to Wilson's disease. Bearn (1960) suggested two possible explanations for the clinical differences which he found in the central (eastern) European Jews in his series: 1) a different mutant gene, which might code for a modified gene product in central European patients rather than for no gene product as in the other group of patients or 2) the presence of a modifying suppressor gene in the

affected individuals. In the present series, there are three families in which one parent is of central-eastern European origin and the partner is of western European origin. All three patients in these families had an early age of onset and were clinically the same as other patients with an early age of onset. If different mutant genes are involved, they are probably at the same genetic locus. The 'central European allele' would appear to be recessive to the 'western European allele'. If such is the case, and if the western European allele, for example, does not code for any product, then the heterozygotes might differ in the two groups. Since the rate of incorporation of ^{64}Cu into ceruloplasmin is the most basic defect known to date, a more pronounced defect might be expected in heterozygotes of western European origin when compared with those of central-eastern European origin. Only one parent of central-eastern European origin, from a 'mixed origin' marriage was studied with ^{64}Cu. Her rate of incorporation of ^{64}Cu into ceruloplasmin was considerably reduced and was similar to that of several heterozygotes of western European origin. However, there is too little evidence to exclude the hypothesis of different alleles. The second hypothesis, suggesting the presence of a suppressor gene, might also be expected to produce a difference in the heterozygotes, which, as we have stated, does not appear to exist. If this hypothesis were correct, the three patients of mixed origin matings would, by chance, not have inherited the modifying gene from the parent of central-eastern European origin.

A more general difference in genetic backgrounds can reasonably account for the observations. For example, genetically determined

differences of the liver might cause it to be less susceptible to the toxicity of excess copper in individuals of central-eastern European origin. This in turn would allow a later age of onset, which appears to be correlated with the neurological form of the disease. Alternatively, dietary differences may be the modifying factor, in view of the early age of onset in the Orientals, who have a relatively high copper diet (Tu, 1963; Arima and Kurumada, 1962b).

In this series, there were more affected males than females. This sex difference has been found in most other series and is probably a real difference. The reason for it is unknown.

The three patients with normal or near-normal ceruloplasmin levels will be discussed in Part 2 to follow.

The present data supports a recessive mode of inheritance for Wilson's disease, using methods of analysis for both complete and incomplete ascertainment. Part of the material is believed to be completely ascertained, particularly that from the urban centres, and part is incompletely ascertained particularly the out-of-province referrals. For the most part, there is little tendency to include preferentially sibships with multiple affected individuals, therefore our ascertainment can be considered close to complete. There is very close agreement between the expected and observed genetic proportion when the 'a priori' method of analysis for complete ascertainment is used. Assuming single ascertainment, which is a possibility for part of our data since there was a

tendency to include only those patients first diagnosed during the period of our study, recessive inheritance is also supported.

The frequency of consanguineous marriages among the parents was relatively low in this series, as in a series from England (Walshe, 1967) but unlike the high frequencies found in the New York series (Bearn, 1953; 1960) and Japanese series (Arima and Kurumada, 1962b). These differences do not necessarily reflect a difference in the gene frequency in the populations. In the New York series, the parents appear to be from genetic isolates (Bearn, 1953) and probably the coefficient of inbreeding is high in these groups. The marriages in the population from which the present series is drawn appear less likely to be consanguineous since most of the families have several generations of Canadian or American ancestors who have not lived in racial isolates. For the Japanese series, a high frequency of consanguinity is expected among the parents as it is high even in the general population. No information has been given on the racial origins of the series from England. Estimations of the gene frequency are uncertain because the frequency of consanguineous marriages in the general population from which these patients were derived is unknown. This figure is perhaps most reliable for Japan because there is probably a relatively even distribution of consanguineous marriages in this population. Assuming a consanguinity rate of four per cent among the Japanese, the gene frequency as estimated from the present data is two to four times that found in Japan. Considering the possible inaccuracies involved in the estimates, this difference is small and probably does not

indicate a real difference. The gene frequency, q, estimated from our series, is 0.9 to 1.9 per 1000, the disease incidence 0.9 to 3.6 per 1,000,000 and the corresponding incidence of heterozygotes is one per 265 to 537. The prevalence of patients is consistent with the higher estimate for the gene frequency so even this figure may be too low an estimate. Estimates of prevalence increase as diagnosis is improved. Although the gene frequency is probably not unusually high in Japan, the disease incidence is apparently about ten times higher, presumably because of the extent of inbreeding in the population.

Studies of Heterozygotes and Other Relatives:

Our study indicates that the ceruloplasmin level of the heterozygote fluctuates within a relatively narrow range. This was also true for normal individuals, as reported in Section I. A low level of serum ceruloplasmin is a constant feature in some heterozygotes. This differs from the findings reported by Arima and Kurumada (1962a), of fluctations from normal to low levels in several heterozygotes. Our method of analysis has ensured that there is neglibable variation due to technical error. The differences between the results of the two studies suggests either that the fluctation in the Japanese data is due to technical factors or that the difference between the Japanese heterozygotes and those from the present series is real.

In this predominantly western European series, about nine per cent of heterozygotes have a ceruloplasmin level below the 95 per cent

lower confidence limit for the general population. About six per cent are below the 99 per cent limit, where one expects, by definition 0.5 per cent of the general population to fall. In actual practice, the only healthy individuals found to date with such low levels may be heterozygotes for the Wilson's disease gene. One of the two individuals in our control series who had low levels of ceruloplasmin showed an abnormal rate of incorporation of ^{64}Cu into ceruloplasmin, decreased for part of the study as in known heterozygotes. His identification as a heterozygote for the Wilson's disease gene is still uncertain.

An investigation of the ceruloplasmin levels of numerous relatives has shown that the occurrence of low ceruloplasmin levels in heterozygotes is strongly familial. Low ceruloplasmin levels in related individuals can be seen in several families and kindreds in the literature, for example families Sm. and Q. of Sternlieb et al. (1961), relatives of case 1 of Cartwright et al. (1960), family A of Soothill et al. (1961), and the families of cases 1 and 3 of Canelas et al. (1963). In the latter report, however, the investigation was probably not adequate to distinguish heterozygotes from asymptomatic affected homozygotes and copper determinations appear to be too high, probably because of contamination. The data from all of these families suggest that familial, probably genetic factors are involved in the production of an abnormally low serum ceruloplasmin level in certain heterozygotes. Further evidence that the factors are indeed genetic is provided by the father of the

proband and his monozygotic twin in family W, both of whom have low ceruloplasmin levels.

Studies of relatives following the oral or intravenous administration of ^{64}Cu were useful in distinguishing heterozygotes from normal homozygotes. After oral administration of ^{64}Cu, three of the four presumably heterozygous parents, as well as several other relatives, showed a decreased rate of incorporation of ^{64}Cu into ceruloplasmin compared with that of three other relatives, including one parent, presumed to be homozygous normal. The calculation of R values (Sternlieb et al., 1961b) allowed similar conclusions to that of visual comparison of the plotted incorporation curves in distinguishing heterozygous individuals. There are several plausible explanations for the finding of a normal rate of ^{64}Cu into ceruloplasmin in a parent of a patient. 1) Her test results may have been affected by the occurrence of her menstrual period during the test. 2) Some heterozygotes show a rate of ^{64}Cu incorporation which does not distinguish them from normal homozygotes. This appears to be true from the data of Sternlieb et al. (1961b). While her value was unlikely to be that of a heterozygote, it was calculated from two hour data rather than the higher of values at one and two hours. 3) This parent may actually be a normal homozygote in whose gamete a mutation of a gene at the Wilson's disease locus occurred and was transmitted to her affected daughter. This explanation seems unlikely in view of the relatively high frequency of heterozygotes in the population compared with the rarity of a mutational event at any one locus. The small amount of data in the atypical kindreds

indicates that individuals with low serum ceruloplasmin levels are probably heterozygous for the gene for Wilson's disease, but that individuals with normal serum ceruloplasmin levels in some cases show a reduced rate of ^{64}Cu into ceruloplasmin typical for heterozygotes. Studies in which ^{64}Cu is administered intravenously are probably more effective in distinguishing heterozygous individuals because effects of individual differences in intestinal absorption of the ^{64}Cu are eliminated. Tracer studies were carried out in most individuals (Aspin and Sass-Kortsak, 1965) but the response curves are probably the same as with higher doses of total copper in heterozygotes and normal homozygotes. This may not be true for patients in whom the liver is already loaded with copper. A graphic analysis of the data showed a clear distinction between heterozygous parents and individuals believed to be homozygous normals. The 'normal' category included some sibs of patients with Wilson's disease who were considered normal because their rates of incorporation of ^{64}Cu into ceruloplasmin were as high as in two normal controls. More data on normal controls would be useful and may be possible to obtain as refinements of technique allow the use of lower doses of the radioactive isotope. The incorporation curve of one parent was almost as high as that of the 'normal' group. Paternity was not excluded by a study of seven blood groups. His response probably represents the extreme in the range for heterozygotes. This response, between six and twelve hours after administration of ^{64}Cu, falls in the range of the other known heterozygotes. The collection of more data on heterozygotes should help resolve the questions raised by data from this individual.

The Possibility of Genetic Homogeneity in Wilson's Disease:

If the heterozygotes in the three atypical kindreds in the present series are actually genetically distinct from those in the more usual families, several hypotheses can be suggested to explain the findings. These are as follows:

1) the gene for Wilson's disease decreases the ceruloplasmin levels in all heterozygotes and the atypical kindreds are those in which the ceruloplasmin levels already tend to be rather low.

2) a different allele for the Wilson's disease gene occurs in the atypical families.

3) a modifier gene, closely liked to the Wilson's disease locus, has a pronounced effect on the ceruloplasmin level.

4) a modifier gene exists, as in the previous hypothesis, but is not closely linked to the locus for Wilson's disease.

The data from the present series can be examined in the light of these hypotheses. Let us assume that the mutant gene for Wilson's disease is I^W, occurring at a locus I which controls the incorporation of copper into ceruloplasmin. The individuals with Wilson's disease are therefore the genotype $I^W I^W$.

Evidence for the reduction of the ceruloplasmin level by a single I^W allele is unconvincing. The mean ceruloplasmin level of the

heterozygous parents studied was not significantly different from the mean of the general population, either when the parents from the atypical kindreds were included or omitted. The distribution of ceruloplasmin levels in the general population, which is approximately Gaussian, suggests multifactorial influences. Studies in normal families indicate a familial influence and twin studies indicate that genetic influences are present. In the small number of sibships in which both normal and heterozygous sibs have been identified by studies with ^{64}Cu, the heterozygotes do not appear to have consistently lower ceruloplasmin levels. From the distribution of ceruloplasmin levels in heterozygotes in the typical families and from these studies within sibships, it appears that the ceruloplasmin levels are not appreciably reduced, if at all, by the presence of one I^W allele. Even if the atypical kindreds tended to have lower ceruloplasmin levels because of other factors in their genetic backgrounds, the levels would have to be reduced by 50 per cent or more to get such low levels in the heterozygotes. Furthermore, the distribution of ceruloplasmin levels in the atypical kindreds would not be clearly bimodal, as found. The first hypothesis does not appear to be satisfactory.

The second hypothesis would provide a situation analagous to that in cystinuria, in which one type of cystinuria is completely recessive and the other type is detectable in the heterozygous state (Harris et al., 1955). If a different allele, I^W, occurs at the Wilson's disease locus, then all heterozygotes as identified from ^{64}Cu studies

should have low levels of ceruloplasmin. In each of kindreds W and M, there is one relative of those studied with ^{64}Cu who appeared to be heterozygous for the Wilson's disease gene yet had a normal ceruloplasmin level. Assuming a common mechanism in all three kindreds, the postulated allele I^W must occasionally fail to produce an abnormally low ceruloplasmin level. In the three kindreds, there are eight matings of normal individuals with relatives assumed to be heterozygous, either on the basis of ^{64}Cu studies or a low level of ceruloplasmin. Three of their offspring had low ceruloplasmin levels, instead of the 13 in 26 expected according to this hypothesis. The data from the atypical kindreds do not provide good support for the hypothesis of different alleles.

According to the third hypothesis, a gene at a second locus influences the ceruloplasmin level in the atypical kindreds. Let us designate this locus as C, a structual gene for all or part of the apoceruloplasmin, that is ceruloplasmin without its copper. The mutant allele, which we shall call C^L, could produce a different apoceruloplasmin, consequently a different ceruloplasmin, with a reduced oxidase activity compared with that of the usual ceruloplasmin or could reduce the amount of apoceruloplasmin produced. This allele, C^L, may reduce the ceruloplasmin level somewhat in a C^LC^N individual when no I^W allele is present, but we shall have to assume that it is the combination of the heterozygous state of C^L and I^W in an individual which overtaxes the ceruloplasmin mechanism resulting in a low ceruloplasmin level. If, in heterozygotes for the Wilson's disease gene, the genes at other loci are distributed as in the general

population, then 6.1 per cent of normal individuals, as well as heterozygotes for I^W, would carry one C^L allele and its frequency, as calculated from the Hardy-Weinberg law, would be 0.03. This hypothesis can satisfactorily explain the findings in all three atypical kindreds. In kindred B, the alleles C^L could be linked and there is no evidence of crossing over in the family. In kindred W, these alleles are linked, occurring in the father, uncle, and brother of the proband. In the heterozygous aunt with a normal level, crossing over could have occurred so that her genotype is I^WC^N/I^WC^N. None of her children would have low ceruloplasmin levels, as is the case. In kindred M, I^W and C^L could have occurred in separate paternal grandparents so that the father has the genotype I^WC^N/I^WC^N therefore a normal ceruloplasmin level, a paternal aunt has the genotype I^WC^L/I^WC^N and a low ceruloplasmin level, and a paternal uncle is the same as the aunt. Since this uncle was distinctive in having less reduction of ceruloplasmin than other atypical relatives in this family he could have either the genotype I^WC^N/I^WC^L or I^WC^N/I^WC^N, if the C^L or I^W alleles singly in the heterozygous state have a small effect in reducing the ceruloplasmin level. If the genetic background of the uncle is such that his ceruloplasmin level would otherwise have put him at the lower end of the normal distribution, then the presence of either allele could reduce his ceruloplasmin level just sufficiently to put his ceruloplasmin level in the low range. Assuming normal I^WC^N/I^WC^N marriage partners for the aunt and uncle under discussion, none of their offspring would have low ceruloplasmin levels but

half would be heterozygous for the Wilson's disease gene. In fact, the uncle has seven and the aunt has eight adult offspring who were tested and all have normal ceruloplasmin levels. None of these individuals were available for tests with ^{64}Cu. The probable genotypes for members of the three atypical kindreds are shown in Table 10.

If the gene for Wilson's disease is also present in families N and R (Fig. 7) ascertained from the general population study, then the hypothesis should also be applicable in these families. However, it must be emphasized that these families may have low ceruloplasmin levels for a different reason. No studies with ^{64}Cu have been carried out in family R and the one individual studied in family N showed a normal rate of ^{64}Cu incorporation into ceruloplasmin early in the study and a response typical of heterozygotes for the Wilson's disease gene for the remainder of the study. If we assume that these families contain atypical heterozygotes for Wilson's disease, then in family N, I^W and C^L appear to be linked and the expected pattern of inheritance is found, with the exception of one aunt (11-3). Her ceruloplasmin level is normal and almost twice as high as other atypical members of the kindred. Crossing over may have occurred in a parental gamete such that she has the genotype I^WC^N/I^wC^N. Her daughter, who has a very low ceruloplasmin level, must then have inherited a C^L allele from her father. This is not unlikely when one considers that 6.1 per cent of normal individuals are assumed to carry the gene. The father refused to have a blood sample taken so this mating leaves unanswered questions. In family

TABLE 10

PROPOSED GENOTYPES OF INDIVIDUALS IN ATYPICAL KINDREDS

Kindred	Individual[a]	Genotype	W.D. locus[b]	Ceruloplasmin level
B	II-1, III-1,4 to 7	$I^{W_C N}/I^{W_C N}$	N	normal
	II-2 to 5, III-2,3,8, IV-1	$I^{W_C N}/I^{W_C L}$	H	low
	III-9	$I^{W_C N}/I^{W_C L}$	A	nil
W	II-1,3,6, III-3,4	$I^{W_C N}/I^{W_C N}$	N	normal
	II-4,5; III-5	$I^{W_C N}/I^{W_C L}$	H	low
	II-2	$I^{W_C N}/I^{W_C N}$	H	normal
	III-1,2	$I^{W_C N}/I^{W_C N}$ or $/I^{W_C N}$	N or H	normal
	III-6	$I^{W_C N}/I^{W_C L}$	A	nil
M	II-1	$I^{W_C N}/I^{W_C L}$, $I^{W_C N}/I^{W_C N}$ or $/I^{W_C L}$	N or H	norm.(?)[c] norm.(?) or low
	II-2,3,5	$I^{W_C N}/I^{W_C N}$ or $/I^{W_C L}$	H or N	normal
	II-4	$I^{W_C L}/I^{W_C N}$	H	low
	II-6	$I^{W_C N}/I^{W_C N}$	H	normal
	III-1 to 7	$I^{W_C N}/I^{W_C N}$, $I^{W_C N}/I^{W_C N}$ or $/I^{W_C L}$	H or N	normal(?) or normal
	III-8 to 18, 27-32	$I^{W_C N}/I^{W_C N}$ or $/I^{W_C N}$	N or H	normal
	III-19 to 26	$I^{W_C N}/I^{W_C L}$ or $/I^{W_C N}$	N or H	normal
	III-33 to 36	$I^{W_C N}/I^{W_C N}$	A	nil

[a] See Figs. 12 and 13.

[b] N - normal homozygote; H - heterozygote; A - affected homozygote.

[c] Normal or slightly low, depending upon genetic background.

R, the limited data suggest linkage of I^W and C^L with no crossing over. However no ^{64}Cu studies could be carried out to confirm the presence of I^W.

The fourth hypothesis is similar to the previous except for specifying no linkage or at least no close linkage. The assumption of close linkage appears to fit the data well as only one or two crossovers could be detected. There are a total of 24 offspring of matings of a normal individual with a relative assumed to carry both the C^L and I^W alleles. Three of these offspring have low ceruloplasmin levels. The expected number of such offspring is 4.5 of 24 assuming linkage and six of 24 assuming independent segregation of the two alleles. Therefore the data are in good agreement with either the hypothesis of no linkage or of close linkage. A valid assessment of the degree of linkage will require knowledge of the genotype at the Wilson's disease locus for more individuals in the pedigrees presented.

There still remain four isolated cases of low ceruloplasmin levels, all of which lie above the 99 per cent lower confidence limit for the general population and are therefore higher than those in the atypical kindreds. Two of the relatives were children whose levels were low only after correction for age. The other two were a father previously mentioned as borderline and a sib who appears to be a heterozygote by studies with ^{64}Cu. Perhaps these individuals represent the lower end of a normal distribution shifted downward due to small effects of the I^W allele.

Since a single genetic hypothesis can explain the findings in the family studies, the origin of the proposed allele C^L is of interest. The parents with atypical relatives in kindreds B and W are both of western European origin, and specifically German. Kindred M has lived in Canada for many generations and is probably of western European origin. Its surname also occurs in the ancestry of kindred W, so these people may be of German origin and perhaps even have common ancestry with kindred W. The atypical families N and R, ascertained through the population survey and possibly carrying the same allele, are of Scottish and English-German origin respectively. These data suggest that the modifying allele may occur only in western Europeans, although we may have had insufficient data from families of central European origin. The racial origin was not reported for the previously mentioned atypical families from the literature, except for those of Canelas et al. (1963) in which one family was reported to be of Italian descent. In a Russian series of 20 unspecified relatives of patients (Mittel'shtedt and Bauman, 1964), two sibs have very low ceruloplasmin levels but these are probably asymptomatic affected homozygotes. Several parents in three series of Chinese and Japanese families probably had low ceruloplasmin levels (Tu, 1963; Arima and Kurumada, 1962a; Fukuda, 1965a) but the lower limits of normal were not clear in these series. A careful study of more atypical kindreds, including a study of their racial origin, is required before the hypothesis presented here can be accepted.

Since our evidence is consistent with the existence of a modifier gene in certain atypical families with Wilson's disease, we can examine data which may provide evidence on its mode of action. Such evidence is mainly of a negative type. If the modifier gene exerts its effect directly on the product of the Wilson's disease gene, I^W, then a correlation between the rate of incorporation of ^{64}Cu into ceruloplasmin and the serum ceruloplasmin level might be expected. In our series, some of the lower rates of incorporation of ^{64}Cu into ceruloplasmin were shown by heterozygotes with ceruloplasmin levels well within the normal range. In the studies of orally administered ^{64}Cu reported by Sternlieb et al. (1961b), some heterozygotes with normal ceruloplasmin levels have a much slower rate of incorporation of ^{64}Cu into ceruloplasmin than those with low ceruloplasmin levels, although a significant correlation was found in an analysis of all the data. An exceptionally high rate of incorporation of ^{64}Cu into ceruloplasmin was found in the sister of one of our patients. She also had a ceruloplasmin level above the upper 99 per cent confidence limit and was not pregant, not taking hormones, and was free of known infection. Therefore, assuming that these two features are causally related, in some cases the rate of copper incorporation can influence the final level of circulating ceruloplasmin.

The nature of the change brought about by the I^W allele will be discussed in a later section, but for the present we shall assume that it exerts its effect on some phase of incorporation of copper.

It does not involve the structural locus for apoceruloplasmin, which we have called C. If the variant allele C^L, the modifier gene, promotes low ceruloplasmin levels, it could do this by producing a structurally altered ceruloplasmin, or more precisely, apoceruloplasmin. The resulting ceruloplasmin might have much lower oxidase activity than that of the normal ceruloplasmin. Starch gel electrophoresis was carried out in a system which has previously detected homogeneity of ceruloplasmin, and no difference was found between the mobility of normal ceruloplasmin and that of the atypical heterozygotes. If the reduced ceruloplasmin activity in atypical heterozygotes results from a structural alteration in apoceruloplasmin, the difference does not therefore involve molecular size or charge. Since the staining of the ceruloplasmin on the gel depends upon its oxidase activity, a ceruloplasmin variant which lacks oxidase activity would not be detected.

Inhibition studies did not show a difference in the response of sera from the atypical heterozygotes. The variable lag period will be discussed later. A differential response to inhibitors has been found for isozymes of pseudocholinesterase (Kalow and Genest, 1957). This method would not detect a variant which lacked oxidase activity.

If a variant ceruloplasmin was present in the atypical heterozygotes, we were not able to detect it. Another possibility is that no apoceruloplasmin is produced by the allele C^L. This could result from a nonsense mutation in the C^L allele such that coding is stopped at the mutant point and no apoceruloplasmin is formed. A combination of

reduced incorporation of copper due to the presence of I^W and a reduced amount of apoceruloplasmin could result in the low ceruloplasmin levels of the atypical heterozygotes. A mutation in a regulatory gene, which causes reduced apoceruloplasmin formation, would also show the dominant pattern of inheritance found in atypical heterozygotes in the present series.

No special mechanism, with the possible exception of close linkage, is required to explain the pattern of inheritance in family B, and similarly in normal families R and N, assuming they too are atypical families possessing the I^W allele. If there is close linkage between the I and C loci, then they could be adjacent on the chromosome. One deletion involving parts of both the I and C loci would reduce the amounts of both gene products in the heterozygotes. If human genetic systems are similar to those of bacteria, the concept of the operon might be invoked. If I and C are within one operon, or within a genetic region under synchronized control, then families B, R, and N could have an extremely polar nonsense mutation near the operator region, such that neither gene product is formed (Newton et al., 1965). The application of such systems to humans must be done with recognition of the pitfalls involved (Epstein, 1964). These explanations, while following the current vogue, are very much in the realm of speculation and probably introduce additional complexity into the situation because of their application to only part of the data from atypical families.

The Interpretation of the Inhibition Studies:

Sera from heterozygotes and from patients showed the same response as did sera from normal individuals to the inhibitory action of sodium azide and iproniazid. The apparent inhibition with iproniazid has been shown to be partly due to decolorization of the oxidation products, at least when DPPD is used as substrate (Curzon and Cumings, 1966).

An increase in the lag period was observed with decreasing levels of ceruloplasmin and increasing concentrations of the inhibitor. This increase in lag period did not alter our results as the rate of the reaction was measured when linear. Usually the lag period had ended during the warm-up interval of the reaction mixture; if the lag exceeded this period, as it did with the combination of the lowest ceruloplasmin concentration and highest inhibitor concentrations, then the rate was not measured until after the lag period had ended and the rate was constant. The lag period is probably due to ascorbic acid in the serum (Aprison and Grosz, 1958). The coloured oxidation product of PPD can be reduced to its colourless form again by the ascorbic acid. The coloured product remains and accumulates only after the reducing activity of the ascorbic acid is expended. The time required to reach this point will vary inversely to the concentration of ceruloplasmin present. Walshe and Carpenter (1965) have reported that sera from patients with Wilson's disease show significantly greater inhibition with sodium azide and iproniazid as inhibitors. Their assay method is one which measures the amount of colored product formed after a constant period of incubation (Walshe,

1963c). The results of such a method are affected by variations in the lag period. Walshe and Carpenter noted that "the degree of inhibition produced by either inhibitor varied inversely to the enzymatic concentration both for normal and Wilson's disease serum". They attempted to correct for this by multiple regression analysis on an electronic computer and still found significant differences, however the correction was apparently not adequate. Our findings suggest that the differences found are due only to the lag period effect, as an increased lag period in the presence of inhibitor does give an apparently lower percentage of inhibition. The presence of an inhibitor in sera from Wilson's disease has been suggested in another study by Walshe (1963c). The effect of these apparent inhibitors was greatest when a high serum to pure ceruloplasmin mixture was used. This can again be explained by lag period effects: pure ceruloplasmin raises the total ceruloplasmin concentration of the mixture enough that the lag effect is small, but with a lower ratio of pure ceruloplasmin the lag period is longer.

We have found that mixtures of sera from normal subjects and from patients with Wilson's disease have the expected oxidase activity. There does not at present appear to be any valid evidence for the presence of an inhibitor in the sera of patients with Wilson's disease, or for a difference in the response of ceruloplasmin from patients with Wilson's disease to the inhibitors sodium azide and iproniazid.

PART 2

A Survey of Patients with Normal Levels of Serum Ceruloplasmin

While a deficiency of ceruloplasmin is a frequent finding in patients with Wilson's disease, a small percentage of proven cases have normal ceruloplasmin levels. In a series of 111 patients with clinical signs of Wilson's disease, four patients, or 3.6 per cent, had normal ceruloplasmin levels (Sternlieb and Scheinberg, 1963). In the present series of 33 patients including asymptomatic patients, three have normal ceruloplasmin levels. This represents eight per cent of the 25 families, as two patients with normal ceruloplasmin levels occurred in one family.

A study of all reported patients with Wilson's disease and normal serum ceruloplasmin levels was undertaken, particularly to determine if familial factors are involved.

MATERIALS AND METHODS

Patients with normal ceruloplasmin levels, including two of our own, have been presented in review articles by Kurtzke (1962) and by Scheinberg and Sternlieb (1963). These patients form the main part of the present series. One new patient from our own series, and two reported by other authors have been added. The combined list consists of 16 patients from 15 sibships.

The following information was obtained from the original references, where possible: sex, age of onset, clinical type of the disease, consanguinity of the parents, racial origin, and ceruloplasmin levels in the parents and sibs. Genetic information was seldom available from the original references and was obtained, where possible, by personal communication from the authors.

RESULTS

The clinical features of the 16 patients collected from the literature and from our data are shown in Table 11. Genetic data are summarized in Table 12.

Some of the patients included in the review articles (Kurtze, 1962; Scheinberg and Sternlieb, 1963) have been excluded from the present series. From the review by Kurtzke, case numbers X and XI were omitted because the ceruloplasmin levels, as expressed in optical density units, were about one half of the lower limit of the normal range and were therefore definitely low; case number XII was omitted because the ceruloplasmin level was low except at parturition when it approached the normal range. Case numbers 4, 6, 8, 10, 11, 13, and 14 of the present series are included in both review articles. Our case number 14 was duplicated in both review articles: cases VI and VII of Kurtzke and cases 10 and 12 of Scheinberg and Sternlieb. Our cases 1 and 7 are new cases added from the literature. Case 15 is from our own series and has not been published previously.

Some of the patients on this list have ceruloplasmin levels somewhat below the usual normal limit, but have been included because the levels are higher than those usually found in Wilson's disease.

TABLE 11

CLINICAL DATA ON PATIENTS WITH

A NORMAL LEVEL OF SERUM CERULOPLASMIN

Case	Sex	Age of onset (years)	Clinical type (N[a] or H[b])	Ceruloplasmin level	Reference
1. (Y.S.)	M	12	N	17.1 mg./100 ml.	Arima et. al. (1960)
2. (M.S.)	F	4	H(sev.)	27.5 mg./100 ml.	ibid.
3.	M	10	asymp.	17.5 mg./100 ml.	Combes, in Scheinberg and Sternlieb (1963)
4.	M	35	N, H(sev.)	'normal'	Enger (1959)
5. (R.E.)	M	15	H	16.4 mg./100 ml.	German and Bearn (1961)
6. (V.C.)	F	20	N, H(mod.)	20.8 mg./100 ml.	ibid.
7.	M	31	N	25 mg./100 ml.	Holtzman et al. (1967)
8.	M	32	N, H(sev.)	17.5 mg./100 ml.	Kurtzke (1962)
9.	M	28	H(sev.)	25 mg./100 ml.	Schaffner, (1965)
10. (L.E.)	F	14	N	27 mg./100 ml.	Rosenoer and Franglen (1959)
11a. (Rb.Mo.)	M	10	H(sev.)	24 mg./100 ml.	Sass-Kortsak et al. (1959)
11b. (R.Mo.)	M	11	H	26.6 mg./100 ml.	ibid.
12. (Y.F.H.)	F	6	asymp.	0.254 Ravin units	Tu et al. (1962)
13. (F.T.)	M	11	H(slight)	28 mg./100 ml.	Walshe (1960)
14. (C.W.)	F	13	H	'normal'	Walshe (1960); Sherlock (1960)
15. (S.C.)	M	38	N	18.0 mg./100 ml.	Present study

[a] Neurological [b] Hepatic

TABLE 12

GENETIC DATA ON PATIENTS WITH WILSON'S DISEASE AND A NORMAL LEVEL OF SERUM CERULOPLASMIN

Case No.	Racial Origin	No. of sibs		Parents		
		Normal	W.D.[a]	Consang.[b]	Cerul. level Father	Mother
1	Japanese	3,n[c]	1(M, 18 yrs.) 16.3 mg./100 ml.	1c.	n	n
2	Japanese	-	2(M,F)-probable	2c.	n	n
3	Anglo-Saxon	3	3 6,6,<1 mg./100 ml.	no	dec.	n
4	unknown	3(1?)	-	1c.	-	-
5	N.A. Ind./Negro, Span./Negro	5,n	-	no	n	n
6	French/Span., French/N.A. Ind.	1,n	-	1c.	n	dec.
7	Negro	7,n	-	no	-	n
8	Italian	2	1(F, 27 yrs.) low cerul.	no	-	-
9	Puerto Rican	3	-	?	-	dec.
10	Polish (Jewish)	1(?)	-	no	-	-
11	Irish	3	a,b-sibs	2c.	n	n
12	Chinese	3	2(F,F) low cerul.	no	n	n
13	English	-	1(M) low cerul.	no	n	n
14	Jewish	-	-	no	n	n
15	Polish(Jewish)	3	-	no	-	-

[a] sex and age in brackets, when known; ceruloplasmin level in next line.
[b] 1c. - first cousins, 2c. - second cousins.
[c] normal ceruloplasmin level.

Thirteen of the sixteen patients had clinical signs of Wilson's disease. Hepatic disease was severe in five patients. In a further five patients, liver disease was recognized but was apparently not severe at the time of reporting the ceruloplasmin level. Case 3 was asymptomatic at the time of first testing when his ceruloplasmin level was 17.5 mg./100 ml., had hepatosplenomegaly and a ceruloplasmin level of 22.3 mg./100 ml. at a second testing one year later, and was diagnosed as a patient on the basis of finding post-necrotic cirrhosis on liver biopsy and a characteristically low ratio of copper -64 in the plasma following oral administration of the isotope (Sternlieb, 1967, personal communication). Case 12 was asymptomatic at the time of first testing, except for mild abnormalities of liver function tests. Unspecified abnormalities of the liver were found at liver biopsy. The ceruloplasmin level was reported to have fallen to typically low levels spontaneously (Tu, 1966, personal communication). This is an unusual and unexplained observation. Case 13 had only hepatosplenomegaly, abnormalities in liver function tests, and a urinary copper excretion of 140 μg. per day at the time of first testing. He was suspected previously of being a heterozygote (Walshe, 1964, personal communication). At the age of 15 years, he had developed Kayser-Fleischer rings (Osborn and Walshe, 1967). His ceruloplasmin level was 17 mg./100 ml. after two years of treatment with penicillamine.

The ceruloplasmin levels of cases 5 and 6 rose considerably during treatment with estrogens (German and Bearn, 1961). Testicular atrophy was found in cases 8 and 9, consistent with recent hyper-estrogenism (Scheinberg and Sternlieb, 1961).

In at least three of the cases, the ceruloplasmin levels declined after treatment with penicillamine. In case 7, the ceruloplasmin level decreased to 8 mg./100 ml. after three and one half months, and to an undetectable level after a further three months of treatment (Holtzman et al.,1967). In case 10, the ceruloplasmin level decreased to 3 mg./100 ml. after one year of treatment (Rosenqer , 1961). In case 14, the ceruloplasmin level was reported to have fallen to "classical Wilson's disease levels" (Sherlock, 1960). In cases 3 and 13, the response to penicillamine has been less dramatic with declines to 17 mg./100 ml. (Walshe, 1964, personal communication) and 16.2 mg./100 ml. (Scheinberg, 1967, personal communication). In case 15, the first ceruloplasmin measurement available (18.0 mg./100 ml.) was made after four years of penicillamine treatment; the ceruloplasmin level was 21.4 mg./100 ml. after eight years of treatment. This patient, while clinically free from all but minor symptoms, is on low doses of penicillamine intermittently, thus he may not be adequately decoppered.

From the genetic data summarized in Table 12, it is apparent that a wide variety of racial origins are represented. The first cousin consanguinity rate including second cousin marriages is 28.6 per cent. While these rates are high, there is no evidence that they differ from the rates in the complete series of patients with Wilson's disease from which they were drawn.

In two cases, 1 and 11, both affected sibs in the families had normal or nearly normal ceruloplasmin levels. In four families, those of

cases 3, 8, 12, and 13, the affected sibs had typically low levels of ceruloplasmin.

All of 17 parents tested had ceruloplasmin levels within the normal limits.

DISCUSSION

Severe liver disease with a subsequent increase in circulating estrogens has been suggested as the cause of normal levels of ceruloplasmin in certain patients with Wilson's disease (Sternlieb and Scheinberg, 1963). The administration of estrogens increases the level of serum ceruloplasmin in some patients with Wilson's disease (German and Bearn, 1961). Two patients of those recorded here had signs of hyperestrogenism. In a further three patients, liver disease was severe and could perhaps have interfered with estrogen metabolism. In the remaining eleven patients, the liver disease was not clinically severe at the time of testing and in some cases was recognized only by tests of liver function. While the effect of estrogens may therefore play an important role in causing an increase in the ceruloplasmin level in some patients, this does not appear to be a suitable explanation in all cases.

There is no evidence to suggest that patients with normal levels of serum ceruloplasmin are genetically different from the more common type of patient with a ceruloplasmin deficiency. The age of onset and racial origin are as varied as in the more common type of patient. The consanguinity rate is probably the same as in other patients

although we cannot exclude the possibility of a higher rate. Patients with both normal and typically low ceruloplasmin levels can occur within one sibship.

Familial factors apparently are involved in the production of normal ceruloplasmin levels in certain patients since two sibs in two different sibships both had normal ceruloplasmin levels. Since only about four per cent of patients have normal levels, the occurrence of more than one such patient in a sibship is unlikely to be a chance occurrence.

In all of the patients who have been treated long enough and on adequate doses of penicillamine, the ceruloplasmin level has decreased, frequently to typically low levels. This could be the normal response to removal of copper from the body. This is investigated in rats in Part 3, to follow. Another possibility is that in some patients with Wilson's disease, the presence of large amounts of copper in the liver enables some of it to be incorporated into ceruloplasmin. Serial determinations of the copper in liver biopsies indicate that penicillamine treatment decreases the liver copper content (Sternlieb and Scheinberg, 1963). The removal of the excess copper stores from the liver could account for the simultaneous decrease in the ceruloplasmin level.

The presence of three clinically unaffected patients with Wilson's disease and normal ceruloplasmin levels in the series of 15 patients is important from a diagnostic viewpoint. Patients with normal

levels occur infrequently and these have usually had well-advanced disease. However, in studies of asymptomatic sibs, a low level of ceruloplasmin will usually, but not always identify the homozygotes for Wilson's disease. A clinical examination for hepatosplenomegaly plus liver function tests identified all three previously considered asymptomatic. The patients have been treated with penicillamine so it is not known if they would have developed typical clinical features of the disease. Case 13 is undoubtedly a homozygote because he developed Kayser-Fleischer rings. Case 3 had a low uptake of ^{64}Cu into ceruloplasmin, however, his ratio of plasma ^{64}Cu levels at 24 hours to two hours was in a region where there is some overlap with heterozygotes (Sternlieb et al., 1961b). Case 12 has not been studied with ^{64}Cu. Cases 3 and 12 could be heterozygotes with mild clinical manifestations of the disease, although no such heterozygotes have been reported among many parents of patients.

This survey has shown that ceruloplasmin levels may occasionally fall within the normal range in patients with Wilson's disease. These increased levels are all below the mean level for the normal population. The patients have, in some cases, had severe liver disease. A resulting increase in circulating estrogens may, in such patients, contribute to the increase in the ceruloplasmin level. The accumulation of large amounts of copper in the liver, which begins early in the disease, may promote ceruloplasmin formation in some patients. The extent to which this occurs is probably under familial influences. As the excess copper in the liver is removed with pencillamine, the ceruloplasmin level falls to low levels.

PART 3

The Effect of D-Penicillamine on the Level of Serum Ceruloplasmin in Rats

This study was undertaken because of the reported observations, reviewed previously from the literature, of patients with Wilson's disease and normal ceruloplasmin levels who had a decrease of their ceruloplasmin levels following penicillamine treatment. Two possible hypotheses for these findings are tenable:

1) These patients are genetically similar to those with a ceruloplasmin deficiency and factor(s) not involving the gene for Wilson's disease have caused an increase in their ceruloplasmin levels. The removal of excess copper by penicillamine treatment might correct the secondary factor and a decrease in the ceruloplasmin level would follow.

2) These patients are genetically different from the more usual patients with a ceruloplasmin deficiency and could have a different allele at the same genetic locus or a different abnormal gene producing a defect further removed from ceruloplasmin synthesis. In this case, the fall in ceruloplasmin level following penicillamine treatment would have to be explained as a normal effect of the drug.

The response of normal individuals would differ according to the above hypotheses. In the first case, normal individuals would not be expected to show a decrease in their ceruloplasmin levels after penicillamine treatment. In the second case, the normal response would be a decrease in the ceruloplasmin level, perhaps more rapid than in the patients with Wilson's disease because of smaller tissue copper stores in the normal individual.

Since the decrease in ceruloplasmin level in patients requires at least several months of penicillamine therapy and since patients with Wilson's disease are the only individuals, with the possible exception of some patients with cystinuria, on such therapy, it was decided to test the hypotheses in rats.

MATERIALS AND METHODS

Assay Procedures:

The ceruloplasmin content of the serum was determined by measuring the oxidase activity with paraphenylenediamine (PPD) as substrate. A method basically similar to that described for the assay of human sera in Section I was found suitable for use with rat sera. The reaction was carried out at 37°C using a 1.0 M acetate buffer of pH 5.2, as for human sera. A 50 µl. aliquot of rat serum was used for each assay. The results were expressed as change in optical density per minute (ΔO.D./min.).

The amount of copper in the urine of untreated rats was measured by direct extraction of the copper with zinc dibenzyldithiocarbamate

(Giorgio, Cartwright, and Wintrobe, 1964). This method gave falsely low results on urine obtained after penicillamine treatment, presumably because of the chemical bonding of some of the copper. Urinary copper excretion following penicillamine administration and the copper content of liver were measured as described by Sass-Kortsak et al. (1959), using the method of Eden and Green (1940). In this method, sodium diethyldithiocarbamate is used to produce a colored compound with copper after wet digestion of the test material.

Determination of Dose of Penicillamine:

Twenty-four hour urinary copper excretion was measured after the administration of varying doses of penicillamine, in order to determine a suitable dose for the study. Hooded rats of the Venning strain, weighing about 120 grams, were used for this test. After a one hour fast, each test rat was given the required dose of D-penicillamine[1] by stomach tube. The penicillamine was administered as a two or five per cent solution in deionized distilled water, prepared immediately prior to administration. Each rat was then placed in a metabolic cage, specially constructed to avoid copper contamination. The cages were constructed of lucite with a styrene grid to support the animal and a nylon mesh screen to retain the feces. All parts of the cages were rinsed with deionized distilled water prior to the test. Urine was collected in acid-washed graduated cylinders. During the 24 hour collection period, the rats were given a 1.5 per cent glucose solution in deionized distilled water, add libitum. The glucose prevented excessive weight loss and promoted a high fluid intake.

[1] All D-penicillamine used for this study was kindly donated by Merck, Sharp and Dohme of Canada Limited.

The effects of a single dose of D-penicillamine on the ceruloplasmin level were determined up to eight hours after administration. Penicillamine was also added to the serum to determine possible direct effects upon the assay procedure.

Experimental Procedure:

Hooded rats of the Venning strain, about 110 to 120 grams in weight at the start of the experiment, were used. Up to four rats were housed in each large cage. Their room was maintained at 21 to 22°C. The rats were fed Purina Laboratory Chow.

The rats were divided into three groups at the beginning of the experiment: five females in a control group, five females in a 'low dose' group receiving 60 mg. D-penicillamine/kg. body weight daily, and six females and two males in a 'high dose' group receiving 120 mg. D-penicillamine/kg. daily. After 11 weeks, the study was terminated in the 'low dose' group. The 'high dose' group was given 240 mg. D-penicillamine/kg. daily for a further four weeks and 360 mg./kg. daily for the last four weeks of the study.

The rats were weighed each Monday and the amount of D-penicillamine required was calculated. The daily dose required for all the rats combined was weighed into each of five test tubes. Each day, the required amount of water was added, plus a drop or more of 1 N NaOH, as needed, to bring the solution to about pH 7. The concentration of the penicillamine solution varied from one to five per cent, depending upon the total dose, so that the total amount of liquid given to a rat did not exceed 2 ml. Penicillamine solution, or a

similar amount of water for control rats, was administered by stomach tube, using narrow plastic tubing attached to a syringe, each morning from Monday to Friday.

Food was removed from the cages at about 5 p.m. each week-day except Friday, and early Monday morning. The penicillamine was administered at least three hours after food (Mondays only) and usually after an overnight fast. Food was replaced in the cages one to two hours after the gavage. Tap water was available ad libitum at all times. The intake of food and water during the last weeks of the study when the rats weighed about 200 g. averaged nine g. Purina Laboratory Chow per rat daily and 30 cc. water per rat daily. The chow contains a specified 18.2 parts per million of copper. The copper content of the drinking water was assayed and found to be 0.51 mg./ml. The total daily intake of copper was therefore about 179 mg. per rat.

Blood was taken from the tail every week or every second week, depending upon the dose of drug. The samples were taken in mid-week after only two or three continuous days of drug administration. This avoided prolonged bleeding from the tail which might otherwise have been encountered, since an increased bleeding time was found after continuous administration of higher doses of D-penicillamine to rats. However, this effect was found to disappear after two days of no drug administration (Merck Institute, personal communication, 1964). The blood was collected in 100mm. long sections of acid-washed capillary tubing. The tubes were sealed with melted sealing wax, and the serum was separated by centrifugation. The tube was broken at the junction of the serum and red cells. The serum

containing portion of the tube was covered at both ends with parafilm and frozen until the assay for ceruloplasmin was carried out.

Pyridoxine, 2 mg. per rat, was injected intraperitoneally every Friday into all rats on a penicillamine dose of 120 mg./kg. or more. Sterile water and equipment were used. This injection was given as a precaution because of the pyridoxine deficiency found by Asatoor (1964) when rats were given 300 to 340 mg./kg. D-penicillamine daily.

After 14 weeks of the investigation as outlined, the two surviving control females and six 'high dose' females were allowed to mate. A male of the same strain was placed with two or three females for five days. The females were about 21 to 22 weeks of age and weighed about 200 g. at the time breeding was started. At this point, the drug was administered daily, seven days per week, at an increased dose of 360 mg./kg. body weight. Daily drug administration and weekly pyridoxine injections were continued until delivery. This part of the study was carried out to determine if penicillamine produced a copper deficiency in the offspring of a mother treated during pregnancy. This is not pertinent to the studies reported here and will not be included in this report.

Drug administration was terminated after all pregnant rats had delivered. After 19 weeks of the study, the rats were killed and samples of the liver were obtained for assay of the copper content. Small portions of the large liver sample were removed, weighed, and dried at 110°C to constant weight after cooling in a dessicator. These figures were used to calculate the liver copper content in terms of dry weight of liver.

RESULTS

Assessment of the Method for Quantitative Assay of Ceruloplasmin in Rats:

The following aspects of the assay method outlined below were studied before it was accepted as being suitable for use with rat sera.

a) Linearity:

The oxidase activity was proportional to the amount of serum (therefore amount of ceruloplasmin) present in the reaction mixture. Results of assays using 25, 50, 75, 100, 125 and 150 µl. of rat serum are shown for the two samples in Fig. 19.

b) Buffer:

The rate of reaction was measured using the following buffers:

phosphate buffer: pH 5.8 and 6.8 at each of 0.1 and 1.0 M.

phosphate - citrate buffer:

pH 6.8 of 0.5 M citrate/1.0M phosphate; pH 5.6 of 0.05 M citrate/0.1 M phosphate, 0.1 M citrate/0.2 M phosphate, and 0.5 M citrate/1.0 M phosphate.

acetate buffer: pH 4.8, 5.2, and 5.6 at 0.5 and 1.0 M. pH 5.5 at 0.1, 0.5, and 1.0 M.

When any oxidase activity was apparent, the non-enzymatic oxidation was also determined by omitting serum from the reaction mixture. In the presence of phosphate and phosphate-citrate buffers, the oxidase activity was very low and was found to be entirely non-enzymatic. Oxidase activity was highest in acetate buffer

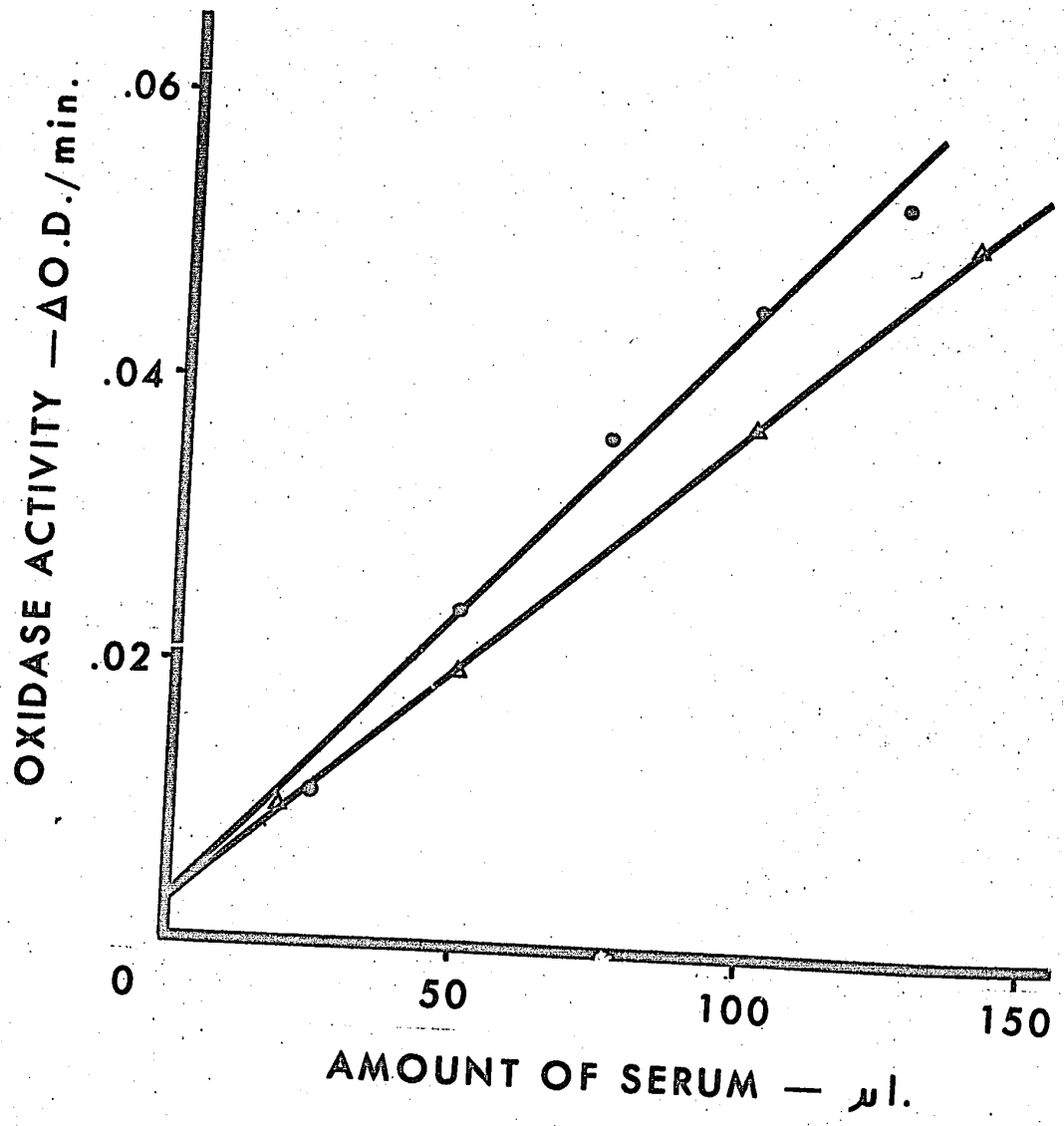

Fig. 19. Rate of oxidase activity in rat sera plotted against amount of serum used. Results of sera from two different rats are shown. Acetate buffer, 1.0 M, pH 5.2; 37° C.

of 1.0 M concentration. The activity was slightly higher at pH 5.6 than at pH 5.2, as seen from the following results in two rat sera, respectively:

pH 5.2: .0106, .0165 ΔO.D./min.

pH 5.6: .0181, .0188 ΔO.D./min.

Some of this difference is due to higher non-enzymatic oxidation at pH 5.6. The optimum pH may be a little higher than the pH 5.2 found for oxidase activity in human sera by this method (Section I) but because the difference was slight, buffer of pH 5.2 was also used for the rat sera.

c) Variation due to Experimental Error:

Duplicate determinations were carried out on 21 random sera over a number of days. A mean difference of 0.0007 ΔO.D./min., considering differences in either direction, were found. Since this difference was small relative to the total activity duplicate determinations were seldom made. The testing on two days of previously frozen sera from three rats, whose blood had been taken on two occasions one week apart, indicated little day-to-day technical variation. In the six samples, the mean differences for each rat between the two days of sampling were 9, 19, and 21 times a large, respectively.

Selection of Dose of Penicillamine:

The response of the 24 hour urinary copper excretion to a single dose of D-penicillamine is shown in Table 13. The copper excretion rose

TABLE 13

24-HOUR URINARY COPPER EXCRETION AFTER A SINGLE DOSE OF D-PENICILLAMINE TO RATS

Dose of D-penicillamine (mg./kg. body wt.)	No. of rats	No. of copper determinations	Urinary Cu excretion (µg/24 hrs.)
0	5	5	3.7
60	4	2	15.4
120	4	2	25.3
360	4	4	33.0

rapidly and linearly with increasing dose up to a dose of 120 mg./kg. body weight, then rose more slowly at a dose between 120 and 360 mg./kg. Patients with Wilson's disease usually take 1 g. penicillamine daily, equivalent to 14 mg./kg. for a 70 kg. man and 20 mg./kg. for a 50 kg. adolescent. The original doses selected were 60 and 120 mg./kg. These doses are three and six times, respectively, the higher (adolescent) human dose rate. The urinary excretion was increased five and eight-fold respectively over the control excretion, a lower response than that of the twenty fold increase in normal humans taking 1 g. penicillamine daily (Sass-Kortsak et al., 1961). The higher dose rates (240 and 360 mg./kg.) gave about a ten-fold increase in urinary copper excretion.

There was no immediate effect of a single dose of 360 mg./kg. D-penicillamine upon the ceruloplasmin level. In two rats, the values obtained before treatment, and one half and three hours after penicillamine administration were .0219, .0208, .0211 ΔO.D./min. and .0229, .0255, .0238 ΔO.D./min. respectively. In another rat, the pre-treatment value and those at two and four hours were similar: .0266, .0232, and .0241 ΔO.D./min., respectively. When added directly to rat serum, there was no demonstrable change in the rate of oxidase activity. A lag period of about 30 seconds was observed with a concentration of 10^{-4}M penicillamine. The lag period was increased to one half hour at 10^{-3} M and longer at 10^{-2}.M. These results are similar to those observed in human sera in Section II, Part 1.

Levels of Serum Ceruloplasmin During Penicillamine Treatment:

The results of the ceruloplasmin determinations for 15 rats are shown in Figs. 20, 21, 22 and 23. The ceruloplasmin assay was not carried out on all the serum samples collected during the early part of the study when it could be seen that the levels were not changing rapidly. Three rats did not survive the first 51 days of treatment and were excluded from the study. Two of these were control rats, receiving only water, and one was in the 'low-dose' group. An autopsy was carried out on each of these rats and extensive pneumonia was found. This was believed to be due to inspiration of some of the fluid during gavage. A third control rat died after 51 days of treatment and was included in the study.

The pronounced rises in the ceruloplasmin level in some of the rats, particularly during the early weeks of treatment, probably indicated a response to infection, such as pneumonia, aggravated by the gavage procedure. The ceruloplasmin levels in the control rats showed little change during the 92 days of study. The most pronounced changes in the ceruloplasmin levels are the decrease shown by rats in the 'high dose' group between 51 and 72 days of treatment with 240 mg. penicillamine/kg. daily and an increase in the levels after about 20 further days of treatment on a higher dose of 360 mg. penicillamine/kg. daily.

The mean values for the ceruloplasmin levels of each group expressed as change in optical density per minute ($\triangle$O.D./min.) are shown in Table 14. The ceruloplasmin levels of two female rats after

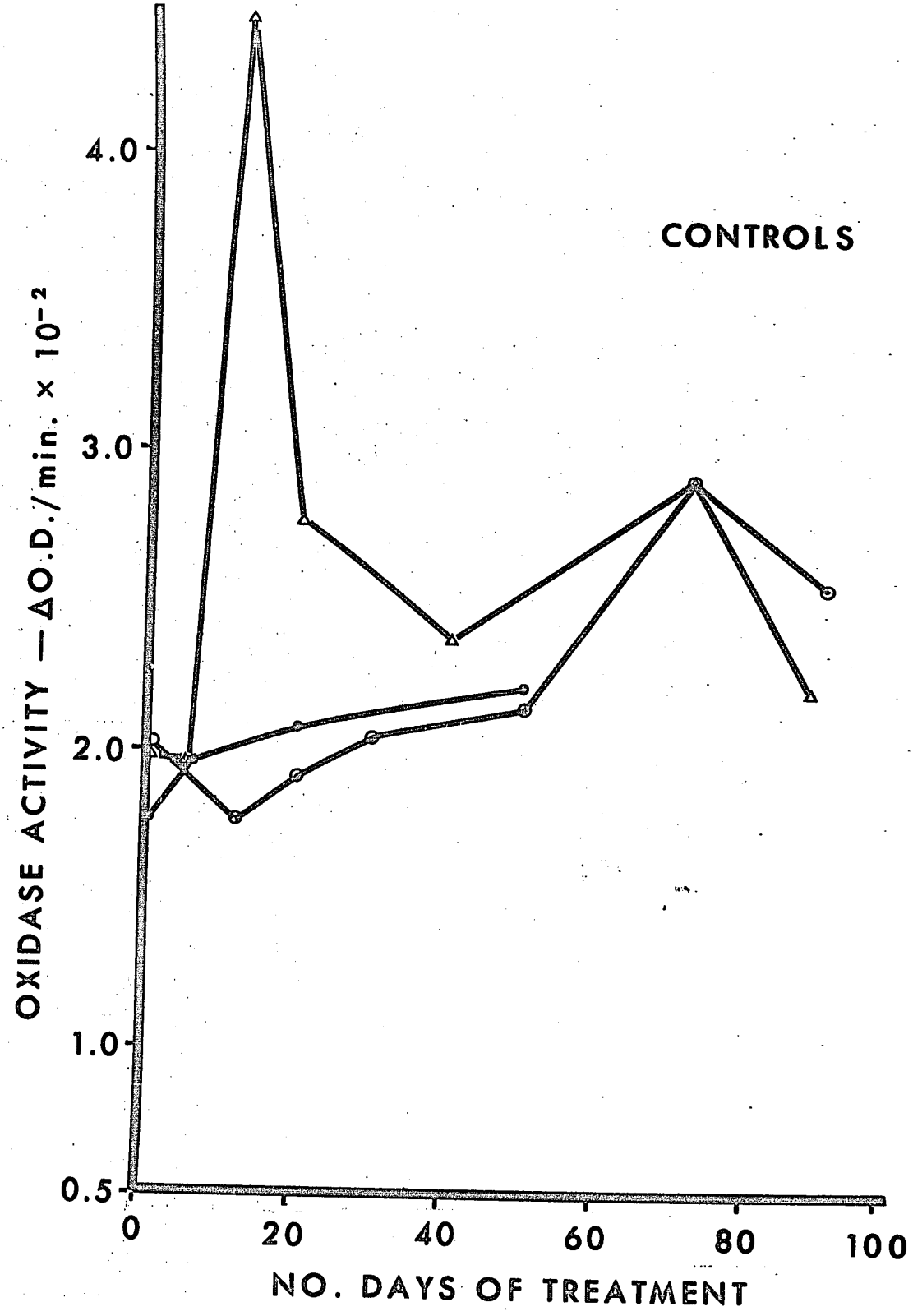

Fig. 20. Oxidase activity in serum of three control rats (female), given no penicillamine.

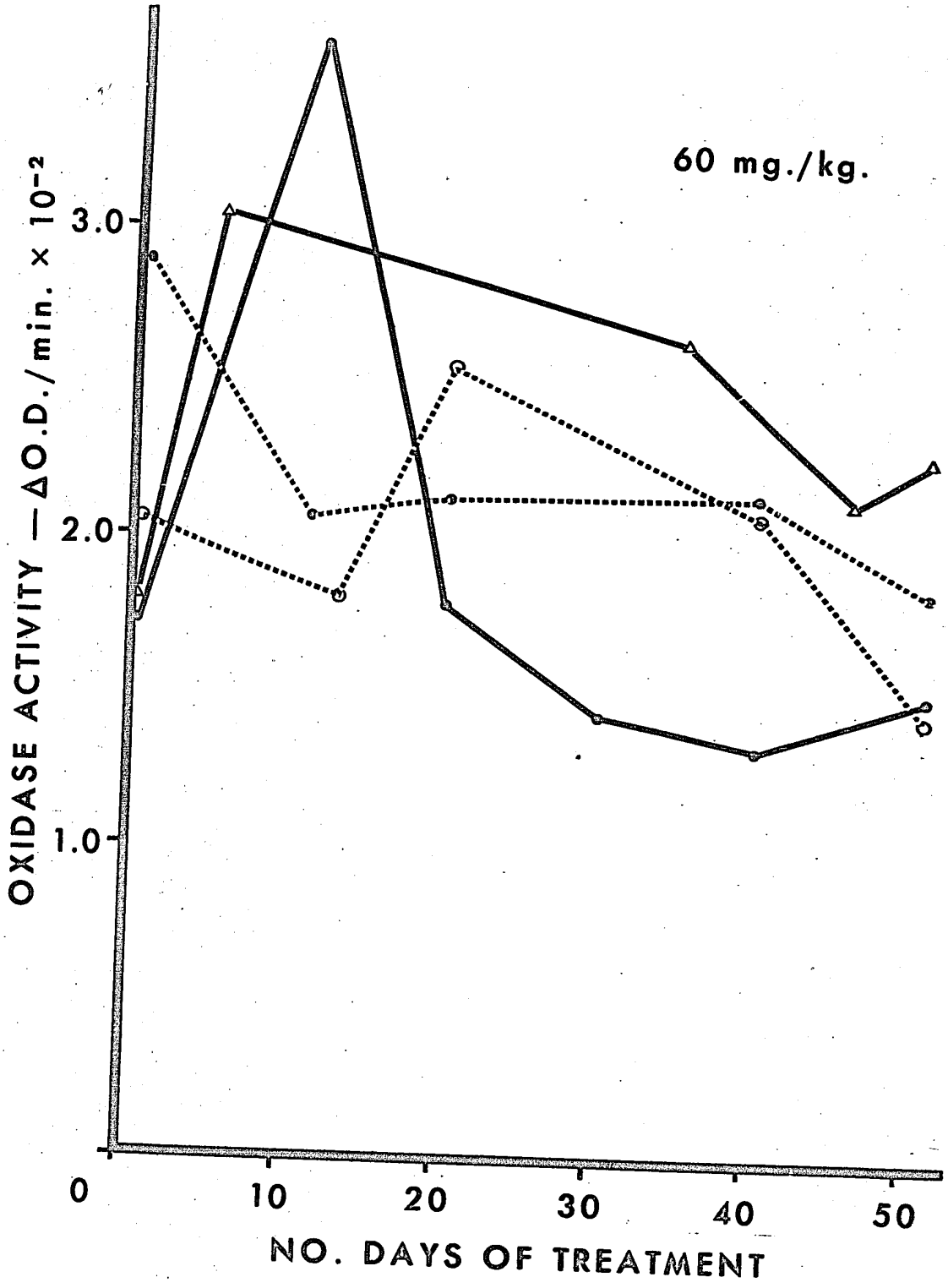

Fig. 21. Oxidase activity in serum of four 'low dose' rats (female), given 60 mg./kg. body weight D-penicillamine daily. Solid and dotted lines are used to help distinguish individual rats.

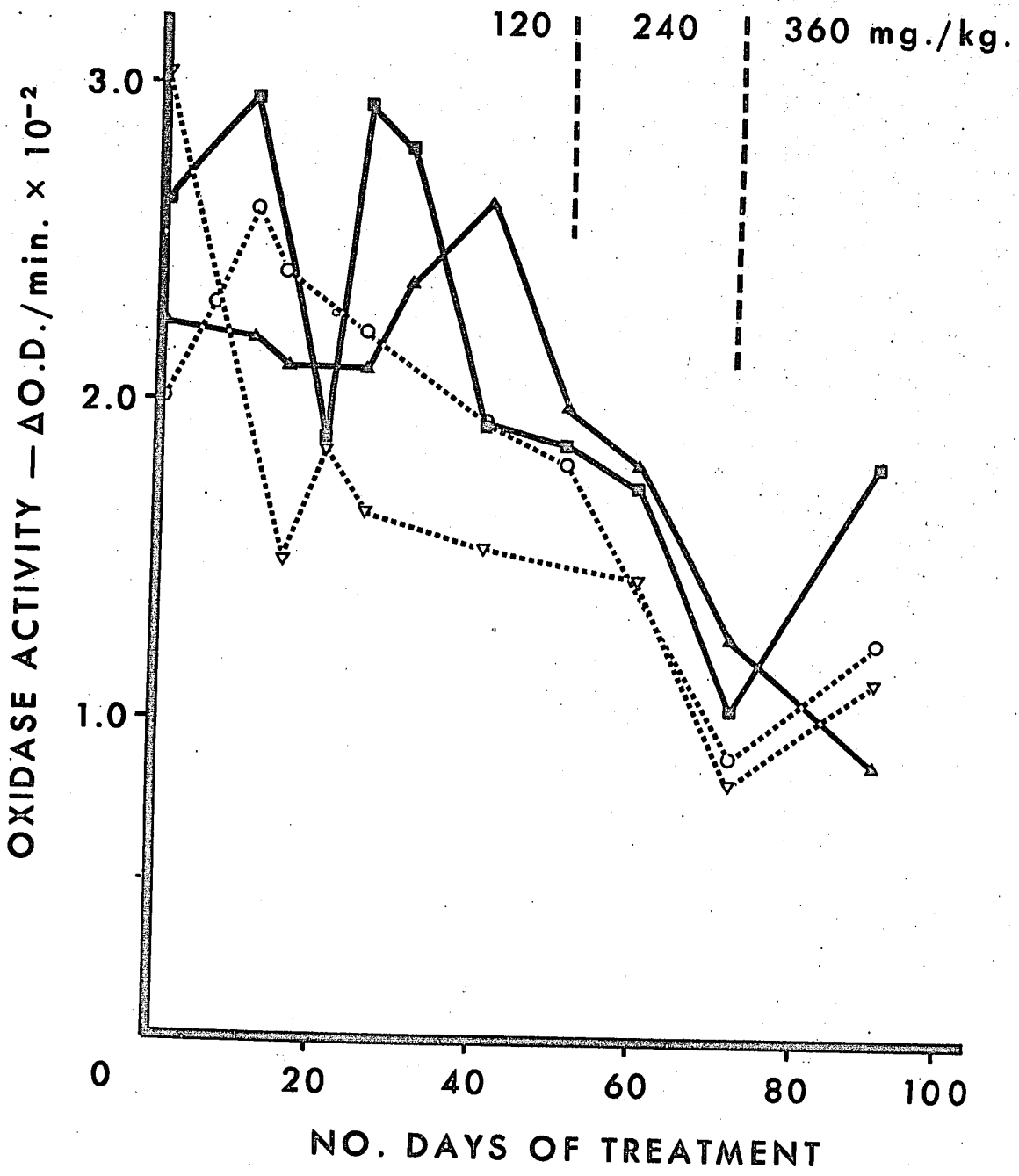

Fig. 22. Oxidase activity in serum of four 'high dose' rats (female), given 120, 240, and 360 mg./kg. body weight D-penicillamine daily, during periods indicated. Solid and dotted lines are used to help distinguish individual rats.

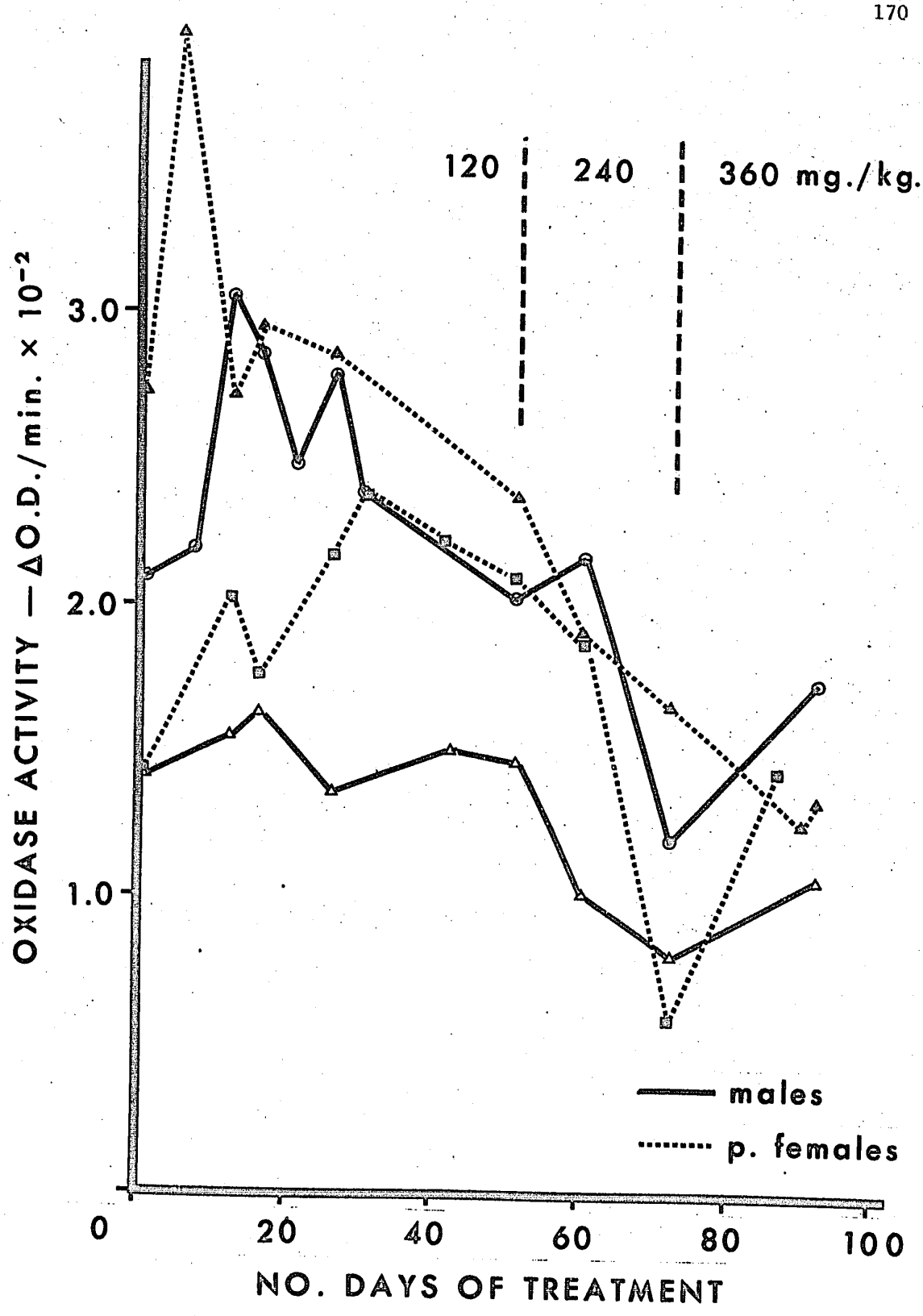

Fig. 23. Oxidase activity in serum of four 'high dose' rats (two males, two females which became pregnant after 70 days of treatment), given 120, 240, and 360 mg./kg. body weight D-penicillamine daily, during periods indicated. Solid lines - male rats; dotted lines - female rats. Final value for female rats was obtained one or two days post-partum.

TABLE 14

CERULOPLASMIN LEVELS (OXIDASE ACTIVITY) OF RATS

AFTER TREATMENT WITH D-PENICILLAMINE

Group	No. of days	No. of rats	Oxidase activity (Δ O.D./min. x 10^{-2}) mean	S.D.
Control	0	3	1.907	0.145
	51	3	2.233	0.114
	75-92	4	2.628	0.296
Low Dose	0	4	2.112	0.545
	51	4	1.775	0.390
High Dose	0	8	2.211	0.563
	51	8	1.898	0.295
	72	8	1.024	0.334
	89-91	6	1.313	0.381

90 days of treatment in the 'high dose' group (Fig. 23) were excluded from analysis because the blood samples were taken one or two days post-partum. The control values (Fig. 20) were similar throughout the course of the study. There may have been a slight rise with time but there were too few surviving animals to draw any firm conclusion. The ceruloplasmin levels before treatment in each of the 'low dose' (Fig. 21) and 'high dose' groups (Figs. 22, 23) were similar. The pre-treatment values were used for statistical comparisons so that each group served as its own control. This seems reasonable because of the relative stability of the levels with time in the control rats. Although the mean ceruloplasmin level was lower after 51 days of 60 mg./kg. daily of penicillamine the difference was not significant ($t = 1.00$, d. f. $= 6$, $P > 0.3$). There was no significant difference between the pre-treatment mean and the mean after 51 days of 120 mg./kg. daily ($t = 1.39$, d. f. $= 14$, $P > 0.1$). The mean ceruloplasmin level of these eight rats was significantly lower than that prior to treatment after a further 21 days (total of 72 days of treatment) on a dose of 240 mg./kg. daily ($t = 5.13$, d. f. $= 14$, $P < 0.001$), but the reduction was less pronounced after a further 18 to 20 days on a still higher dose of 360 mg./kg. daily ($t = 3.28$, d. f. $= 12$, $P < 0.01$). The difference between the ceruloplasmin level after 52 days of treatment and the final value was not significant ($t = 1.51$, d. f. $= 12$, $P > 0.1$), however a rise in level occurred in five of the six male and non-pregnant female rats. When the pre-treatment ceruloplasmin levels of all 15 rats in the study (mean ± S.D.: 212.4± 48.9 Δ O.D./min.) were used as control values,

the differences were slighly greater but the conclusions remained the same.

The copper content of the liver was assayed at the end of the experiment in both surviving control rats and in the eight rats of the 'high dose' group. The liver copper content of four rats of the same age and strain and maintained under the same conditions as the control rats were included with the results of the control rats because of the poor survival rate of the latter. The results, shown in Table 15, show that the penicillamine significantly reduced the amount of copper in the liver, whether calculated on a wet weight or dry weight basis.

DISCUSSION

All the results of the ceruloplasmin assays in this study have been expressed as change in optical density per minute. This is entirely satisfactory since comparison of values can conveniently be made. A valid calibration would require purified rat ceruloplasmin. However, if we assume that for both human and rat ceruloplasmin the relation between oxidase activity and the optical density at 610 mμ. is the same, then the same conversion factor can be used. For ceruloplasmin assayed in human serum, we found the conversion factor at 37°C to be 730. The appropriate factor for use with rat serum is, therefore, 730 x 3 = 2190. Multiplication by three adjusts for the use of only 50 μl. aliquots of rat serum, compared with 150 μl. aliquots of human serum. The mean ceruloplasmin level in this strain of rats is therefore about 46 mg./100 ml.

TABLE 15

COPPER CONTENT OF THE LIVER IN CONTROL RATS AND RATS TREATED WITH D-PENICILLAMINE FOR UP TO 92 DAYS

Treatment group	No. of rats	Copper Content (µg./g. liver) mean	S.D.	t	P
Liver - wet weight:					
Control	6	5.50	1.05		
High dose	8	3.68	0.42	4.91	<0.001
Liver - dry weight:					
Control	6	20.27	4.22		
High dose	8	12.75	1.03	4.48	<0.001

The presence of penicillamine in the serum did not alter the rate of the oxidase reaction, but did cause an increased lag period in in vitro studies. In this study, blood samples were always drawn about 22 hours after the previous administration of penicillamine to avoid possible undetected direct effects on the assay procedure.

The ceruloplasmin levels, as indicated by the serum oxidase activity, did not show any consistent change during the 90 day period of the study, with one exception. In one of the three rats, a very pronounced rise in the ceruloplasmin level during the early weeks of the study was probably due to acute infection. Pneumonia aggravated by the gavage was present in several rats autopsied at this time. There was no pronounced increase in ceruloplasmin level attributable to age as observed in albino rats of the Wistar strain during the ages of 25 to 80 days (Legrand and Kayser, 1960). The rats in the present study were about 50 to 125 days of age during this first period and may have already passed the time for the age effect to be apparent in this strain. Any increase in ceruloplasmin level with age would have tended to underestimate the significance of the findings when pre-treatment values for each group were used as the control.

Pre-treatment values for each group were used for the statistical evaluation of treatment effects within each group. After 51 days of treatment with 60 or 120 mg. D-penicillamine/kg. body weight, there was a possible slight decrease, although not statistically significant,

in the ceruloplasmin levels. All of the eight rats tested showed a pronounced decrease in their ceruloplasmin levels during the next period of 21 days of treatment with a doubled dose of D-penicillamine, that is 240 mg. per kg. daily. The mean value at the end of this period showed a highly significant decrease from the pre-treatment level. Possibly this change would have occurred if the dose had not been increased but would have required a longer time. This question cannot be answered by the present study. The mean ceruloplasmin level was significantly lower than the pre-treatment mean after the continuous daily administration of 360 mg./kg. of penicillamine for a further 20 days. or after 90 days, in total, of treatment. Continuous drug administration was required during this period because of concommitant teratological studies.

The mean ceruloplasmin level after 90 days of treatment was higher than the mean level after 72 days of treatment, however the difference was not significant. This increase may therefore have been caused only by random fluctations. However, since six of the eight rats in this group showed a rise in the ceruloplasmin level, the increase may have been real but not of sufficient magnitude to appear significant in the small numbers of animals in this study. There were no signs of acute infection in the rats after 90 days of treatment, as determined at autopsy. The ceruloplasmin levels of the two rats which were pregnant during the last phase of the study did not appear to respond differently from those of the non-pregnant animals. The pregnant animals were, however, excluded from the statistical analyses because the ceruloplasmin level is known to

remain increased in humans during the post-partum period. The two rats which showed the highest levels of ceruloplasmin at 72 days apparently continued to respond to the penicillamine with an even lower ceruloplasmin level at 90 days. This suggests a possible explanation for the rise in level observed in the remaining rats during the 72 to 90 day period. There may be a minimum threshold level to which copper stores, reflected by ceruloplasmin formation, can fall before an adaptive response occurs. When the ceruloplasmin level reaches about one half of its normal level, more copper may be absorbed from food. The amount of ceruloplasmin formed is probably a reflection of the amount of copper available in the liver, so perhaps the amount of copper in the liver triggers the physiological adaption.

The treatment with penicillamine had caused a marked decrease in the copper content of the liver of the rats to about two thirds of the copper content in untreated rats.

A relatively high dose of penicillamine was required to produce a 50 per cent reduction in the serum ceruloplasmin level of the rats in this study. The effective doses of penicillamine, 240 and 360 mg./kg. daily, administered to rats in the 'high dose' group were 17 and 26 times, respectively, the usual human adult dose of 1 g. D-penicillamine daily, or about 14 mg./kg., when considered as dose per kg. of body weight. The daily copper in the diet of the rats was estimated to be about 180 µg. Rats in the 'high dose' group were therefore receiving about 115 and 173 moles of penicillamine, respectively, per mole of copper in the diet. Assuming a

daily dietary intake for an adult human of from two to four mg. of copper, an adult patient on penicillamine therapy receives from 106 to 213 moles of penicillamine per mole of copper. On this basis, the dose administered to rats was similar to the usual dose for humans. However, the penicillamine was administered to the rats on an empty stomach and presumably removes tissue copper, as discussed previously in the literature review, and not primarily dietary copper. The comparison based on the dietary intake of copper may, therefore, not be valid.

The small amounts of data available from studies in humans also suggest that the ceruloplasmin level of a normal individual cannot be readily decreased penicillamine therapy. The usual therapeutic dose of penicillamine administered to patients with Wilson's disease probably does not alter the ceruloplasmin level when administered to normal individuals. The ceruloplasmin and copper levels were followed at about three-month intervals in a child being treated for cystinuria with 750 mg. D-penicillamine daily (Sass-Kortsak, 1967, unpublished observations). The levels over a three-year period, during which time this boy was one and one half to four and one half years of age, are shown in Figure 24. The patient had frequent respiratory infections, particularly during the first year of treatment, as reflected in sharp peaks in the copper and ceruloplasmin and copper levels. Rapid increases were followed by declines at the time of infections. A slight decline in the ceruloplasmin level with age was expected during this period and our previous studies indicated a normal decrease of about 2.3 mg./100 ml. in the ceruloplasmin level

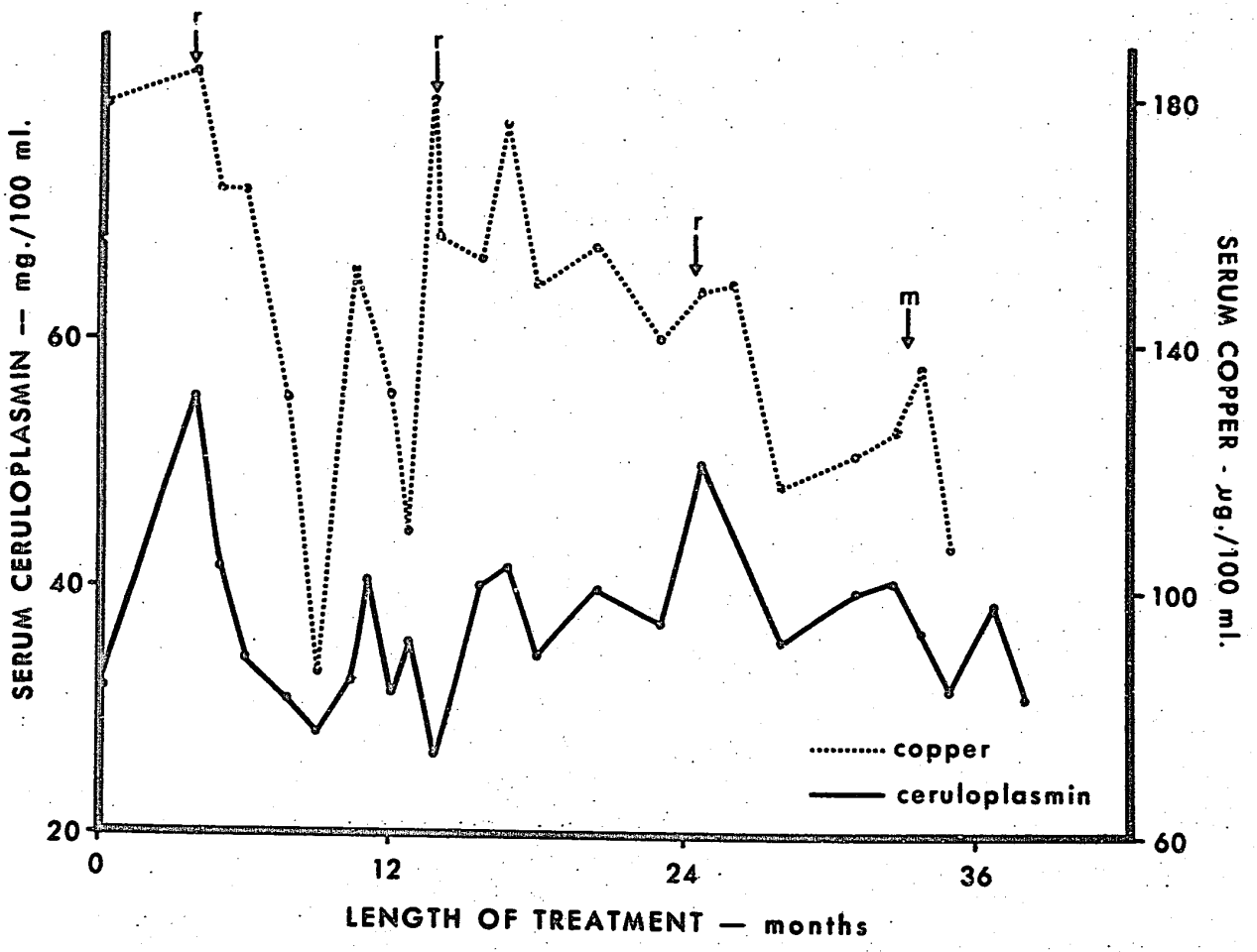

Fig. 24. Serum ceruloplasmin and copper levels in a patient with cystinuria treated for over three years with D-penicillamine (0.75 g. daily). Patient was 18 months of age at start of treatment. r - respiratory infection; m - measles.

between two and four years of age (Cox, 1966). This rather small change may have occurred, but it is difficult to be sure because of the effects of repeated infections. This patient has cystinuria and a renal defect which may alter his metabolism of penicillamine, however, we have no evidence of this. In four schizophrenic patients studied by Walshe (1964), the ceruloplasmin levels were reported unchanged after one year of taking 1200 mg. penicillamine daily.

The results of these studies in rats and humans who do not have Wilson's disease suggest that the ceruloplasmin level is not changed by the daily dose of penicillamine commonly administered to patients with Wilson's disease. In such patients, the ceruloplasmin level declines from normal to negligible levels within several months. A very high dose of penicillamine brought about a decline in the ceruloplasmin level only to about one half of the normal level in rats, at which point physiological changes may have occurred which allowed greater retention of copper from the diet with a subsequent slight increase in the ceruloplasmin level.

Our first hypothesis regarding patients with normal levels of serum ceruloplasmin appears to be supported: such patients are probably not different from those with a ceruloplasmin deficiency and a factor, or factors, not involving the gene for Wilson's disease probably caused an increase in their ceruloplasmin levels. The most important factor involved in increasing the ceruloplasmin level may be the high levels of excess copper available in the liver. This concept will be discussed further.

CONCLUSION

Hypotheses for the Genetic Defect in Wilson's Disease:

The available evidence indicates that Wilson's disease is caused by a defect in copper metabolism.

The disease is probably not caused by ceruloplasmin deficiency (Scheinberg and Gitlin, 1952) even thought this is a frequent feature of the disease. Some patients with proven Wilson's disease have normal levels of ceruloplasmin, while some heterozygotes have decreased ceruloplasmin levels yet are clinically normal. No chemical differences between purified ceruloplasmin from a patient with a normal level and ceruloplasmin from a normal individual could be found in studies including an analysis of peptide fingerprints from all but about a ten per cent undigested core of the ceruloplasmin molecule (Holtzman et al., 1967). Increasing the ceruloplasmin level in patients by administration of ceruloplasmin (Bickel, 1961) or by estrogen administration (German and Bearn, 1961) does not result in clinical improvement. In the review of literature on ceruloplasmin, the conclusion was reached that no physiological role for ceruloplasmin has yet been found, if indeed there is one.

Lack, in the liver, of a copper-concentrating enzyme which also mediates the incorporation of copper into ceruloplasmin was proposed (Walshe, 1963a; Osborn, Roberts, and Walshe, 1963). Recent evidence indicates that the reduced uptake of ^{64}Cu into the liver is a secondary consequence of the disease process and that there is apparently no defect in copper concentration (Osborn and Walshe, 1967). These

authors have now proposed that the defect lies in the incorporation of copper into ceruloplasmin, and that ceruloplasmin is an essential precursor for biliary excretion of copper.

A block in the transfer of copper into ceruloplasmin has been suggested as the primary defect producing Wilson's disease, on the assumption that ceruloplasmin-incorporated copper is the only form in which copper is available for the synthesis of cytochrome oxidase (Broman, 1964). The basic problem in the disease, then, would actually be a deficiency of copper where required. However, patients who have been decoppered by chelating agents can become clinically normal, with no evidence of impairment of mitochondrial function from lack of so essential an enzyme as cytochrome oxidase.

A defect in an amino acid-mediated copper transport system across membranes, apparently an active transport system for amino acids (Harris and Sass-Kortsak, 1967), was suggested by Neumann and Silverberg (1966). The uptake of ^{64}Cu into the liver is normal in asymptomatic patients (Aspin and Sass-Kortsak, 1966; Osborn and Walshe, 1967), so there is probably no abnormality in the transport of copper into the liver cell. Abnormalities of membrane transport may be produced in Wilson's disease as a secondary effect of excess copper in the tissues (Peters, 1966).

The previously described studies using ^{64}Cu have demonstrated that the incorporation of copper into ceruloplasmin is seriously impaired early in the course of Wilson's disease. A considerable body of evidence also supports the concept that biliary excretion of copper

is impaired. Evidence reviewed in Section 1 indicates that the incorporation of copper into ceruloplasmin is not a prerequisite for the biliary excretion of copper, as has been proposed.

A New Hypothesis:

The hypothesis which I shall now present is that the individuals homozygous for the Wilson's disease gene are unable to produce an enzyme with two functions:

1) to incorporate copper into ceruloplasman, or into its subunits

2) to incorporate copper into a form suitable for biliary excretion, thus preventing copper accumulation.

Ceruloplasmin can be reconstituted in vitro from apoceruloplasmin and ionic copper, but under non-physiological conditions (Aisen and Morell, 1965). An enzyme could be required in vivo for the efficient and preferential incorporation of copper. Other metals, for example iron and zinc, require such enzymes, called chelatases, for their incorporation into protein precursors. A typical example is that of heme formation from iron and protoheme, a simple chemical reaction which nevertheless requires the enzyme iron synthetase, or ferro-chelatase, in vivo (Phillips, 1967). The action of this enzyme has been studied in detail. While a porphyrin structure is involved in the case of heme and is not suggested for ceruloplasmin, there are several ways in which the proposed copper chelatase might function. The chelatase could incorporate the proposed ligand-bound

structure of eight copper ions (Broman, 1964; Blumberg, 1966) into ceruloplasmin and into a compound secreted in the bile. Probably such a ligand-bound ionic cluster could form spontaneously under chemical forces. Alternatively, the chelatase might assemble subunits of ceruloplasmin, each containing some of the copper ions for the completed ceruloplasmin molecule. Such subunits might even constitute the form in which copper is excreted via the bile. Failure to remove such subunits, because of absence of the chelatase, could stop their production through a feedback mechanism, thus impairing biliary excretion of copper and allowing copper to accumulate in the liver in other forms. The form in which copper is excreted in the bile is unknown, so these possibilities are entirely speculative.

Data on patients with normal ceruloplasmin levels have been presented. The reduced incorporation of ^{64}Cu into ceruloplasmin reported in one such patient (Sass-Kortsak et al., 1959), may have been due to the difficulty in getting ^{64}Cu into the liver, since the patient had an extremely high liver copper concentration. Data are required for other patients with normal levels of ceruloplasmin and especially for heterozygotes in these families. If the incorporation of ^{64}Cu is in fact impaired in such patients, suggesting that their genetic defect also involves the proposed copper chelatase, then the hypothesis must account for their normal ceruloplasmin levels. It has been shown that familial factors may be involved in the production of normal ceruloplasmin levels in patients. The elevated ceruloplasmin levels in these patients could arise from increased non-enzymatic

incorporation of copper into ceruloplasmin, with such non-enzymatic incorporation influenced by genetic and environmental factors. The reaction taking place could be as follows:

$$\text{apoceruloplasmin} + Cu^{2+} \rightleftharpoons \text{ceruloplasmin}$$

The non-enzymatic formation of ceruloplasmin would be encouraged by an increase in either substrate, that is by: 1) an increase in the concentration of apoceruloplasmin or 2) an increase in copper concentration. Estrogen administration can increase the level of ceruloplasmin in some patients with Wilson's disease (German and Bearn, 1961) and may do this by increasing the production of apoceruloplasmin. Increased estrogenic activity due to impaired estrogen degradation in the damaged liver has been suggested as the cause of normal ceruloplasmin levels in Wilson's disease (Scheinberg and Sternlieb, 1963). This may be true in those patients who had definite evidence of increased estrogenic activity from liver damage. In other patients, perhaps the majority, an excessively high level of copper in the liver may promote the non-enzymatic incorporation of copper into ceruloplasmin. Genetic factors may be involved in determining the effectiveness of alternate pathways for incorporation not involving the proposed chelatase. Several lines of evidence suggest that high liver copper levels may be responsible for the excess ceruloplasmin level: 1) The ceruloplasmin level of rabbits, as well as the liver copper content, is increased when rabbits have copper added to their normal diet (Gaballah et al., 1965). An increased ceruloplasmin level has been reported in a child with copper

intoxication (Holtzman, Elliott, and Heller, 1966), although this could be a normal response to inflammation rather than a response to the excess copper. 2) In at least three patients with Wilson' disease, discussed in Section 2, Part 2, the ceruloplasmin level has been in the normal or near normal range prior to treatment and has fallen to very low levels after extensive decoppering with chelating agents. The studies in normal rats treated with penicillamine, as reported here, are somewhat ambiguous but suggest that with large doses of penicillamine, the ceruloplasmin level can be decreased, although not as much as in patients with Wilson's disease. The few studies on humans who do not have Wilson's disease indicate that penicillamine treatment does not alter the ceruloplasmin level. Patients with normal ceruloplasmin levels are as severely affected as the more typical types of patient, which may indicate that there is little non-enzymatic excretion of copper from the bile under any circumstances.

According to the hypothesis presented, a lack of ceruloplasmin is not detrimental per se, as appears true in decoppered patients and in certain heterozygotes for Wilson's disease. During evolution, ceruloplasmin may have lost its once important physiological role and may now be maintained only as a consequence of the dual role of the chelatase required for normal copper excretion.

While this hypothesis appears to fit well with all the findings in Wilson's disease, there is at present no direct proof for its existence. An enzyme-mediated interaction of ionic copper with

purified apoceruloplasmin can be sought using a liver homogenate, then sub-fractions of the homogenate. Isolation of a copper chelatase would be the ultimate goal. Determining the role of such a chelatase, if found, in the biliary excretion of copper presents a larger task. The isolation of pure bile is not easy and excretion products can rapidly be broken down by the bile. However chromatographic separation of copper-containing excretion products in the bile of normal individuals and patients with Wilson's disease would be a useful step. Ultimate proof of the role of the enzyme in biliary excretion would perhaps have to await isolation and purification of the copper chelatase.

SUMMARY

1. A revised method for determining the concentration of ceruloplasmin in serum by measuring the rate of oxidation of p-phenylenediamine was developed. This method has high precision and reproducibility and is unaffected by a variable lag period.

2. Serum ceruloplasmin levels of 309 normal unrelated individuals were determined by the previous method. The mean level for 163 normal adults in this series was 30.4 ± 5.0 mg./100 ml. The mean copper level in 125 of these sera was 108.1 ± 20.6 μg./100 ml.

3. The serum ceruloplasmin level of 117 normal children and serum copper levels of 112 normal children each showed a negative regression on age. The high levels of early childhood declined to normal adult levels by about 12 years of age. The levels of adolescent males were lower than those of adults. Appropriate factors were obtained for the adjustment of ceruloplasmin and copper levels in children and adolescent males to corresponding adult values.

4. The ceruloplasmin levels of adults showed little variation from day to day and were not influenced by body weight or the menstrual cycle.

5. A study of ten normal families indicated that famial factors influence the ceruloplasmin level. In two families, ascertained through the study of normal individuals, low ceruloplasmin levels showed a regular pattern of inheritance.

6. Analysis of the serum ceruloplasmin levels in nine pairs of monozygotic and nine pairs of dizygotic twins suggested that hereditary factors are important in determining the ceruloplasmin level.

7. A rapid screening test, convenient for large-scale testing and easily carried out without laboratory facilities, was developed particularly for the study of large kindreds in which Wilson's disease had occurred. The test detects individuals with a ceruloplasmin level below the acceptable limit of normal for any specified age.

8. Twenty-five probands with Wilson's disease were found to have 14 affected sibs. Two main groups of patients were distinguishable. In 16 families in which one or both parents were of western European origin, the majority of patients developed symptoms of Wilson's disease, usually hepatic, before 16 years of age. Patients in five families in which both parents originated in eastern Europe, and some of which were Jewish, had a later age of onset and predominantly neurological form of the disease. The difference could be due to different alleles at the Wilson's disease locus, to less specific genetic differences, or to dietary differences.

9. Analysis of sib data indicated agreement with an autosomal recessive mode of inheritance, as previously accepted. The frequency of consanguinity among parents of patients was 0.12. The gene frequency was estimated to be from 1.9×10^{-3} to 9.3×10^{-4} and the incidence of heterozygotes from one in 265 to one in 537.

10. The ceruloplasmin level for a given heterozygote remains relatively constant, whether normal or low, as shown by studies in 11 individuals presumably heterozygous for the Wilson's disease gene. Based on a study of 33 presumed heterozygotes, it was found that 6.5 per cent of unrelated heterozygotes have a ceruloplasmin level below the 99 per cent confidence limits for the normal population.

11. In 13 kindreds in which Wilson's disease had occurred, the ceruloplasmin level was measured in 112 relatives other than parents. An additional 116 relatives were screened for low ceruloplasmin levels by the rapid screening test. Three kindreds appeared to be atypical in that the heteroyzgotes could usually be recognized by a low level of ceruloplasmin. The most satisfactory explanation was that the atypical kindreds carried two alleles, at different genetic loci, which acted together to reduce the ceruloplasmin level. The proposed loci were designated as I, the Wilson's disease locus which controls copper incorporation into ceruloplasmin, and C, a structural gene locus for ceruloplasmin. The mutant allele of the C gene may be present primarily in individuals of German ancestry.

12. Studies of the rate of incorporation of ^{64}Cu into ceruloplasmin in patients with Wilson's disease and their relatives showed that three non-overlapping groups could be distinguished. These groups were believed to be homozygous normal, heterozygous, and homozygous abnormal at the Wilson's disease locus. Studies in the atypical kindreds indicated that individuals with low ceruloplasmin levels were heterozygotes.

13. Ceruloplasmin of the atypical heterozygotes did not differ from that of normal individuals either in its mobility in starch gel electrophoresis or in its response to the inhibitors sodium azide and iproniazid.

14. Clinical and genetic information was obtained on patients with Wilson's disease and a normal ceruloplasmin level in 15 families, mainly collected from the literature. The patients did not constitute a

homogeneous group. Severe liver disease was not a prerequisite for an increased level of ceruloplasmin. A greatly increased concentration of copper in the liver may have facilitated ceruloplasmin production in these individuals. Familial factors were implicated in the production of normal ceruloplasmin levels in some of the patients.

15. The effects of D-penicillamine on the ceruloplasmin level in the presence of normal metabolism of copper was investigated in rats. During 92 days of treatment, the ceruloplasmin levels of eight rats decreased to about half of the pre-treatment values on a daily dose of up to 360 mg./kg. body weight. This dose is over 20 times as high, on a body weight basis, as the dose usually administered to adult patients with Wilson's disease. It was concluded that the ceruloplasmin level could probably not be reduced by penicillamine therapy as extensively in the normal individual as in certain patients with Wilson's disease and a normal ceruloplasmin level. The normal ceruloplasmin level in such patients might therefore be caused by an excessively high copper concentration in the liver.

16. A new hypothesis for the genetic defect in Wilson's disease was presented: the patients lack a 'copper chelatase', an enzyme which incorporates copper into ceruloplasmin and into a form suitable for its biliary excretion.

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APPENDIX

CASE MATERIAL FOR STUDIES OF WILSON'S DISEASE

Proband	Sex	Age of onset (years)	Parents		Sibs	
			Consang.[a]	Racial origin[b]	Normal	W.D.[c]
D.B.	F	9	-	Polish, Germ.	2	-
R.Be.	M	24	-	Irish, Eng.	- (2 dec.)	1F(20)
B.Bl.	M	8	-	Eng.(?), Ital.	8	1M(9-as.)
F.Bu.	M	11	-	Eng., Irish	2	-
M.Ch.	F	13	1c.	Eng.	2	-
S.C.	M	38	-	Polish(J)[d]	2	-
R.Co.	M	9	-	Eng.	1	-
V.D.	M	10	-	French, Fr./N.A. Ind.	2	-
B.K.	M	11	-	Russian/Pol.,Eng.	2	-
M.L.	M	13	-	French	8	1F(2-as.)
B.Ma.	F	3(as.)	1c.	Eng.(?)	8	2M(7?,10)
V.Mc.	F	23	-	Polish	1 (1 n.t.)	-
B.McI.	F	8	-	Germ., Eng.	6	-
J.Mi.	M	25	-	Czech.	3	-
Rb. Mo.	M	10	2c.	Irish	3	1M(12)
L.M.	F	20(as.)	-	Anglo-Saxon(?)	-	2M(9,11) 1F(15?)
J.O.	M	12	-	Ital./Eng.,Polish	3	1M(6-as.)
J.Or.	M	9	3c.	Ital.	2	-
O.R.	F	33	-	Ukranian	1	-
M.T.	F	16	-	Russian(J)[d]	3	-
E.To.	F	12	-	Italian	2	-
F.Wa.	M	9(as.)	-	Irish	-	2M(9?,13)

Proband	Sex	Age of onset (years)	Parents		Sibs	
			Consang.[a]	Racial origin[b]	Normal	W.D.[c]
K.W.	F	9	-	Germ., Eng.	1	-
R.Wo.	M	9	-	Chinese	3	-
F.Y.	M	13	1c.	Irish	1	2F(11?,9?)

[a] 1c - first cousins, 2c - second cousins, etc.

[b] Parents same where one listed; father given first if different; stroke indicates mixed origin of parent.

[c] as. - asymptomatic; ? - probable Wilson's disease, but not confirmed.

[d] (J) - Jewish