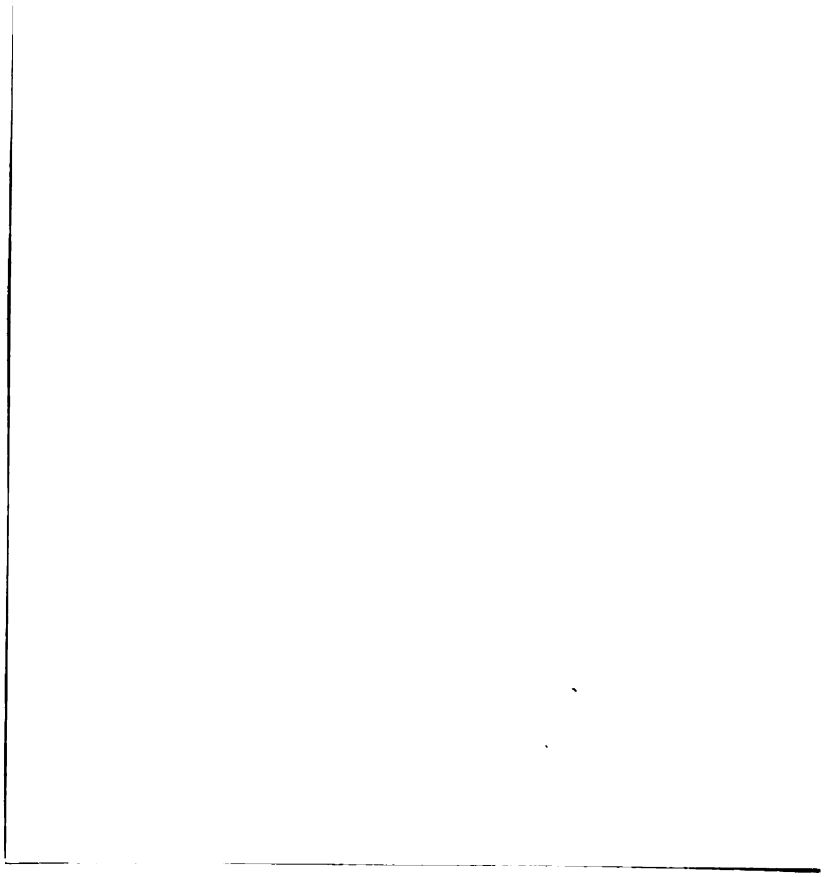


THE BINDING OF METAL IONS BY PROTEINS IN NORMAL AND ABNORMAL HUMAN BLOOD SERUM

By

Robert Bastian Foy

A THESIS

Submitted to the School of Advanced Graduate Studies of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Chemistry

1960

ACKNOWLEDGMENT

The author wishes to express appreciation and gratitude to Doctor Hans A. Lillevik for guidance and counsel.

Grateful acknowledgment is given to Edward W. Sparrow Hospital, Lansing, Michigan for the use of certain facilities made available during this investigation.

To My Wife Jayne

VITA

The author was born June 14, 1928. His secondary education was completed in 1946 at Cass City High School, Cass City, Michigan. In 1950 he was graduated from Central Michigan University, Mt. Pleasant, Michigan, with the Bachelor of Science Degree. He was admitted to the Graduate School of Michigan State University in September, 1953 and has been in attendance since. The Master of Science Degree was completed in 1955.

Employment experience consists of positions as: Medical Technologist from 1950 to 1952 with the Clinical Laboratory at Hurley Hospital, Flint, Michigan; Graduate Teaching Assistant 1953 to 1955 in the Department of Chemistry, Michigan State University; and Technical Director of Laboratories since January 1955 at the Edward W. Sparrow Hospital, Lansing, Michigan.

He is a Senior member of the American Chemical Society and a member of the American Association of Clinical Chemists.

THE BINDING OF METAL IONS BY PROTEINS IN NORMAL AND ABNORMAL HUMAN BLOOD SERUM

By

Robert Bastian Foy

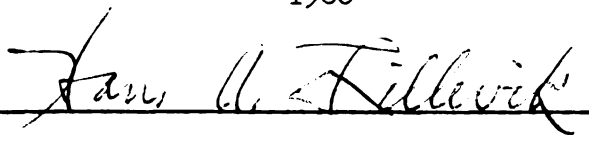
AN ABSTRACT

Submitted to the School of Advanced Graduate Studies of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Chemistry

1960

Approved 

Robert Bastian Foy

AN ABSTRACT

Human blood serum contains a variety of proteins and metal ions. The functions of these serum components are varied and numerous. Some of the metals in serum, (e.g. copper, iron, zinc, calcium and magnesium) are considered to be protein bound, at least in part.

The determination of serum concentrations of certain metals and proteins is often an aid to diagnosis and treatment in many pathological states. In some cases abnormal changes in the serum content of specific metal-protein complexes have occurred.

The study of metal binding by protein fractions of normal and abnormal sera has been accomplished by several means. These include techniques of ultrafiltration, equilibrium and compensation dialysis as well as serum protein electrophoresis after the administration of metal radio-isotopes. Adherence to physiological conditions was not always apparent nor experimentally possible.

The present investigation was undertaken to study more suitable methods for the detection and analysis of certain metals among the protein fractions of normal and abnormal human sera. Since some question has been raised regarding the role of copper in multiple sclerosis this disease was included for study as the principal abnormal group. Other pathological states studied included multiple myeloma, liver cirrhosis, glomerulonephritis and severe burns.

Paper electrophoresis was utilized for the separation of serum protein fractions. Several organic dyes were employed in the histochemical analyses for metals among the various protein fractions. Dialysis studies aided in the evaluation of these dyes as histochemical

staining reagents.

Alizarin red was most useful for detection of calcium on paper electrophoretograms after correction was made for the presence of protein. Calcium was determined to be similarly distributed in all protein fractions of sera from normal subjects and multiple sclerosis patients. However, it was principally located in albumin and the beta and gamma globulins.

Iron analyses by use of bathophenanthroline dye showed that this metal was present to some extent in all protein fractions, mainly the alpha and beta globulins. This was true for both normal and abnormal serum proteins. No interference due to the presence of protein with formation of iron-dye complex was apparent.

Magnesium and zinc appeared to be distributed among all serum protein fractions. However, the main site of magnesium binding was in albumin, whereas that of zinc was in the beta globulin fraction. A satisfactory reagent was not found for separate identification of these metals on paper electrophoretograms.

Albumin and beta globulin contributed most to the binding of copper when paper electrophoretograms of normal and multiple sclerosis sera were treated with alizarin blue dye. Protein did not interfer with these results.

Sera from other pathological states suggested that calcium, magnesium, zinc and copper binding was dependent to some extent upon the amount and kind of abnormal proteins present.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

Human blood serum contains numerous proteins as well as a variety of metal ions. Metals such as copper, iron, magnesium, calcium and zinc are thought to exist in serum largely in the form of metal-protein complexes. Some of these serum metals have known biochemical and physiological function. For example, calcium and magnesium are factors in neuromuscular excitability and calcium also has an important role in blood coagulation. Other instances of metal participation in body functions are those of the metal-protein complexes serving as enzymes. Some examples are listed in Table I.

TABLE I

SOME SERUM-METALLO ENZYME SYSTEMS[1]

Enzyme	Metal
Alkaline phosphatase	Mg
Choline esterase	Mg, Ca
Verdoperoxidase	Fe
Aldolase	Fe, Cu, Zn
Carbonic anhydrase	Zn

[1]References, 38, 89.

Still another form of metal combination with protein are the serum metalloproteins, such as transferrin (iron) and ceruloplasmin (copper) which are involved in transport functions.

Pathological manifestations in humans are often reflected by the biochemistry of the blood. Decreased concentrations of serum iron, zinc and/or copper have been observed in certain anemic states. In some instances specific metal-protein changes have been demonstrated. For

example, in Wilson's disease a reduced concentration of serum protein-bound copper was found (22).

Multiple sclerosis, a neurological disorder, has not been adequately evaluated as to etiology, but there are indications that copper may be significant in the pathology of this disease (8, 45).

The extent to which a serum metal ion is bound to protein may be estimated by several techniques. These include ultrafiltration, equilibrium dialysis and compensation dialysis. Isolated human serum protein fractions, obtained by salt or alcohol precipitation methods, have been utilized to secure data regarding their relative metal-ion binding capacity.

Recently a selective staining method for the location of serum metal-protein complexes upon paper electrophoretograms was described (58). It is suggested that if histochemical techniques could be made accurate and reliable, one would have a simplified approach to the study of serum metal-protein combinations.

The purpose of this study was to investigate methods for the location and distribution of calcium, magnesium, copper, iron and zinc metal ions among the protein fraction(s) of both normal and pathological human blood sera. Special attention was given to multiple sclerosis sera. Methods of study included protein separation and analysis by paper electrophoresis, quantitative chemical analyses, selective histochemical staining, dialysis and photometric techniques.

II. HISTORICAL

In connection with an investigation concerning the binding of calcium, magnesium, iron, copper and zinc to human serum protein fractions it would be desirable to review previous methods and findings. Electrophoresis development and a background for the work with multiple sclerosis sera will also be considered.

A. Electrophoresis in Stabilized Media

The entire field of chromatography and subsequent zone electrophoresis received its impetus from Tswett's (87) original experiments on the separation of plant pigments by adsorbents contained in a column. Other workers brought out different elaborations of this technique, such as new solvents for elution(77) and frontal analysis (57). Some investigators applied electrical potentials across the column ends (19,86).

Integration of previous researches led to the development of zone electrophoresis. This involves the migration of charged components as individual zones upon a supporting medium and gives the advantage of securing more complete resolution. However, mobilities and isoelectric points are not generally evaluated by this method.

One of the first reports regarding electrophoresis on paper was given by König (54) in 1937. The first application to protein separations appeared in 1939 when von Klobusitzky and König (49) described the resolution of a yellow chromoprotein from snake venom. During the decade that followed paper chromatography and various electrochromatographic methods appeared to overshadow paper electrophoresis development. The introduction of moving boundary electrophoresis by Tiselius (93) in 1937 competed with the development and acceptance of the paper work.

In the late 1940's various techniques for paper electrophoresis were independently suggested from several laboratories. The simplest apparatus consisted of a circuit which was completed by dipping the ends of an electrolyte moistened paper into electrode vessels. The problems of supporting the paper to prevent "buffer pooling" and surface evaporation were two disadvantages of this setup.

Attempts to surmount these difficulties led to three basic constructions. One method (21, 55) placed the moistened paper strip between two glass plates which were sealed to minimize evaporation. A second construction, known as the gallows type (26, 32) provided support to the strip by suspending it over a glass rod with the paper ends extended downward and outward so as to dip into the electrolyte solution. A third was the box-type apparatus (10, 18) that kept the paper horizontal while giving minimum contact to the migration surface. The use of glass rectangles or raised pointed projections provided support. A stretching frame or the application of weights was also utilized to keep the paper in a taut, level position.

For control of temperature and surface evaporation, the glass plate method seemed quite effective. However, it was improved by other setups that provided a cover and the optional use of refrigeration. Nonpolar immiscible liquids such as chlorobenzene (18), heptane (10), or carbon tetrachloride (21) were used as sealing agents. McDonald (64) also employed hydrogen and helium as water vapor saturated gas sealants.

B. Metal-Ion Serum Protein Combinations

1. Calcium.- Rona and Takahashi (79) suggested in 1911 that calcium was, in part, bound to serum proteins. Making use of in-vivo compensation dialysis in dogs, Greene and Power (39) estimated protein bound calcium to be 35 to 45 per cent of the total serum calcium. By ultrafiltration methods, Nicholas (68) observed that 36 per cent of the calcium in human serum was non-diffusible. Watchorn and McCance (100) using a similar procedure, found a value of 43 per cent for human sera. Todd (94) concluded from ultrafiltration experiments that about 47 per cent of normal human serum calcium was diffusible. Using alizarin red as a stain for calcium on electrophoretograms of human serum, LeDuc (58) suggested that albumin and gamma globulin contributed equally in binding two thirds of the total bound calcium. The remainder was distributed in the other globulin fractions. On the other hand, Prasad and Flink (75) reported that albumin bound 50 to 55 per cent of the non-ultrafilterable calcium. The remainder was mainly bound to beta-globulin, while the alpha and gamma globulins contributed only slightly to calcium binding. Prasad and Flink (74) had previously determined that 43 to 55 per cent of the total serum calcium was bound by protein.

Various equilibrium studies with isolated human and equine serum proteins (25, 58, 65, 101) have indicated that both albumin and the globulins bind calcium reversibly, with a greater tendency by the globulins to form an irreversible combination.

The amount of circulating calcium, bound to protein, is known to vary with total serum protein concentration and pH of blood (36, 65, 74). Therefore, discrepancies in the reported levels of protein bound calcium

may, in part, be attributable to variations in experimental conditions.

2. Iron.- In 1925 Fontes and Thivolle (34) demonstrated conclusively, by experimentally induced anemias, the presence and importance of iron in equine serum. Two years later Barkan (4) and Warburg and Krebs (99) observed that a portion of the same serum iron was neither dialyzable nor ultrafilterable. Others (35, 48) also noted the similar behavior of serum iron. In 1945 Holmberg and Laurell (41) discovered that a component of human serum formed a pink-colored complex with iron. They considered iron to be albumin bound as did Eisler, et al.(27). Barkan and Schales (5) previously suggested the combination to be with globulin proteins, since iron was found with this fraction after one-half saturation of serum with ammonium sulfate.

In 1946 Schade and Caroline (81) reported that a beta protein in human serum was capable of combining with iron. Koechlin (53) in 1952 isolated a beta iron-combining protein from human plasma. Cohn (17) indicated that the protein possessed a chemically specific site for interaction with iron and also showed (16) that iron formed a complex with isolated human beta globulin. By using radioactive iron, several groups of workers (12, 44, 67, 98, 104) have also concluded that the iron binding protein migrates as a beta-globulin. Neale (67) employed the radioactive isotope, in conjunction with paper electrophoresis, for his study of the iron-binding serum protein. He demonstrated that the abnormal proteins of multiple myeloma, nephrosis and hypogammaglobulinemias in no way affected the normal iron-binding ability of beta-globulins. Working with isolated human plasma protein fractions Surgenor, et al (88) indicated that beta-globulin bound, not only iron but, copper and zinc as well.

Le Duc (58) also showed iron to be associated with beta-globulin of histochemically stained paper electrophoretograms of human serum.

3. Magnesium.- Of the various metal ions involved in serum protein combinations, magnesium has been least investigated. Magnesium is an essential body constituent but little is known regarding its metabolic role. Intracellular fluids are rich in this element and perhaps too much has been assumed concerning its metabolism because of its similarity to calcium. Chemical methods for its determination have not been completely satisfactory.

In 1931 Greene and Power (39) used in vivo compensation dialysis of canine blood to calculate magnesium binding. Of the total magnesium, they found 35 to 45 per cent to be protein bound. A year later Watchorn and Mc Cance (100) showed by ultrafiltration techniques, that only 25 per cent of total human serum magnesium was bound; in contrast to 43 per cent for calcium. According to Cantarow and Schepartz (13) about 15 to 30 per cent of human serum magnesium appeared to be protein bound. They also indicated that this value rose in hyperthyroidism and dropped in hypothyroidism. No further significance has been attached to these changes.

In 1955, Lillevik, et al (60), reported magnesium present in all protein fractions as indicated by paper electrophoresis of human serum and subsequent staining with titan yellow. At the same time, LeDuc (58) found that purified human serum albumin retained 20 to 25 per cent of its magnesium after equilibrium dialysis. Copeland and Sunderman (20) determined the magnesium binding for the serum proteins of 17 human subjects and found that albumin bound 0.0128 mEq./gm while the globulins bound only 0.0081 mEq./gm.

Starch block electrophoresis was employed by Prasad and co-workers (76) to separate human serum proteins. Magnesium chloride was added to the individual isolated fractions and the amount of magnesium bound to each was then calculated from ultrafiltration data. Albumin bound 60 to 65 per cent of the magnesium in normal subjects. Alpha-2 globulin bound magnesium in normal subjects, in patients with multiple myeloma, other hyperglobulinemias and hypoproteinemic patients. They found that beta globulin bound magnesium only in cases of hypoproteinemia.

4. Zinc.- By conductivity measurements Pauli and Schon (70) in 1924 demonstrated an association of equine albumin with zinc. Cohn and coworkers (16) reported in 1950 that the addition of zinc ions to human plasma aided in fractionation of the proteins. By their respective aforementioned techniques Klotz, et al (52) and Tanford (90) demonstrated the ability of bovine albumin to combine with zinc ions.

Gurd and Goodman (40) determined that a totally reversible combination existed between isolated human serum albumin and zinc ions. Cohn (17) indicated that several different human plasma proteins underwent reversible reactions with zinc. Surgenor, et al (88) reported that 3 per cent of human plasma beta-globulin was capable of binding divalent cations, among which was zinc.

The first precipitate formed upon adding zinc ions to human serum was found by Ressler, et al (78) to contain albumin and gamma globulin. In 1956 Valle (95) stated that 35 per cent of the protein bound zinc in human serum existed as a metallo-globulin. The remaining 65 per cent appeared loosely bound to the other protein fractions.

Employing electrophoresis on canine blood serum after the administration of radioactive zinc, Wolff, et al (104) found zinc-65 principally

associated with the albumin and alpha globulins. Little radioactivity was observed in the beta and gamma globulin fractions. An inability of albumin to bind zinc appeared to be the principal factor involved in pathological states related to reduced serum zinc levels. They observed this effect in patients with pernicious anemia, chronic hemorrhage, malignant tumors and anemias caused by chronic infection.

By means of paper electrophoresis and histochemical staining techniques, LeDuc (58) suggested in 1956 that zinc was present in the gamma globulin fraction of human serum proteins.

5. Copper.- In 1927 Warburg and Krebs (99) showed that the copper of avian and mammalian serum was loosely bound and that its binding was influenced by pH. They felt that the loosely bound copper represented total serum copper, whereas Abderhalden and Moller (1) pointed out that serum copper was not dialyzable. Boyden and Potter (11) concluded that all bovine serum copper was protein bound and further suggested that more than one form of organically bound copper existed in such sera.

In 1936 Eisler, et al (27), observed the migration of copper during serum protein electrophoresis. They regarded copper as being bound to albumin. However, Holmberg and Laurell (42) stated in 1947 that serum copper coprecipitated with the globulins upon one-half saturation with ammonium sulfate.

Polarographic analysis by Tanford (90) demonstrated the formation of a complex between bovine serum albumin and divalent copper. In 1955 Klotz and co-workers (52) substantiated this finding through the use of absorption spectra analysis. However, a similar complex was not apparent to them when human serum albumin was studied.

Wolff, et al (103), found that immediately after administration of radioactive copper, its distribution was essentially the same for all human serum protein fractions after paper electrophoresis. Bearn and Kunkel (6) studied the localization of orally given radioactive copper in human serum protein fractions, after starch block electrophoresis. Serum samples taken immediately after ingestion showed a rapid uptake of copper by albumin in normal subjects, cirrhotic and Wilson's diseased patients. Samples taken after longer time intervals exhibited localization of copper in the alpha-2 globulins for both the normal and the cirrhotic. Patients with Wilson's disease did not show this shift.

Holmberg and Laurell (43) isolated a copper bearing protein (ceruloplasmin) from human serum in 1948. They characterized it as an alpha-2 metallo-globulin. The same year Cohn (15) demonstrated that copper formed a blue-green complex with beta-globulin of human serum, the same protein which binds iron. Koechlin (53) observed that this complex appeared to involve one molecule of protein and one or two atoms of copper.

LeDuc (58) found copper to be iso-migratory with gamma globulin upon staining human serum paper electrophoretograms with diethyldithiocarbamate.

Lahey, et al (56) noted a high degree of correlation between human serum copper levels and the amount of alpha-2 and beta-globulins. There was no significant relationship between serum copper levels and other serum protein fractions. Thompson and Watson (91) and Cummings, et al (22), employed sodium sulfate fractionation of human serum proteins in their studies upon copper binding. They obtained an average per cent distribution of serum copper as follows: albumin 8 to 10 per cent,

alpha-globulins 10 to 31 per cent, beta-globulin 42 to 55 per cent and gamma-globulin-19 to 25 per cent.

C. Multiple Sclerosis

Multiple sclerosis (M.S.) is a disease involving widespread patches of demyelinated fibers of the central nervous system. This nerve cell destruction leads to various neurological manifestations, depending on the site of the lesion.

Although the early 19th century findings were misinterpreted by Charcot, Scheinker (82) credited him with the first complete and pathological account of the disease.

Fog (33) reported in 1951 that serum protein levels of M.S. patients were normal whereas Saifer, et al (80) as well as Volk and co-workers (97) found that in 23 cases, 85 per cent had lowered serum albumin levels and slightly increased alpha-2 globulin. Jones, et al (47) in 1954, studied the albumin levels of 130 patients and likewise reported lowered values. On the other hand, Dobin and Switzer (24) applied serum protein studies as well as various liver function tests to 58 M.S. patients and found no significant evidence of hepatic dysfunction.

Moving boundary electrophoretic analysis of M.S. serum proteins in 1954 by Bernsohn and Cochrane (9) revealed low albumin and elevated alpha-2 globulin content. Other globulin components were not significantly abnormal. Total protein average values by Kjeldahl analysis were 7.36 and 7.20 gm/100 ml of serum respectively for M.S. patients and normal subjects. Most of the electrophoresis patterns of M.S. patients exhibited a "double peak" effect in the alpha-2 region.

In 1958, Vlad, et al (96) published data relating serum protein levels to the state of activity in lesions in the M.S. patient. During a quiescent phase they noted normal protein concentrations. When alpha-2 or beta globulin levels were elevated this predicted the onset of a demyelinizing process whereas when elevated gamma-globulin content was observed the patient was regarded as being in a sclerosing stage.

Serum cholesterol and other lipids have been studied in M.S. patients, but, as with the proteins, no conclusive agreement has been reached. Fog (33), Wilmont and Swank (102), Bernsohn and Cochrane (9) and Chiavacci and Sperry (14) all concluded that blood cholesterols in M.S. patients were normal. However, Dobin and Switzer (24), Pichler and Reisner (73) and Frisch (37), reported that levels of both total and esterified blood cholesterol were high. A recent report by Persson (71) indicated that in male M.S. patients, blood lipids were higher than normal, whereas only a few female patients had elevated values. He found no correlation between lipid levels and patient age or stage of the disease.

Some consideration has been given to the possibility of a trace metal deficiency being involved in the pathogenesis of M.S. A naturally occurring demyelinating disease of young lambs was related to a copper deficiency in the Australian pasture (7). Bennetts and Chapman (8) found in 1937 that copper levels of the blood, milk and liver of the diseased lambs were low. Feeding copper to the lambs arrested the ataxic condition. Prevention was aided by dietary administration of copper to the ewes prior to dropping. A similar disease of lambs occurs in many parts of Great Britain. Abnormal assimilation was

thought to be involved, since tissue levels in pregnant ewes and ataxic lambs were low, while normal amounts of pasture copper were found (45, 84). Additional dietary copper produced similar therapeutic results as in Australian sheep.

In 1948 Mandelbrote and co-workers (62) analyzed the blood and urine of 26 M.S. patients for copper content. They were unable to find conclusive abnormalities.

Cumings and associates (22) found that the percent of total copper distributed among the globulins of normal serum was as follows: alpha- 31 per cent, beta - 42 per cent and gamma - 19 per cent. For M.S. patients the distribution shifted to : alpha - 19 per cent, beta - 60 per cent and gamma globulin - 16 per cent.

III. EXPERIMENTAL

A. Equipment

Electrophoresis Apparatus.- The Durrum-hanging strip, inverted V-type cell, including a power supply unit, was obtained from the Spinco Division of Beckman Instruments Company, Palo Alto, California. The entire cell and cover were constructed of lucite and utilized platinum wire electrodes. Schleicher and Schull No. 2043A filter paper strips (3.0 x 30.6 cm) served as supporting media. Schleicher and Schull filter paper No. 470 (5.1 x 31.8 cm) served as buffer wicks. Figure 1 illustrates the apparatus.

Analytrol Scanner.- Dyed paper electrophoretograms were analyzed by means of a Spinco recording densitometer. Solution absorbancy measurements were made with either a Coleman Model-14 or a Beckman Model-DU spectrophotometer.

Oven.- Electrophoresis paper strips were dried in a Despatch convection oven (Minneapolis, Minnesota).

Analytical Balance.- Chemicals and dyes were weighed on a Voland semi-automatic balance (New Rochelle, New York).

Dialysis Apparatus.- A rotating, multiple specimen dialyzer with visking dialysis bags was obtained from Oxford Laboratories, San Francisco, California.

B. Materials and Reagents

Serum.- Ten to twenty ml. of blood was collected by venipuncture from each subject.* Following blood clotting at room temperature the serum was pipetted off and placed in sterile glass tubes. Samples were stored in the frozen state at -5°C. Before analysis they were thawed by a 5 to 10 minute immersion in a 37°C. water-bath.

Electrophoresis Buffer.- Veronal buffer of pH 8.6 and 0.075 molarity was prepared by dissolving 5.52 g. diethyl barbituric acid (N.F.-Merck) and 30.8 g. sodium diethyl barbiturate (N.F.-Fisher) in sufficient distilled water to make two liters of solution.

Protein Stain.- The dye bath was composed of 100 mg. bromphenol blue (Harleco No. 859) dissolved in sufficient absolute methanol (Mallinckrodt) to yield one liter of solution.

Acetic Acid 5 per cent (v/v) Rinse Solution.- This was obtained by diluting 100 ml. of glacial acetic acid (Baker) to two liters with distilled water.

Protein Analysis.- Reagents for the biuret method were prepared as directed by Ferro and Ham (31).

Standard protein solution (7.2 g./100 ml.) from human serum was obtained from Dade Reagents, Inc., Miami, Florida.

Biuret reagent was made by dissolving 3.0 g. crystalline cupric sulfate pentahydrate (C.P.-Mallinckrodt), 12.0 g. sodium potassium

---

*We are grateful to Dr. Gabriel Steiner at the Detroit Multiple Sclerosis Center for providing us with sera from his patients.

tartrate (C.P.-Baker) and 2.0 g. potassium iodide (C.P.-Mallinckrodt) in about one liter of distilled water. Six hundred ml. of 10 per cent (w/v) carbonate-free sodium hydroxide was added; the solution was then diluted to 2 liters with distilled water and stored in a polyethylene bottle.

Sodium sulfate-22.6 per cent (w/v) was prepared by dissolving 226 g. of the anhydrous chemical (C.P.-Mallinckrodt) in warm distilled water and adjusting the volume to one liter. The solution was kept at 37°C.

Cholesterol Analysis.- Total serum cholesterol was estimated with Kiliani's reagent according to the method of Shibata and Hasegawa (85).

Stock reagent was made by dissolving 10 g. ferric chloride-hexahydrate (C.P.-Cenco) in 100 ml. glacial acetic acid. Before use, 0.25 ml. was diluted with 25 ml. of concentrated sulfuric acid (C.P.-Baker).

Standard cholesterol solution contained 200 mg. recrystallized cholesterol (Matheson-5177) in 100 ml. glacial acetic acid.

Calcium Analysis.- Total serum calcium levels were quantitatively determined by the method of Bachra, et al (2).

Potassium hydroxide (1.25N) was prepared by appropriate dilution of a saturated solution of the chemical (Mallinckrodt).

A 0.012M stock solution of disodium ethylene-diamine tetraacetate was obtained by dissolving 4.5 g. Eastman No. 6354 salt in distilled water and diluting to one liter. Before use 5 ml. of stock was diluted to 100 ml. with water.

Cal-Red indicator was composed of 25 mg. 2-hydroxy-1-(2-hydroxy-4-sulfo-1-napthylazo)-3 napthoic acid (Scientific Service Laboratories, Dallas, Texas) dissolved in water to a volume of 25 ml. It was stored

in a polyethylene container and kept under refrigeration. Standard calcium solution (10.0 mg/100 ml.) prepared from human serum was secured from Dade Reagents, Miami, Florida.

Iron Analysis.- Bathophenanthroline reagent (0.001 M) was made by dissolving 332 mg. of 4,7 diphenyl-1-10-phenanthroline (G. F. Smith No. 108) in absolute isopropanol (Shell Corporation) and diluting to 1 liter with the same solvent.

Thioglycolic acid solution, 80 per cent (v/v) was obtained by dilution of 80 ml. of the chemical (Fisher-No. A319) with 20 ml. distilled water.

Sodium acetate, saturated. This reagent was prepared by adding 60 g. sodium acetate, trihydrate (Baker-C.P.) to 100 ml. water. After shaking and allowing to stand overnight the supernatant fluid was collected.

Hydrochloric acid, 0.2N. Dilution of 20 ml. 1 N hydrochloric acid with 80 ml. absolute isopropanol provided this reagent.

Trichloroacetic acid, 30 per cent (w/v) was obtained by adding sufficient distilled water to 30 g. of the Mallinckrodt A.R. chemical to yield 100 ml. of solution.

Iron standard (2 ug./ml.) was prepared by dilution of 10 ml. of stock iron standard (Harleco No. 2572) to 500 ml. with distilled water.

Total Serum Magnesium Analysis.- Trichloracetic acid, 10 per cent (w/v) was prepared by adding an amount of water to 10 g. of the Mallinckrodt (A.R.) chemical necessary to provide 100 ml. of solution.

Dilute magnesium standard consisted of 1 ml. of 0.1 N stock magnesium standard (Harleco No. 3633) and 10 ml. of 0.02 M calcium chloride

diluted to 100 ml. with 10 per cent trichloracetic acid. Titan yellow 0.05 per cent (w/v) was made by dissolving 0.05 g. (Harleco No. 1835) of the solid in sufficient distilled water to yeild 100 ml. of solution.

Sodium hydroxide, 2.5 N, was obtained by appropriate dilution and titration of a saturated solution.

Calcium Stain.- The stock solution for calcium staining of paper electrophoresis strips contained 1.0 g. Alizarin red-S (Fisher-Na-418) per liter of aqueous solution. The working stain was made by diluting 20 ml. stock with distilled water, adding 0.5 ml. 1 N hydrochloric acid and finally diluting to 1 liter with distilled water.

For elution 0.4 N sodium hydroxide was prepared from a carbonate-free saturated solution.

Magnesium-Zinc Stain.- A stock solution (0.5 per cent w/v) was made by dissolving 500 mg. 1-(1-hydroxy, 2-napthylazo)-5-nitro-2-napthol,-4-sulfonic acid, sodium salt (Eastman Kodak No. L6361) in sufficient distilled water to give one liter.

Elutions were accomplished with 0.4 N sodium hydroxide prepared as described in the calcium procedure above.

Copper Stain.- Alizarin Blue S (Harleco No. 112) served as the histochemical reagent for copper detection. A working solution was prepared fresh from 0.5 g. of dye dissolved in 500 ml. distilled water. One ml. of 1 N sodium hydroxide was added and the solution made up to 1 liter with water.

Acid for elution was obtained by dilution of 43 ml. concentrated hydrochloric acid to one liter with distilled water.

Miscellaneous Stains.- A saturated solution of dithizone (Eastmand Kodak No. 3092) in 95 per cent ethanol was used during preliminary studies for the location of copper and zinc. Pinkish-stained areas indicated the presence of zinc while pale brown appeared where copper was located.

Quinalizarin solution (Eastman Kodak No. 2787), 0.05 per cent (w/v) in 0.1 N sodium hydroxide was employed in the first attempts to locate magnesium. Dark blue areas on a light blue background appeared where magnesium was detected.

A 0.1 per cent (w/v) Dithio-oxamide (Eastman Kodak No. 4394) solution in 70 per cent (v/v) ethanol was utilized in the study of paper staining for copper. A pale greenish-black color on a white background indicated the location of copper.

C. Methods

Paper Electrophoresis and Staining.- Paper wicks were put into place and the strip holder, loaded with 8 paper strips, was assembled on the cell. Nine hundred ml. of veronal buffer were added to the cell. Another 100 ml. of buffer was used to wet the paper strips and wicks. The ends of each paper strip were gently pressed against the wick with a glass rod. The cell was covered and sealed around the edges with masking tape. Fifteen minutes was allowed for equilibration and then the buffer was brought to the same level in each electrode compartment by tilting the entire assembly to one end for 30 seconds. Six microliters of serum were placed on each paper strip with the aid of a wire capillary applicator. Access to the strips was gained through the top of the cell cover by removing, one at a time, individual masking tape

seals. Immediately upon completing sample application, current was applied. The electrophoretic separation proceeded for 16 hours under a constant current of 2.5 milliamperes and a difference of potential ranging from 60 to 70 volts. Runs were carried out at room temperature, which averaged from 20 to 22°C. At the end of a run the filter paper strips were immediately oven dried at 120 to 130°C. for 30 minutes.

After numbering, strips 3 and 6 were transferred to a staining rack and immersed in absolute methanol for 30 minutes to eliminate buffer salts. A 30 minute immersion in methanolic bromphenol blue followed by three 6-minute rinses in 5 per cent (v/v) acetic acid completed the staining process for location of proteins. The strips were briefly blotted and then dried at 120 to 130°C. for 15 minutes.

The dried strips were exposed for 15 minutes to vapors of concentrated ammonium hydroxide. A stable, bluish-purple color developed on a white background for the areas containing protein. Each strip was analyzed for zone densities by means of the Analytrol instrument (Recording Densitometer). Densitometric curves and integration of the areas under each were simultaneously obtained. Percentage values for each protein fraction were secured by computation of the integration data. Results of duplicate analyses were combined and averaged for each protein fraction. The values for serum protein distribution are recorded in Tables VI, VIII, and X.

Total Serum Protein Determination.- The Ferro and Ham (31) biuret technique for total serum protein was utilized. The procedure is based on the intensity of color developed between peptide linkages and alkaline copper sulfate. The determination involved photometric comparison

of the protein content of the unknown serum with known serum, which was previously standardized by Kjeldahl analysis.

Dilutions of unknown and standard sera were prepared by mixing 0.5 ml. of each with 9.5 ml. of 0.85 per cent (w/v) sodium chloride. Eight ml. of biuret reagent was added to the following: First, 2 ml. sodium chloride solution (blank); second, 2 ml. of unknown serum dilution; and third, 2 ml. of standard serum dilution. All tubes were mixed by several inversions. After 30 minutes the absorbancies at 540 mμ of the unknown and standard were obtained. The equation for the calculation of total serum protein content is given in Appendix I. The results of serum protein analyses are reported in Tables VII, IX, XI, and XVIII.

Serum Cholesterol Determinations.- The method of Shibata and Hasegawa (85) was followed for the estimation of total serum cholesterol. Duplicate 0.1 ml. serum samples were mixed with 8 ml. glacial acetic acid and centrifuged to obtain the protein-free supernatant fluid. Four ml. of clear supernatant were then mixed with 2.0 ml. Kiliani's working solution and allowed to stand for 15 minutes. A Cholesterol solution standardized by the method of Schoenheimer and Sperry (83) was treated in a similar manner. The absorbancies of unknown and standard at 550 mμ were obtained. A brown-violet color developed and its intensity followed Beer's law up to concentrations of 400 mg./100 ml. An equation for the calculation of total cholesterol appears in Appendix I. The results of the analyses are recorded in Table XXIV.

Total Serum Calcium Analysis.- The method of Bachra, et al (2) was followed for the determination of total serum calcium. Duplicate 0.5 ml.

serum samples were placed in separate 10 ml. Erlenmeyer flasks. Two drops of Cal-Red indicator and 0.5 ml. of 1.25 N potassium hydroxide were added immediately before titration. Samples were titrated with 0.0006 M disodium ethylene-diamine tetra acetate from a wine-red color to a blue end-point. Standard serum samples were determined in like manner. The method of calculation appears in Appendix I. The results of the analyses are given in Tables XII, XIII, XVII-A and XVIII.

Total Serum Iron Determination.- Iron determinations on serum were performed according to the method of Peters et al (72). Two ml. samples of unknown and iron standard (2 ug./ml.) were pipetted into separate 15 ml. centrifuge tubes. A blank containing 2 ml. of water was also prepared. Three ml. of 0.2 N hydrochloric acid and 1 drop of 80 per cent thioglycolic acid were added to each tube. The contents were mixed and the tubes were allowed to stand at room temperature for 30 minutes. One ml. of 30 per cent trichloracetic acid was added to each tube and the contents mixed. After standing at room temperature for fifteen minutes, the tubes were centrifuged at 2,000 r.p.m. for 10 minutes.

Four ml. aliquots of each supernatant were transferred to appropriately labeled cuvettes. Five-tenths ml. of saturated sodium acetate was added and the contents mixed. After adding 2 ml. of 0.001 M bathophenanthroline and mixing the contents of each tube, they were allowed to develop at room temperature for 10 minutes. The absorbancies were obtained at 535 mμ. An example of the calculation of results from these data may be found in Appendix I. The results are tabulated in Table XVIII.

Total Serum Magnesium Analysis.- A modification of the Natelson method (66) for serum magnesium was used.

Two ml. of serum, 2 ml. distilled water and 4 ml. 10 per cent (w/v) trichloracetic acid were mixed in a 15 ml. centrifuge tube and allowed to stand at room temperature for five minutes. Four ml. of the protein-free supernatant, obtained by centrifugation at 1500 r.p.m. for five minutes, was transferred to a cuvette. A standard was prepared with 2 ml. of dilute magnesium standard solution (2 meq/L) and 2 ml. of water. The blank consisted of 2 ml. water and 2 ml. 10 per cent trichloracetic acid. One ml. of 0.05 per cent (w/v) Titan Yellow was added to each tube. At 30 second intervals, 2 ml. of 2.5 N sodium hydroxide was added to each tube and the contents mixed. The absorbancies at 540 mμ were obtained exactly five minutes after the addition of sodium hydroxide. A sample calculation appears in Appendix I. The analytical results are entered in Table XVIII.

Paper Strip Analysis for Metal Ions.- In order to relate metal-ion distribution to the various protein fractions, a method was devised whereby the paper strips were sectioned into areas corresponding to the zone where a given protein fraction was previously identified. This was accomplished by aligning the stained or unstained strip for metal analysis with one of the protein-stained strips. Vertical sections of the strip for metal analysis were then taken; the width in centimeters and order taken for each was recorded. At the same time a section of paper was taken from an area of strip where no serum had been applied and utilized as a control (blank). From an eluate of the latter, a factor was obtained to correct for the amount of dye contributed by the

paper alone. Making use of the corrected absorbancies, relative percentages of dye distribution for each protein-metal area were calculated. The method of percentage computation is described in Appendix I.

Calcium.- The histochemical use of Alizarin Red-S was developed by Dahl (23) in 1952.

Strip No. 2 of each run was used for the location of calcium. The strips were pre-rinsed in absolute methanol for thirty minutes and stained in fresh-0.02 per cent (w/v) Alizarin Red-S for ten minutes. Excess dye was removed by rinsing in distilled water; the strips were blotted and dried at room temperature. Red-orange areas on a pale pinkish-white background appeared in those zones bearing calcium. Strip segments corresponding to protein zones were eluted with 5 ml. of 0.4 N sodium hydroxide for 20 minutes. After removal of the paper segments the absorbency of each eluate was measured at 550 mμ relative to a water blank. The results may be seen in Tables XII, XIII and XVII-A.

Iron.- To locate iron, strip No. 4 of each run was utilized. In this case the staining and elution was carried out in one step. Four ml. 0.2 N hydrochloric acid and one drop 80 per cent (v/v) thioglycolic acid were added to each strip-segment corresponding to protein zones. The contents of the tubes were mixed and allowed to stand at room temperature for sixty minutes after which 0.5 ml. of saturated sodium acetate and 1 ml. bathophenanthroline reagent were added. The tubes were mixed and again allowed to stand ten minutes. The paper sections were removed and the absorbancy at 535 mμ of the red ferrous-complex was recorded. A distilled water blank was used. These results are

recorded in Table XIV and XVII-B.

Magnesium and Zinc.- This method was developed to utilize the colorimetric reagent presented by Liddell and Williams (59). Strip number 7 was used for the location of both magnesium and zinc. Each strip was pre-rinsed in absolute methanol for 30 minutes and then immersed in fresh 0.01 per cent (w/v) dye solution for ten minutes. The excess dye was drained off and the paper strip blotted and dried. A pink-red stain on a white background appeared where magnesium and/or zinc was present. Elution of stained segments was accomplished by immersion in 5 ml. of 0.4 N sodium hydroxide for 20 minutes. The paper sections were removed and the absorbancy at 515 mμ was obtained. These findings are tabulated in Tables XV and XVII-C.

Copper.- The dye used by Markowitz, et al (63) was employed for copper detection on paper electrophoretograms.

Paper strip No. 5 was utilized in the location of copper. Immersion of the strips in methanol for ten minutes preceded the staining and then they were submerged in Alizarin Blue-S for ten minutes. Excess dye was removed by rinsing in distilled water. After blotting, the strips were dried at 90°C. for ten minutes. Dark blue areas on a light blue background appeared only where copper was most concentrated. Strip segments were each treated with 5 ml. of 0.5 N hydrochloric acid and allowed to stand at room temperature for twenty minutes. After the paper segments were removed the absorbancy at 500 mμ of each eluate was obtained. Hydrochloric acid solution (0.5 N) was used as a reagent blank. The results of these analyses are entered in Tables XVI and XVII-D.

D. Dialysis Experiments

Pooled Serum Study.- Thirty-four ml. of pooled normal human sera were divided in two equal portions. One portion (17 ml.) was dialyzed against four liters of 0.075 M veronal buffer at pH 8.6 and with sodium chloride added to make the total ionic strength 0.15 M. The other portion was stored in the refrigerator until the dialysis was completed. Dialysis continued at 0 to 5°C. for 52 hours, with one change of fresh buffer after 26 hours. Quantitative analyses by the aforementioned methods were performed upon both the non-dialyzed and dialyzed samples for total protein, calcium, iron and magnesium. Paper electrophoretic analyses and histochemical staining of each portion gave the distribution of protein, calcium, iron, magnesium-zinc and copper. These results appear in Tables XVIII, XIX, XX, XXI, XXII, and XXIII.

Individual Control Serum Study.- One ml. of serum from each of six control subjects was placed in separate dialysis membranes. These were dialyzed against four liters of 0.075 M veronal buffer at pH 8.6 with sodium chloride added to make a total ionic strength of 0.15 M. Dialysis was allowed to proceed for 18 hours at 0 to 5°C. Paper electrophoretic analysis of each dialyzed serum was performed. Individual paper electrophoresis strips were stained for protein, calcium, iron, magnesium-zinc and copper. The resulting data are recorded in Tables XIX, XX, XXI, XXII, and XXIII.

TABLE II

SERUM PROTEIN DISTRIBUTION BEFORE AND AFTER DEEP-FREEZE STORAGE[1]

Subject	Albumin[2]		Globulins[2]							
			Alpha-1		Alpha-2		Beta		Gamma	
	Before	After	Before	After	Before	After	Before	After	Before	After
3	69.0	65.7	3.1	4.0	7.5	9.2	8.2	10.0	12.2	11.1
5	67.9	63.5	2.8	1.7	6.7	9.6	9.8	10.9	13.7	14.3
7	67.8	63.0	2.6	2.4	5.4	7.9	10.2	10.2	14.0	16.5
8	70.6	63.2	3.3	4.0	5.4	6.8	9.1	10.0	11.6	16.0
17	66.2	68.3	3.5	3.5	6.7	6.6	8.3	9.0	15.3	12.6
20	72.9	68.5	2.3	2.5	4.6	6.1	9.4	9.0	10.8	13.9
Mean	69.0	65.4	2.9	3.0	6.1	7.7	9.0	9.9	13.0	14.1

[1]Stored at minus 18°C. for 2 to 3 months.

[2]Determined by paper electrophoresis and given as percent of total.

TABLE III

VARIOUS PAPER ELECTROPHORETIC ANALYSES OF NORMAL HUMAN SERUM PROTEINS[1]

Reference	Albumin	Globulins			
		Alpha-1	Alpha-2	Beta	Gamma
(28)	63.7	3.7	10.0	10.5	12.0
(29)	67.0	3.3	6.9	9.1	13.5
(46)	68.9	2.7	7.3	9.0	12.0
(58)	60.2	3.7	8.3	12.8	15.0
This work	66.4	3.4	6.4	9.6	14.2

[1]Given as per cent of total protein (mean value).

TABLE IV

AGE AND SEX OF NORMAL SUBJECTS AND MULTIPLE SCLEROSIS PATIENTS

Subjects			Patients		
	Age	Sex		Age	Sex
1	38	Female	A	47	Female
2	19	Female	B	51	Female
3	30	Female	C	54	Female
4	48	Female	D	36	Female
5	20	Female	E	43	Female
6	41	Female	F	36	Female
7	39	Female	G	35	Female
8	37	Female	H	33	Female
9	43	Female	I	34	Female
10	21	Female	J	42	Female
11	19	Female	K	37	Female
12	23	Female	L	29	Female
13	19	Female	M	44	Female
14	20	Female	N	50	Female
15	21	Female	O	45	Female
16	18	Female	P	33	Male
17	20	Female	Q	38	Male
18	28	Male	R	47	Male
19	32	Male	S	34	Male
20	32	Male	T	33	Male
21	26	Male	U	36	Male
22	29	Male	V	--	Male
23	24	Male			
24	27	Male			
25	21	Male			
26	25	Male			

TABLE V

EFFECT OF AGE AND SEX UPON SERUM PROTEIN DISTRIBUTION AMONG NORMAL SUBJECTS AND MULTIPLE SCLEROSIS PATIENTS[1]

Group	Albumin	Globulins			
		Alpha-1	Alpha-2	Beta	Gamma
Normals					
Males	66.4	3.4	6.1	10.2	13.9
Females	66.4	3.4	6.6	9.2	14.4
Ages 18-21	66.0	3.6	6.9	9.4	14.1
Ages 23-30	65.4	3.6	6.5	9.9	14.5
Ages 32-48	67.7	3.0	5.8	9.4	14.1
M. S. Males	66.6	2.9	7.6	9.0	13.8
M. S. Females	68.9	2.8	6.3	9.1	12.9

[1]Given as Percent of Total Protein.

TABLE VI

PROTEIN DISTRIBUTION OF NORMAL SERA BY PAPER ELECTROPHORESIS[1]

Subject	Albumin	Globulins			
		Alpha-1	Alpha-2	Beta	Gamma
1	65.8	3.3	5.9	8.0	17.0
2	70.4	3.6	6.1	9.7	10.2
3	69.0	3.1	7.5	8.2	12.2
4	64.7	2.8	6.5	11.1	14.9
5	67.9	2.8	6.7	8.9	13.7
6	69.2	3.1	5.3	6.7	15.7
7	67.8	2.6	5.4	10.2	14.0
8	70.6	3.3	5.4	9.1	11.6
9	65.4	3.2	7.1	9.8	14.5
10	61.0	3.4	7.5	9.6	18.5
11	63.2	3.5	5.8	9.7	17.8
12	65.7	4.1	7.2	8.5	14.5
13	67.0	4.0	6.4	8.9	13.7
14	68.4	3.3	7.3	8.1	12.9
15	65.1	3.1	7.7	10.9	13.2
16	65.2	3.8	7.3	9.9	13.7
17	66.2	3.5	6.7	8.3	15.3
18	63.4	5.0	6.5	9.9	15.3
19	65.5	3.8	6.2	10.6	13.9
20	72.9	2.3	4.6	9.4	10.8
21	65.6	2.9	7.2	10.2	14.1
22	66.7	2.9	6.7	9.1	14.6
23	66.3	3.7	5.6	12.4	12.0
24	65.5	3.8	6.2	10.9	13.6
25	65.5	4.7	7.0	9.9	12.9
26	65.2	2.5	5.1	9.3	17.8
Range	61.0-72.9	2.3-4.0	4.6-7.8	6.7-12.4	10.2-17.8
$\bar{X}$	66.4	3.4	6.4	9.6	14.2
S	2.4	0.6	0.9	1.2	2.0
$\bar{X}\pm S$	64.0-68.8	2.8-4.0	5.5-7.3	8.4-10.8	12.2-16.2

[1]Given as per cent of total protein.

TABLE VII

PROTEIN DISTRIBUTION (g./100 ml.) OF NORMAL SERA[1]

Subject	Total[2]	Albumin	Globulins			
			Alpha-1	Alpha-2	Beta	Gamma
1	6.9	4.54	0.23	0.41	0.55	1.17
2	7.0	4.93	0.25	0.43	0.68	0.72
3	7.3	4.75	0.26	0.57	0.68	1.04
4	7.1	4.60	0.20	0.46	0.79	1.06
5	7.0	4.75	0.20	0.47	0.62	0.96
6	7.2	4.91	0.22	0.38	0.48	1.13
7.	6.9	4.67	0.18	0.37	0.70	0.97
8	6.8	4.81	0.22	0.37	0.62	0.79
9	7.1	4.64	0.23	0.50	0.70	1.03
10	7.4	4.51	0.25	0.55	0.71	1.37
11	7.0	4.43	0.24	0.41	0.68	1.25
12	6.8	4.48	0.28	0.49	0.58	0.99
13	7.0	4.69	0.28	0.45	0.62	0.96
14	7.8	5.33	0.26	0.57	0.63	1.01
15	7.2	4.69	0.22	0.55	0.79	0.95
16	7.2	4.70	0.27	0.53	0.71	0.99
17	7.4	4.90	0.26	0.50	0.61	1.13
18	7.1	4.51	0.36	0.46	0.70	1.09
19	8.1	5.30	0.31	0.50	0.86	1.13
20	8.6	6.26	0.20	0.40	0.80	0.93
21	7.9	5.18	0.23	0.57	0.81	1.11
22	7.0	4.67	0.20	0.47	0.64	1.02
23	7.4	4.91	0.27	0.41	0.92	0.89
24	8.0	5.23	0.30	0.50	0.87	1.09
25	7.6	4.97	0.36	0.53	0.75	0.98
26	8.1	5.28	0.20	0.41	0.75	1.44
Range	6.8-8.6	4.43-6.26	0.18-0.36	0.37-0.57	0.48-0.92	0.72-1.44
$\bar{X}$	7.3	4.84	0.25	0.47	0.70	1.04
S	0.5	0.43	0.05	0.07	0.11	0.16
$\bar{X}\pm S$	6.8-7.8	4.41-5.27	0.20-0.30	0.40-0.54	0.59-0.81	0.88-1.20

[1]Calculated from total protein analysis (column 2 above) and electrophoresis data (Table VI).

[2]Determined by biuret analysis (31).

TABLE VIII

PROTEIN DISTRIBUTION OF MULTIPLE SCLEROSIS SERA BY PAPER ELECTROPHORESIS[1]

Patient	Albumin	Globulins			
		Alpha-1	Alpha-2	Beta	Gamma
A	72.4	2.7	6.0	7.6	11.3
B	65.4	3.5	6.6	10.2	14.3
C	73.2	2.3	4.5	7.5	12.5
D	68.8	1.8	4.5	6.6	18.3
E	73.5	2.9	5.4	6.9	11.3
F	71.7	3.1	6.0	6.7	12.5
G	68.8	2.8	6.0	6.7	15.7
H	80.5	1.0	4.2	6.1	8.2
I	62.4	2.5	7.2	13.6	14.3
J	64.7	3.8	8.4	10.4	12.7
K	63.2	3.0	7.4	11.7	14.7
L	67.8	3.9	5.2	10.9	12.2
M	62.9	3.3	9.5	10.9	13.4
N	71.3	2.8	5.3	8.7	11.9
O	68.8	2.8	8.8	8.8	10.8
P	72.1	2.1	5.8	8.8	11.2
Q	77.0	2.4	5.2	5.7	9.7
R	61.4	4.7	8.9	12.0	13.0
S	61.2	2.6	9.9	11.2	15.1
T	57.1	3.8	12.2	10.3	16.6
U	60.8	2.8	9.2	9.9	17.3
V	65.8	2.0	6.6	7.8	17.8
Range	57.1-80.5	1.0-4.7	4.2-12.2	5.7-13.6	8.2-18.3
$\bar{X}$	67.7	2.9	7.0	9.0	13.4
S	5.8	0.8	2.0	2.1	2.6
$\bar{X}\pm S$	61.9-73.5	2.1-3.7	5.0-9.0	6.9-11.1	10.8-16.0

[1]Given as per cent of total protein.

TABLE IX

PROTEIN DISTRIBUTION (g./100 ml.) OF MULTIPLE SCLEROSIS SERA[1]

Patient	Total[2]	Albumin	Globulins			
			Alpha-1	Alpha-2	Beta	Gamma
A	7.0	5.07	0.19	0.42	0.53	0.79
B	7.6	4.96	0.27	0.50	0.78	1.09
C	7.3	5.35	0.17	0.33	0.55	0.91
D	8.1	5.57	0.15	0.36	0.53	1.48
E	6.6	4.85	0.19	0.36	0.46	0.74
G	7.3	5.04	0.20	0.44	0.49	1.15
H	7.0	5.64	0.07	0.29	0.43	0.57
I	8.5	5.30	0.21	0.61	1.15	1.21
J	7.5	4.85	0.28	0.63	0.78	0.95
K	7.6	4.81	0.23	0.56	0.89	1.12
L	6.8	4.62	0.27	0.35	0.74	0.83
M	7.2	4.53	0.24	0.68	0.78	0.97
N	6.8	4.84	0.19	0.36	0.59	0.74
O	7.6	5.23	0.21	0.67	0.67	0.82
P	7.3	5.26	0.15	0.42	0.64	0.82
Q	7.5	5.77	0.18	0.39	0.44	0.73
R	7.0	4.29	0.33	0.62	0.84	0.91
S	8.6	5.27	0.22	0.85	0.96	1.30
T	8.6	4.92	0.33	1.05	0.89	1.43
U	7.6	4.63	0.21	0.70	0.75	1.32
V	8.5	5.68	0.17	0.56	0.66	1.51
Range	6.6-8.6	4.29-5.77	0.07-0.33	0.29-1.05	0.43-1.15	0.57-1.51
$\bar{X}$	7.5	5.09	0.22	0.52	0.68	1.00
S	0.6	0.35	0.06	0.19	0.18	0.27
$\bar{X}\pm S$	6.9-8.1	4.74-5.44	0.16-0.28	0.33-0.71	0.50-0.86	0.73-1.27

[1]Calculated from total protein analysis (column 2 above) and electrophoresis data (Table VIII).

[2]Determined by biuret analysis (31).

TABLE X

PROTEIN DISTRIBUTION OF VARIOUS PATHOLOGICAL SERA BY PAPER ELECTROPHORESIS[1]

Patient	Albumin	Globulins			
		Alpha-1	Alpha-2	Beta	Gamma
1. Multiple myeloma(M.M.)	29.2	1.8	3.2	4.9	60.9
2. Multiple myeloma(M.M.)	19.4	3.0	5.0	4.6	68.0
3. Multiple myeloma(M.M.)	4.4	1.7	2.0	3.4	88.5
4. Liver cirrhosis(L.C.)	52.5	4.3	5.0	8.5	29.7
5. Severe burns(S.B.)	49.2	9.6	17.0	11.4	12.8
6. Glomerulonephritis(G.N.)	61.6	4.3	11.7	8.4	14.0
7. Mean Control Value(M.C.V.)	66.4	3.4	6.4	9.6	14.2

[1]Given as per cent of total protein.

TABLE XI

PROTEIN DISTRIBUTION (g./100 ml.) OF VARIOUS PATHOLOGICAL SERA[1]

Patient	Total[2]	Albumin	Globulins			
			Alpha-1	Alpha-2	Beta	Gamma
1. M.M.	9.7	2.83	0.17	0.31	0.48	5.91
2. M.M.	8.8	1.70	0.26	0.44	0.40	6.00
3. M.M.	17.8	0.79	0.30	0.36	0.60	15.76
4. L.C.	6.0	3.15	0.26	0.30	0.50	1.79
5. G.N.	5.1	3.14	0.22	0.60	0.43	0.71
6. M.C.V.	7.3	4.84	0.25	0.47	0.70	1.04

[1]Calculated from total protein analysis (column 2 above) and electrophoresis data (Table X).

[2]Determined by biuret analysis (31).

TABLE XII

CALCIUM DISTRIBUTION OF NORMAL SERA BY PAPER ELECTROPHORESIS

Subject	Total[1]	Albumin[2]	Globulins[2]			
			Alpha-1	Alpha-2	Beta	Gamma
1	9.4	52.4	7.9	9.6	11.4	18.7
2	10.3	58.6	6.5	13.8	11.7	9.4
3	9.9	56.4	5.9	12.7	10.5	14.5
4	10.1	61.5	5.8	10.1	11.0	11.8
5	9.5	49.2	7.4	10.8	15.2	17.6
6	10.2	50.6	8.5	8.9	14.2	17.7
7	9.0	50.9	7.4	9.2	13.7	18.9
8	9.4	54.7	8.0	9.6	13.0	14.8
9	9.4	45.2	9.8	10.2	11.6	23.2
10	9.9	50.8	7.7	12.1	12.9	16.5
11	10.1	50.7	7.8	10.0	10.0	21.5
12	9.6	48.7	7.2	11.8	13.5	18.8
13	9.9	60.3	9.7	13.8	11.1	5.1
14	10.9	49.7	11.3	11.8	14.6	12.6
15	9.8	47.9	10.7	14.6	14.6	12.2
16	10.3	45.7	15.3	15.8	15.8	7.4
17	9.7	45.4	9.4	12.2	17.4	15.6
18	9.8	50.1	10.9	14.6	13.7	10.7
19	10.9	45.5	14.0	12.5	14.9	13.1
20	10.0	52.0	9.0	13.5	14.6	10.9
21	10.4	45.6	11.3	17.9	14.6	10.6
22	9.6	42.1	13.4	16.2	16.9	11.4
23	10.0	45.7	13.3	12.3	17.5	11.2
24	9.7	48.5	14.6	13.6	15.3	8.0
25	10.1	46.1	13.8	13.8	14.5	11.8
26	9.5	40.4	13.2	13.2	21.7	11.1
Range	9.0-10.9	40.4-61.5	5.8-15.3	9.2-17.9	10.0-21.7	5.1-23.2
$\bar{X}$	9.9	49.8	10.0	12.5	14.0	13.7
S	0.5	5.1	2.8	2.2	2.5	4.4
$\bar{X}\pm S$	9.4-10.4	44.7-54.9	7.2-12.8	10.3-14.7	11.5-16.5	9.3-18.1

[1]Determined by the chelatometric method(2); given in mg./100 ml. serum.

[2]Given as per cent of total calcium on paper electrophoretogram.

TABLE XIII

CALCIUM DISTRIBUTION OF MULTIPLE SCLEROSIS SERA
BY PAPER ELECTROPHORESIS

Patient	Total[1]	Albumin[2]	Globulins[2]			
			Alpha-1	Alpha-2	Beta	Gamma
A	10.2	51.0	8.8	11.3	12.2	16.6
B	11.7	54.2	8.1	10.0	11.2	16.6
C	9.3	50.5	9.5	7.9	13.9	18.3
D	8.3	46.5	9.7	9.9	11.6	22.2
E	10.6	44.4	8.4	10.2	16.2	20.2
F	11.8	46.7	7.8	15.1	13.6	16.8
G	6.4	47.3	9.5	9.8	13.4	19.9
H	6.7	55.6	5.4	12.0	13.0	14.0
I	--	37.0	10.0	14.9	18.1	20.0
J	--	37.2	10.1	17.6	18.2	16.9
K	10.7	44.2	9.3	11.0	19.1	16.4
L	8.0	46.8	10.0	13.2	13.6	16.3
M	8.0	53.5	10.1	12.0	13.4	11.1
N	9.7	59.2	8.4	9.8	11.1	11.5
O	--	54.3	7.5	13.6	12.0	12.6
P	9.9	57.1	7.5	9.6	13.2	12.5
Q	6.7	50.7	9.7	12.0	10.9	16.7
R	8.8	53.5	11.0	10.7	12.4	12.0
S	12.6	42.2	10.8	14.9	15.1	17.1
T	11.2	46.8	10.0	13.1	13.0	17.1
U	--	48.7	8.5	14.3	12.9	15.7
V	11.3	43.0	8.5	17.0	12.1	19.4
Range	6.4-12.6	37.2-59.2	5.4-11.0	7.9-17.6	10.9-19.1	11.1-22.2
$\bar{X}$	9.6	48.7	9.0	12.3	13.6	16.3
S	1.8	5.4	1.3	2.5	2.3	3.0
$\bar{X}\pm S$	7.8-11.4	43.3-54.1	7.7-10.3	9.8-14.8	11.3-15.9	13.3-19.3

[1]Determined by the chelatometric method (2); given in mg/100 ml. serum.

[2]Given as per cent of total calcium on paper electrophoetogram.

TABLE XIV

INDIVIDUALS IN VARIOUS RANGES OF IRON CONTENT AMONG THE SERUM PROTEIN FRACTIONS[1]

A. For Sera of 26 Normal Subjects[2]

Range[3]	Albumin	Globulins			
		Alpha-1	Alpha-2	Beta	Gamma
0 - 10	20	2	3	7	22
11 - 20	3	3	5	8	2
21 - 30	3	3	7	6	1
31 - 40	0	7	7	3	0
41 - 50	0	5	1	1	0
51 - 60	0	4	1	0	1
Over 60	0	2	2	1	0
Mean %	6.5	37.5	31.0	20.0	5.0

B. For Sera of 22 Multiple Sclerosis Patients[2]

0 - 10	16	4	6	5	22
11 - 20	3	3	6	5	0
21 - 30	2	2	2	4	0
31 - 40	0	3	2	2	0
41 - 50	0	5	2	2	0
51 - 60	0	4	1	1	0
Over 60	1	1	3	3	0
Mean %	8.0	39.0	28.0	25.0	0.0

[1]e.g., 20 out of 26 subjects showed an iron content for their serum albumin fraction to be in the 0 - 10% range.

[2]Given as the number of individuals in each range.

[3]Range is given as per cent of total iron on the paper electrophoretogram.

TABLE XV

INDIVIDUALS IN VARIOUS RANGES OF MAGNESIUM-ZINC CONTENT AMONG THE SERUM PROTEIN FRACTIONS[1]

A. For Sera of 26 Normal Subjects[2]

Range[3]	Albumin	Globulins			
		Alpha-1	Alpha-2	Beta	Gamma
0 - 10	7	1	0	1	2
11 - 20	8	17	14	9	11
21 - 30	10	6	11	15	8
31 - 40	1	2	1	1	4
41 - 50	0	0	0	0	1
Over 50	0	0	0	0	0
Mean %	18.0	18.0	21.0	21.0	22.0

B. For Sera of 22 Multiple Sclerosis Patients[2]

0 - 10	4	0	0	0	0
11 - 20	9	14	10	3	6
21 - 30	4	3	7	14	7
Over 30	0	0	0	0	0
Mean %	15.0	18.0	20.0	24.0	23.0

[1]e.g., 7 out of 26 subjects showed a magnesium-zinc content for their serum albumin fraction to be in the 0 - 10 % range.

[2]Given as the number of individuals in each range.

[3]Range is given as per cent of total magnesium plus zinc on the paper electrophoretogram.

TABLE XVI

INDIVIDUALS IN VARIOUS RANGES OF COPPER CONTENT AMONG THE SERUM PROTEIN FRACTIONS[1]

A. For Sera of 26 Normal Subjects[2]

Range[3]	Albumin	Globulins			
		Alpha-1	Alpha-2	Beta	Gamma
0 - 10	0	9	12	5	9
11 - 20	1	15	9	8	12
21 - 30	5	2	4	8	2
31 - 40	8	0	1	4	2
41 - 50	6	0	0	1	1
51 - 60	2	0	0	0	0
Over 60	4	0	0	0	0
Mean %	42.0	12.0	11.0	20.0	15.0

B. For Sera of 22 Multiple Sclerosis Patients[2]

0 - 10[4]	6	11	8	9	15
11 - 20	0	3	7	3	3
21 - 30	4	4	2	4	0
31 - 40	3	2	2	2	1
41 - 50	3	0	0	1	1
51 - 60	0	0	0	0	0
Over 60	4	0	1	1	0
Mean %	38.0	14.0	20.0	20.0	8.0

[1]e.g., none of the 26 subjects showed a copper content for their serum albumin fraction to be in the 0 - 10 % range.

[2]Given as the number of individuals in each range.

[3]Range is given as per cent of total copper on the paper electrophoretogram.

[4]Three patients had no detectable copper in any fraction.

TABLE XVII

METAL DISTRIBUTION AMONG SERUM PROTEINS OF VARIOUS PATHOLOGICAL SERA FROM PAPER ELECTROPHORETOGRAMS

A. Calcium[1]

Patient[2]	Albumin	Globulins			
		Alpha-1	Alpha-2	Beta	Gamma
1. M.M.	28.6	5.4	9.7	9.6	46.7
2. M.M.	23.3	6.6	10.7	10.0	49.4
3. M.M.	9.0	9.9	5.7	6.4	69.0
4. L.C.	31.9	6.9	12.8	16.2	32.2
5. S.B.	51.0	10.8	20.2	10.1	7.9
6. G.N.	55.2	7.8	13.1	11.8	12.1
7. M.C.V.	49.8	10.0	12.5	14.0	13.7

B. Iron[1]

1. M.M.	15.3	30.6	25.8	23.6	4.7
2. M.M.	15.4-	33.4	20.4	30.8	0.0
3. M.M.	8.4	27.7	27.7	19.3	16.9
4. L.C.	12.3	32.3	20.0	15.4	20.0
5. S.B.	18.1	25.3	10.9	27.6	18.1
6. G.N.	10.7	46.5	17.8	21.4	3.6
7. M.C.V.	6.5	37.5	31.0	20.0	5.0

C. Magnesium-Zinc[1]

1. M.M.	28.0	10.0	7.0	10.0	45.0
2. M.M.	25.5	5.7	9.3	10.5	49.0
3. M.M.	12.5	0	0	12.5	75.0
4. L.C.	18.5	16.7	18.5	9.3	37.0
5. S.B.	27.7	17.0	24.1	16.3	14.9
6. G.N.	28.8	15.6	21.4	17.5	16.7
7. M.C.V.	18.0	18.0	21.0	21.0	22.0

[1]Given as per cent of total metal upon the paper electrophoretogram.

[2]The abbreviations represent the patients described in Table X.

TABLE XVII (Cont.)

D. Copper[1]

Patient[2]	Albumin	Globulins			
		Alpha-1	Alpha-2	Beta	Gamma
1. M.M.	0	31.4	27.4	41.2	0
2. M.M.	23.5	29.5	23.5	23.5	0
3. M.M.	0	37.6	24.8	37.6	0
4. L.C.	0	75.6	12.2	12.2	0
5. S.B.	28.0	9.4	40.0	22.6	0
6. G.N.	0	0	0	0	0
7. M.C.V.	42.0	12.0	11.0	20.0	15.0

[1]Given as per cent of total metal upon the paper electrophoretogram.

[2]The abbreviations represent the patients described in Table X.

TABLE XVIII

ANALYTICAL RESULTS OF POOLED SERA DIALYSIS STUDY

	Before	After
Total Protein (g./100 ml.)	6.10	5.30
Calcium (meq./L.)	4.40	0.00
Iron (meq./L.)	0.067	0.056
Magnesium (meq./L.)	2.58	0.00

TABLE XIX

PROTEIN DISTRIBUTION OF NORMAL SERA BEFORE AND AFTER DIALYSIS[1]

Subject	Albumin[2]		Globulins[2]							
			Alpha-1		Alpha-2		Beta		Gamma	
	Before	After	Before	After	Before	After	Before	After	Before	After
Pool	61.2	64.8	4.2	4.3	9.1	8.3	12.6	9.2	12.9	13.4
3	69.0	69.4	3.1	3.3	7.5	6.9	8.2	9.0	12.2	11.4
5	67.9	67.7	2.8	2.7	6.7	6.9	8.9	9.7	13.7	13.0
7	67.8	67.8	2.6	2.4	5.4	7.0	10.2	7.8	14.0	15.0
8	70.6	70.5	3.3	2.5	5.4	5.5	9.1	8.6	11.6	12.9
17	66.2	70.5	3.5	2.8	6.7	5.7	8.3	7.9	15.3	13.1
20	72.9	72.6	2.3	2.2	4.1	5.4	9.4	8.6	10.8	11.2
Mean	65.2	67.2	3.5	3.5	7.6	7.3	10.8	8.9	12.9	13.1

[1]Dialysis time varied from 28-56 hours.

[2]Given as per cent of total protein.

TABLE XX

ABSORBANCIES OF CALCIUM-DYE ELUATES FROM SECTIONS OF PAPER ELECTROPHORETOGRAMS BEFORE AND AFTER SERUM DIALYSIS[1]

Subject		Albumin	Globulins			
			Alpha-1	Alpha-2	Beta	Gamma
Pool	Before	0.140	0.017	0.027	0.031	0.039
	After	0.087	0.011	0.020	0.024	0.031
3	Before	0.280	0.029	0.063	0.052	0.072
	After	0.122	0.021	0.023	0.018	0.015
5	Before	0.187	0.028	0.040	0.058	0.067
	After	0.145	0.019	0.026	0.036	0.033
7	Before	0.265	0.037	0.046	0.069	0.095
	After	0.102	0.014	0.024	0.025	0.028
8	Before	0.273	0.040	0.048	0.065	0.074
	After	0.170	0.024	0.024	0.032	0.030
17	Before	0.174	0.036	0.047	0.067	0.060
	After	0.231	0.037	0.037	0.042	0.054
20	Before	0.242	0.042	0.063	0.068	0.050
	After	0.244	0.039	0.047	0.049	0.035
Mean	Before	0.222	0.033	0.044	0.059	0.065
	After	0.157	0.023	0.029	0.032	0.032
Difference of means[2]		0.050	0.008	0.012	0.024	0.030
Mean % Calcium[3]		40.3	6.4	9.7	19.4	24.2

[1]Wave length 550 mμ.

[2]Corrected for dilution.

[3]Calculated from difference of means.

TABLE XXI

ABSORBANCIES OF IRON-DYE ELUATES FROM SECTIONS OF PAPER ELECTROPHORETOGRAMS BEFORE AND AFTER SERUM DIALYSIS[1]

Subject		Albumin	Globulins			
			Alpha-1	Alpha-2	Beta	Gamma
Pool	Before	0.017	0.054	0.051	0.033	0.019
	After	0.009	0.029	0.030	0.023	0.013
3	Before	0.027	0.014	0.025	0.036	0.000
	After	0.000	0.021	0.009	0.005	0.000
5	Before	0.000	0.022	0.014	0.018	0.001
	After	0.007	0.025	0.015	0.024	0.010
7	Before	0.000	0.043	0.037	0.028	0.006
	After	0.007	0.030	0.008	0.007	0.000
8	Before	0.007	0.016	0.002	0.000	0.000
	After	0.013	0.033	0.020	0.025	0.010
17	Before	0.000	0.002	0.000	0.052	0.000
	After	0.010	0.021	0.024	0.022	0.016
20	Before	0.032	0.070	0.043	0.047	0.020
	After	0.017	0.023	0.021	0.019	0.000
Mean	Before	0.012	0.031	0.025	0.031	0.007
	After	0.010	0.026	0.018	0.018	0.007

[1]Wavelength 535 mμ.

TABLE XXII

ABSORBANCIES OF MAGNESIUM-ZINC-DYE ELUATES FROM SECTIONS OF PAPER ELECTROPHORETOGRAMS BEFORE AND AFTER SERUM DIALYSIS[1]

Subject		Albumin	Globulins			
			Alpha-1	Alpha-2	Beta	Gamma
Pool	Before	0.051	0.018	0.034	0.040	0.074
	After	0.057	0.022	0.047	0.044	0.080
3	Before	0.033	0.023	0.027	0.032	0.018
	After	0.074	0.039	0.045	0.049	0.044
5	Before	0.072	0.038	0.054	0.043	0.048
	After	0.078	0.050	0.056	0.049	0.053
7	Before	0.030	0.030	0.044	0.036	0.034
	After	0.073	0.016	0.021	0.039	0.032
8	Before	0.017	0.022	0.036	0.036	0.035
	After	0.043	0.016	0.017	0.030	0.021
17	Before	0.010	0.009	0.016	0.009	0.005
	After	0.090	0.030	0.042	0.050	0.058
20	Before	0.007	0.018	0.025	0.023	0.041
	After	0.096	0.027	0.030	0.052	0.042
Mean	Before	0.031	0.023	0.034	0.031	0.036
	After	0.073	0.029	0.037	0.045	0.039

[1]Wavelength 515 mμ.

TABLE XXIII

ABSORBANCIES OF COPPER-DYE ELUATES FROM SECTIONS OF PAPER ELECTROPHORETOGRAMS BEFORE AND AFTER SERUM DIALYSIS[1]

Subject		Albumin	Globulins			
			Alpha-1	Alpha-2	Beta	Gamma
Pool	Before	0.046	0.022	0.032	0.022	0.012
	After	0.036	0.022	0.023	0.020	0.011
3	Before	0.036	0.003	0.013	0.007	0.007
	After	0.012	0.002	0.002	0.000	0.000
5	Before	0.013	0.000	0.001	0.013	0.011
	After	0.020	0.000	0.006	0.010	0.000
7	Before	0.013	0.000	0.000	0.000	0.005
	After	0.000	0.000	0.002	0.000	0.000
8	Before	0.042	0.017	0.016	0.026	0.024
	After	0.043	0.005	0.016	0.010	0.003
17	Before	0.004	0.001	0.000	0.004	0.000
	After	0.012	0.000	0.019	0.011	0.000
20	Before	0.012	0.008	0.003	0.013	0.003
	After	0.034	0.010	0.017	0.023	0.002
Mean	Before	0.024	0.007	0.009	0.012	0.009
	After	0.028	0.008	0.015	0.013	0.003

[1]Wavelength 500 mμ.

TABLE XXIV

TOTAL SERUM CHOLESTEROL LEVELS[1]

Normal Subjects		Patients	
1	160	A	350
2	150	B	260
3	190	C	270
4	250	D	260
5	185	E	240
6	165	F	250
7	170	G	250
8	185	H	235
9	205	I	255
10	195	J	275
11	170	K	310
12	215	L	225
13	265	M	350
14	210	N	300
15	195	P	230
16	195	Q	205
17	200	R	195
18	195	S	260
19	285	T	340
20	155	V	215
21	200		
22	225		
23	208		
24	240		
25	228		
26	258		
Range	150 - 285		195 - 350
$\bar{X}$	204		246
S	33		48

[1]Given as mg./100 ml. serum.

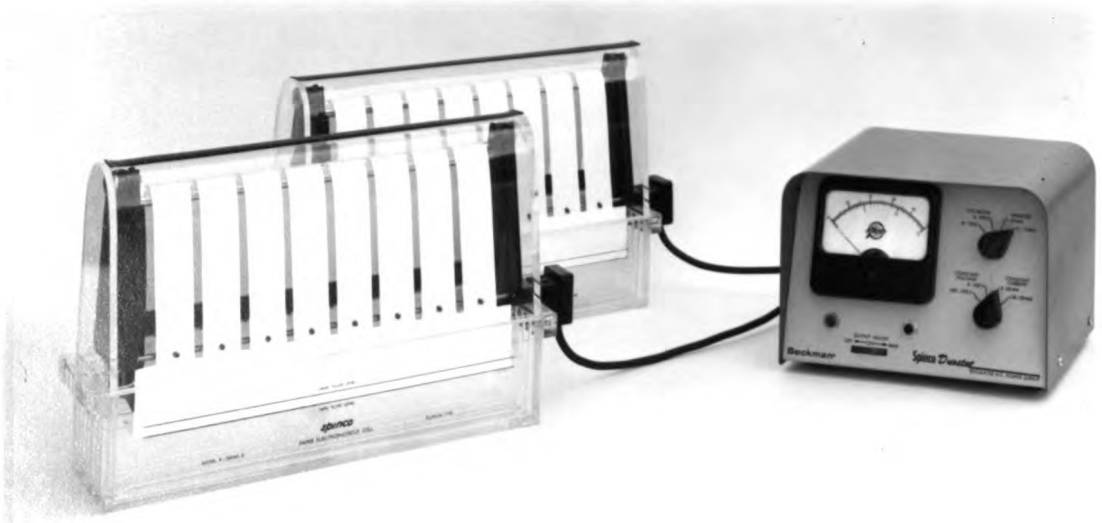

Beckman
Spinco Duostar

FIGURE 2

DENSITOMETRIC TRACING OF A PROTEIN ELECTROPHORETOGRAM OF SERUM FROM A NORMAL SUBJECT

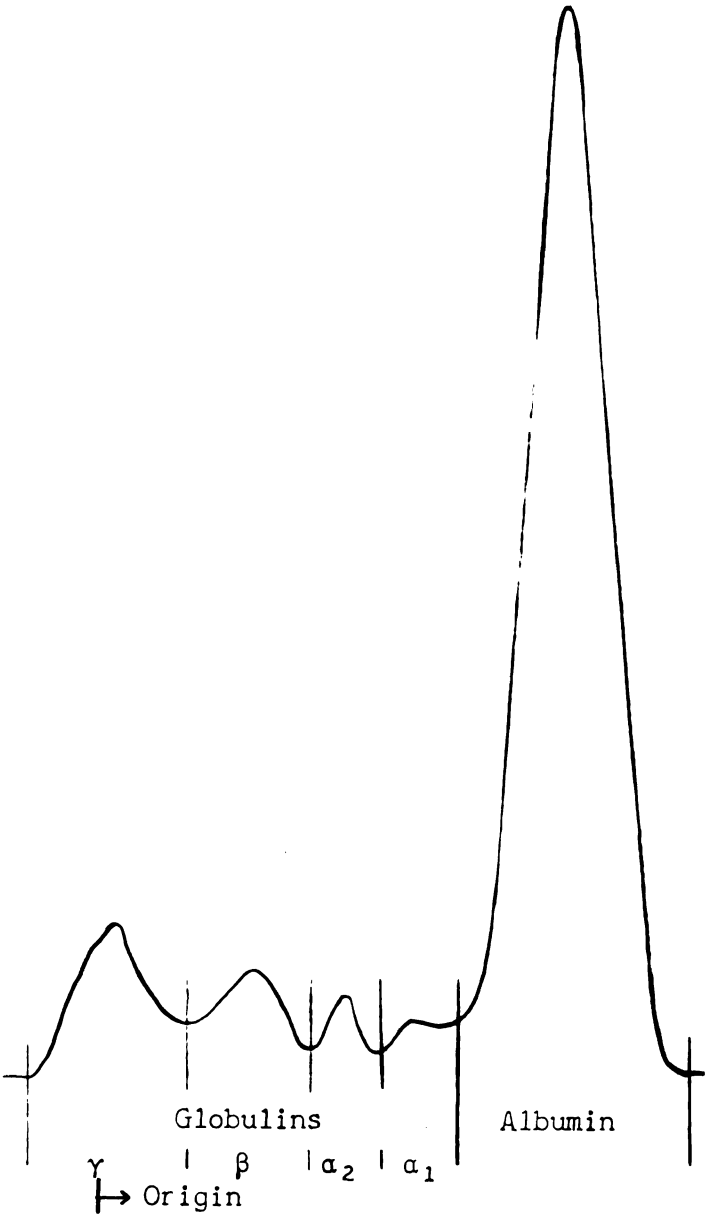


FIGURE 3

DENSITOMETRIC TRACING OF A PROTEIN ELECTROPHORETOGRAM OF SERUM FROM A MULTIPLE SCLEROSIS PATIENT

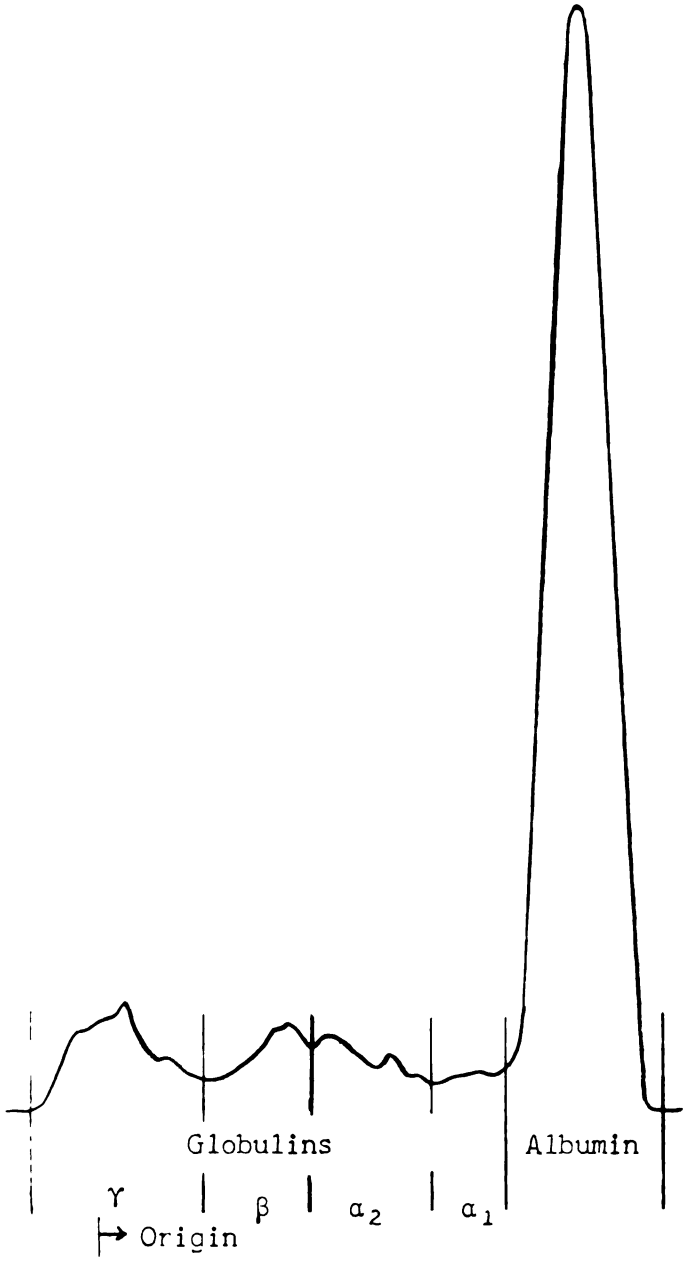


FIGURE 4

DENSITOMETRIC TRACING OF A PROTEIN ELECTROPHORETOGRAM OF SERUM FROM A CIRRHOSIS OF LIVER PATIENT

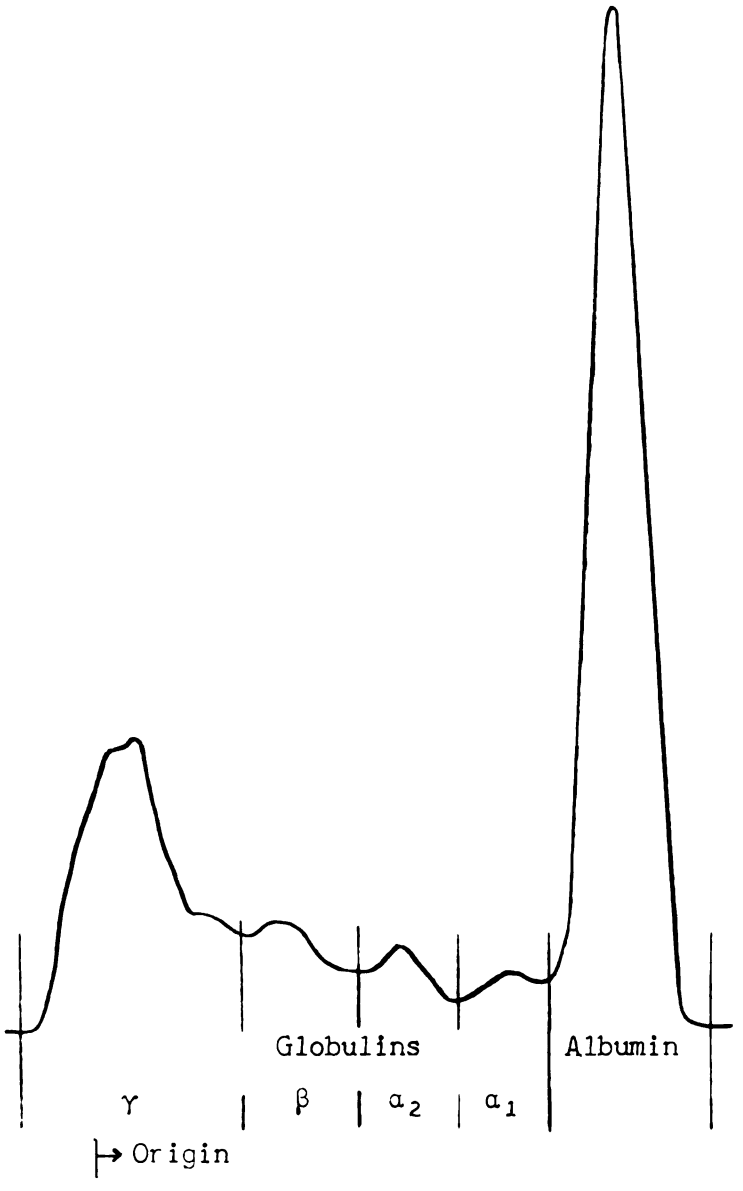


FIGURE 5

DENSITOMETRIC TRACING OF A PROTEIN ELECTROPHORETOGRAM OF SERUM FROM A MULTIPLE MYELOMA PATIENT
(Case #2)

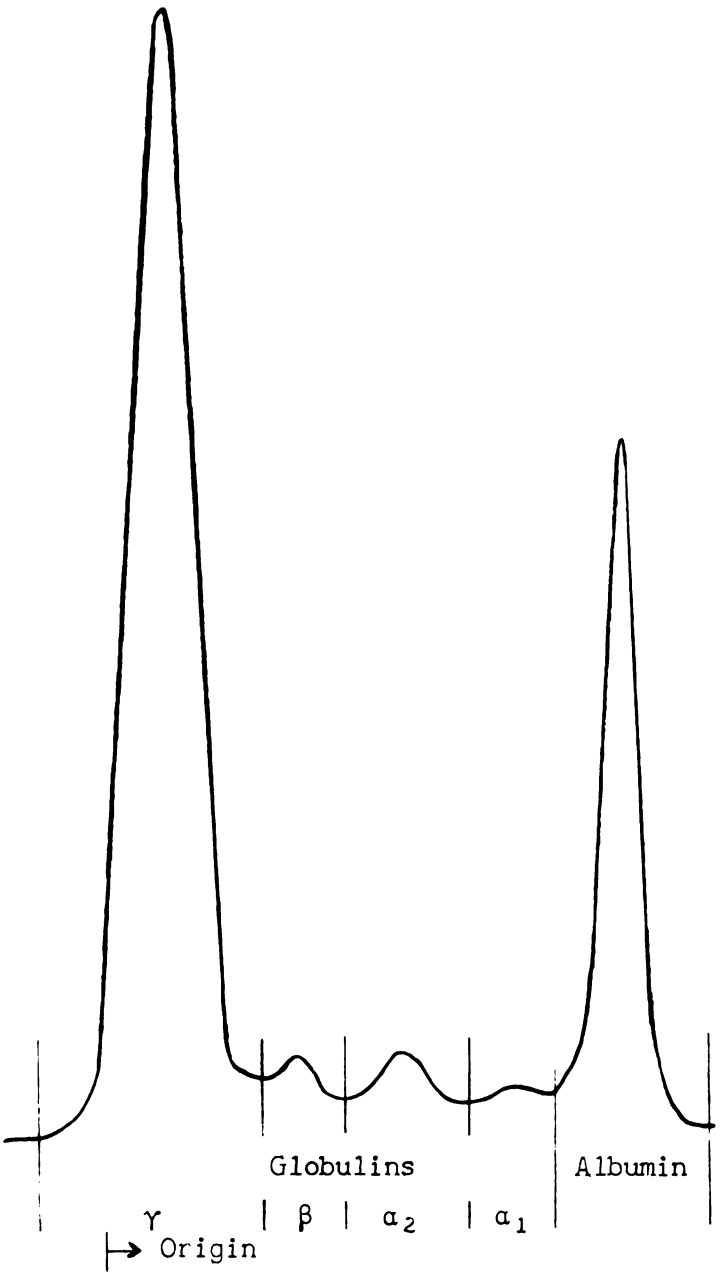


FIGURE 6

DENSITOMETRIC TRACING OF PROTEIN ELECTROPHORETOGRAM OF SERUM FROM A SEVERELY BURNED PATIENT

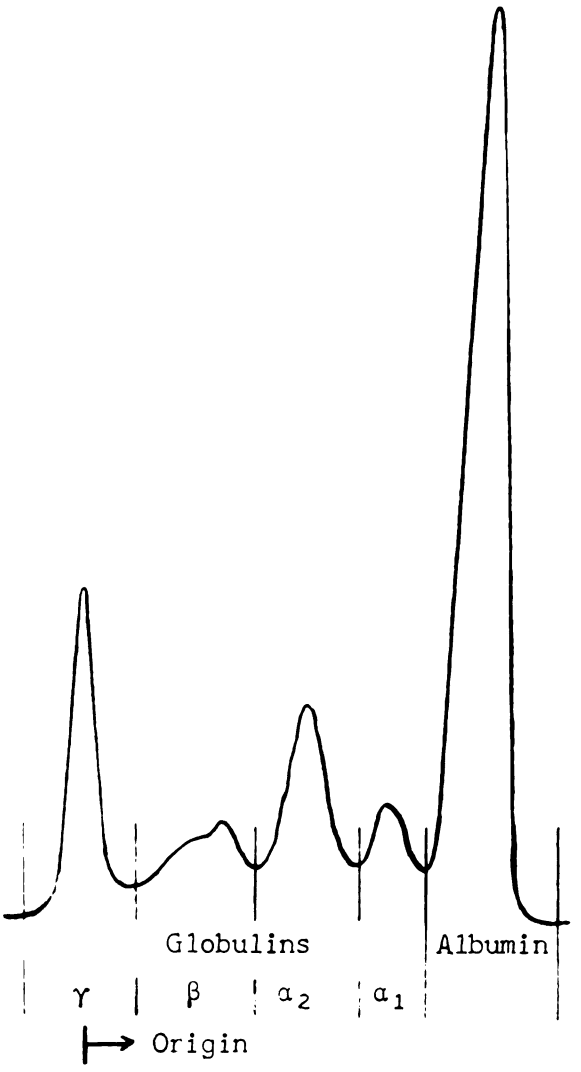
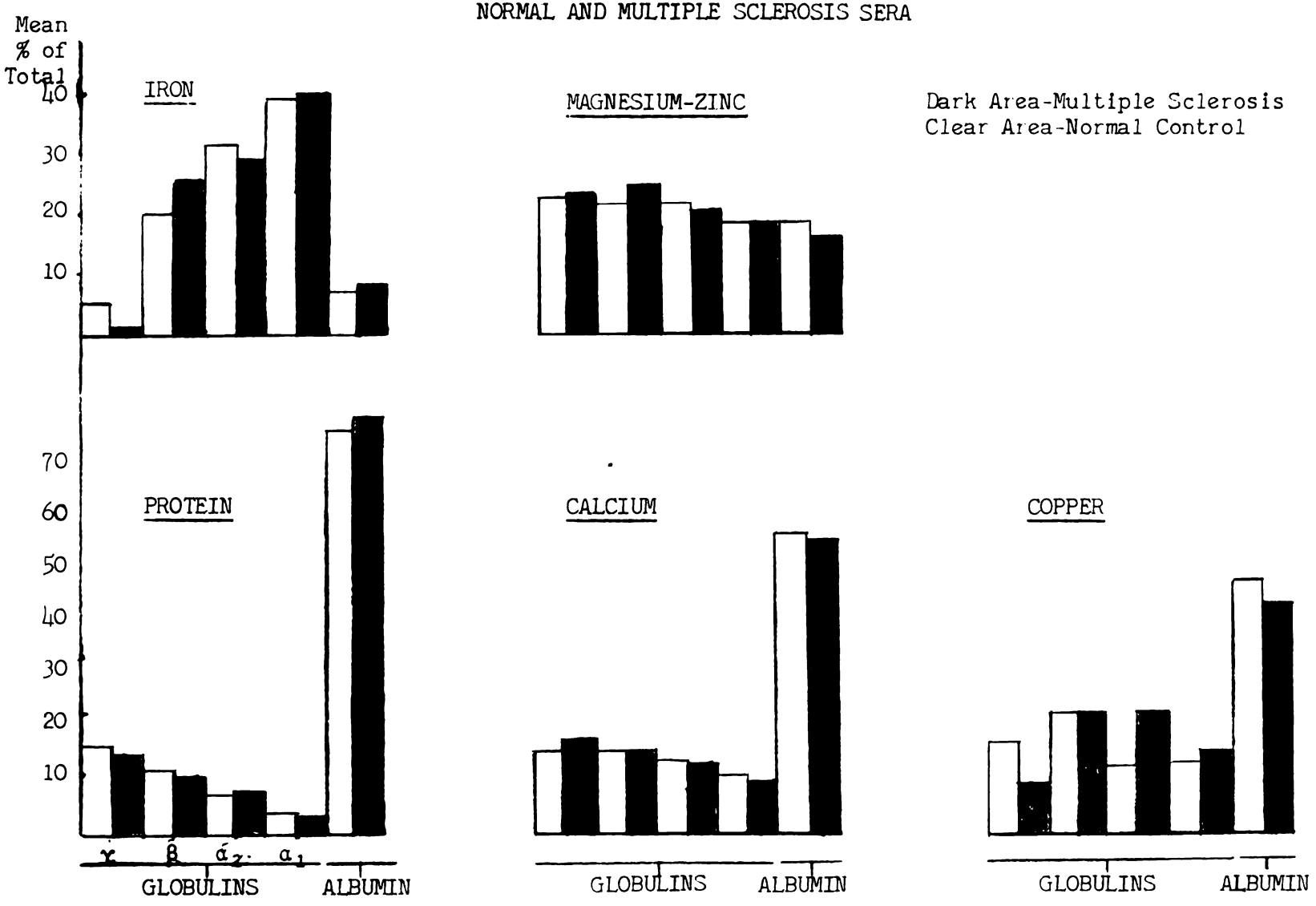


FIGURE 7

A COMPARATIVE DISTRIBUTION OF METALS AMONG PROTEIN FRACTIONS FROM NORMAL AND MULTIPLE SCLEROSIS SERA



IV. DISCUSSION

This section will attempt to evaluate and interpret the results of experimental findings with regard to the binding of metal ions by human serum proteins.

A. Serum Protein Analysis By Paper Electrophoresis

1. Statistical Considerations.- The arithmetic mean ($\bar{X}$) and standard deviation (S) of analytical results were calculated by use of the statistical equations (3):

$$\bar{X} = \frac{\sum X}{N} \quad \text{and} \quad S = \sqrt{\frac{\sum (X-\bar{X})^2}{N-1}}$$

in which X is a single observation and N is the number of observations. The range, $\bar{X} \pm S$, was also determined and appears with the means and standard deviations in the appropriate tables.

Electrophoretic analysis gave the relative amounts of each serum protein fraction as a per cent of the total, e.g., Table V, subject number 1 shows albumin as 65.8 per cent. Total serum protein concentrations were measured in order to convert electrophoretic percentages into grams per 100 ml. of serum. A sample calculation appears in Appendix I.

To obtain the data presented in Table II, paper electrophoretic analyses were performed upon human serum before freezing and after two to three months storage in the frozen state. The information shows that the protein storage changes which occurred fell within the range of standard deviations for these analyses. It was shown previously (58) that cold storage had no significant effect upon the serum concentration

of protein, iron, calcium, copper and magnesium.

Figure 1 illustrates the apparatus employed to perform the paper electrophoretic analyses. To demonstrate the validity of protein analytical results obtained with this equipment, Table III gives the mean normal values found in this study together with those of other investigators (28, 29, 46, 58). Comparison of the values in the table shows reasonable agreement.

2. Serum Protein Distribution.- The data regarding age and sex of normal subjects and multiple sclerosis (M.S.) patients appear in Table IV and show that the proportion of males to females in each group is nearly the same. Table V indicated that there is no significant difference in serum protein distribution between sexes. This was also the finding of Jencks, et al. (46). Although not statistically substantiated, Table V suggests that the per cent of alpha-2 globulin decreases with increasing age. Jencks, et al. (46) observed a similar change and likewise attached no importance to it.

Data regarding serum protein distribution for 26 normal subjects (control sera) are recorded in Tables VI and VII. Similar data for 22 M.S. patients are found in Tables VIII, and IX. Statistical examination reveals no significant differences concerning the distribution of serum protein fractions among M.S. patients and normal controls. This conclusion agrees with the findings of Dobin and Switzer (24) and Fog (33). However, all previous workers have based their conclusions on moving boundary electrophoresis.

Figures 2 and 3 illustrate densitometric pattern tracings of paper electrophoretograms for normal and M.S. serum proteins. The "double

peak" phenomenon exhibited in the alpha-2 globulin fraction by the M.S. pattern was found only for patients Q and V. This qualitative difference was not observed in any control serum. Similar patterns were observed by Bernsohn and Cochrane (9) in 55 per cent of the M.S. patients they investigated. As yet no significance has been attached to this heterogeneity.

Tables X and XI give information about serum protein distribution for patients with other diseases. The abnormal deviations found are similar to those reported by earlier investigators (28, 46, 60). Figures IV, V and VI illustrate typical densitometric tracings of paper electrophoretograms for liver cirrhosis, multiple myeloma and severe burns.

B. Serum Metal Distribution on Paper Electrophoretograms

1. Calcium.- The distribution of calcium among the proteins of normal human serum was obtained by paper electrophoresis and alizarin red staining techniques. The data in Table XII indicate that calcium is bound by all fractions. The results also suggest that albumin binds 45 to 55 per cent of the calcium, while the globulins contribute almost equally to binding the remainder. These conclusions agree with those of Prasad and Flink (75), who found by ultrafiltration methods that albumin held 50 to 55 per cent of the bound calcium, whereas, the rest was bound principally by beta globulin. On the other hand, LeDuc (58) using alizarin red dye, reported that albumin and gamma globulin contributed equally in binding two-thirds of the calcium. The discrepancies involving the use of alizarin red may lie in the fact that an insufficient number of subjects were studied in the latter instance.

Data for the distribution of calcium among the protein fractions of M.S. sera are tabulated in Table XIII. Statistical evaluation reveals no significant differences concerning calcium distribution between M.S. patients and normal subjects.

Table XVII-A shows the distribution of calcium found among the serum protein fractions of several pathological cases other than M.S. The data suggest that calcium binding is dependent upon the amounts and types of protein present in the serum. For example, in glomerulonephritis (patient 6-G.N.) the observed serum protein pattern changes are a consequence of actual protein losses due to kidney damage. Thus no essential difference appears in the calcium distribution between this case and the normal subject (7-M.C.V.). However, in the remaining five pathological cases "abnormal globulins" are present in the serum. In these instances, definite changes occurred with regard to calcium binding by the various protein fractions. Further studies will be necessary to substantiate these results. The conclusions are supported by the findings of Prasad and Flink (75). They observed that increased amounts of gamma globulin in multiple myeloma sera bound appreciable calcium.

2. Iron.- Information regarding the distribution of iron among the serum protein fractions of normal subjects is recorded in Table XIV-A. The data suggest that iron is principally bound by the alpha and beta globulins, with little contribution to iron binding being made by albumin and gamma globulin. Transferrin, a beta globulin, has been proposed as the normal serum protein involved in the transport of iron (16, 61, 88). It is known that the transferrin complex dissociates readily to protein plus iron outside the pH range 7.25 to 7.50 (61). Thus under

the conditions of electrophoresis used in this work, it might not be surprising to find iron distributed among other proteins. Furthermore, a large number of physiological and pharmacological factors (e.g., breakdown and synthesis of iron-porphryin compounds) influence the concentration of serum iron (61) and may conceivably affect its binding to protein. The wide variation in per cent of iron bound by protein fractions of normal subjects listed in Table XIV may reflect these factors.

The data in Table XIV-B show the distribution of iron among the protein fractions of M.S. sera. Table XVII-B supplies analytical results concerning the per cent of iron found in the serum protein fractions of six other pathological cases. Both sets of data do not differ significantly from the distribution obtained for normal subjects. Neale (67) also reported that abnormal serum proteins of other pathological cases do not alter the iron binding ability of normal serum beta globulin.

3. Magnesium-Zinc.- A suitable reagent for separate histochemical analysis of magnesium and zinc on paper electrophoretograms does not appear available. Quinalizarin and dithizone were among the reagents tested in this investigation for such a possibility. Both were previously developed for inorganic ion detection (30). Neither of these compounds were found to be adaptable to techniques of elution from paper and subsequent quantitation.

Paper electrophoretograms stained with quinalizarin demonstrated that magnesium was principally associated with albumin and to a slight extent present in all globulin fractions. When paper electrophoretograms

were stained with titan yellow, Lillevik, et al (60), reported magnesium to be present in all fractions of human serum proteins. During the present investigation this observation could not be confirmed according to the conditions reported (60). On the other hand, Copeland and Sunderman (20) and Prasad and associates (76) reported magnesium to be bound principally by albumin, while the globulins contributed only slightly to magnesium binding. These latter findings were obtained by ultrafiltration of human serum to which magnesium in excess of the physiological amount present had been added.

Dithizone stained paper electrophoretograms revealed a pink color for zinc in the area of the beta globulin fraction. However, LeDuc (58) detected zinc in the gamma globulin portion of paper electrophoretograms. The histochemical approach by LeDuc involved the use of potassium sulfide and sodium nitroprusside. During the course of the present investigation this finding by use of these reagents was not substantiated. Liddell and Williams (59) proposed 1-(1-hydroxy, 2-napthyl azo)-5-nitro, 2-napthol, 4-sulfonic acid as a sensitive reagent for the colorimetric detection of zinc. The dye was also found in the present study to be reactive with magnesium. Since no suitable colorimetric reagent was known for their individual histochemical identification this dye was utilized for a combined analysis of both metals. These results are given in Table XV-A for sera of control subjects, in Table XV-B for those of M.S. patients and in Table XVII-C for samples of selected pathological states. The data suggest that magnesium and/or zinc are bound by all serum protein fractions. These findings agree reasonably well with most previous work (17, 20, 60, 76, 88, 104). No significant differences for magnesium plus zinc distribution are seen between

normal subjects and M.S. patients. Only slight variations are observed for the distribution of these metals in the case of liver cirrhosis (Patient 4).

However, marked alterations are noted in Table XVII-C for the distribution of "magnesium plus zinc" in the serum protein fractions of multiple myeloma patients, numbers 1, 2 and 3. As with the binding of calcium, it appears that serum magnesium and/or zinc binding ability is dependent upon the types and amounts of protein fractions present.

4. Copper.- The qualitative application of dithizone and dithiooxamide as copper stains indicated that albumin was the major site of binding for this metal in normal sera. Neither of these reagents were adaptable to the techniques of elution and quantitation as used in this study. However, alizarin blue (63) was found suitable for this purpose. The findings for serum copper distribution of normal subjects (control sera) after treatment of paper electrophoretograms with alizarin blue appear in Table XVI-A. The data show that the presence of copper among protein fractions is quite variable between normal persons, and may be subject to the same physiological and pharmacological factors as involved with iron.

Further indications are that generally albumin binds more copper than do the globulins. Albumin exhibited a wider range of variability in per cent of copper bound than did the globulins. Finding copper in all protein fractions of normal human serum agrees with the reports of Thompson and Watson (91) as well as Cumings, et al. (22). Both groups found copper in all fractions after sodium sulfate fractionation of human serum proteins.

Other information in the literature (6, 15, 43, 103) is confusing but supports the findings that copper can and does form metal-protein complexes with all human serum protein fractions.

The distribution of copper among serum protein fractins of M.S. patients is given by the data in Table XVI-B. The results are similar to those of normal controls. However, there is an apparent shift of copper from gamma to alpha-2 globulin. There was no correlation seen between those patients with elevated alpha-2 globulins and the apparent shift. In fact, no correlation is evident between per cent of copper found and the amount of protein associated with it. The observation that three patients (M, O and R) had no detectable copper in any serum protein fraction may be attributed to technical difficulties in methodology.

Cumings, et al. (22) who employed salt fractionation, found a greater percentage of copper bound by beta globulin than by any other protein fraction in the serum of normal subjects and M.S. patients. However, for M.S. sera they observed a shift of copper from alpha to beta globulin. The number of observations was limited to ten normals and four patients. These findings were not confirmed by the present investigation when a large sampling was made.

In conjunction with the above findings it is interesting to speculate upon the connection between human multiple sclerosis and the demyelinating disease of lambs and copper metabolism. While copper has been considered necessary for the proper formation of myelin in lambs, no good evidence for associating copper deficiences with multiple sclerosis is at hand (92).

Total serum copper content in M.S. patients has been considered to be normal (62), while the tissue and blood levels of copper in ataxic lambs have definitely been shown to be subnormal (8). It maybe possible in both instances that specific copper binding sites of one or more serum proteins are altered which leads to inadequate copper utilization. The progress of the ataxia in the case of the lambs was arrested by administration of copper in the diet (8). In this instance one might suspect that increasing the total serum copper concentration would allow "saturation" of all copper binding sites among the serum proteins and thus assist in copper utilization. Evidence for such a statement comes from the report by Wolff, et al. (104), that copper was distributed almost equally among all serum protein fractions after administration of radio-active copper. Further support is taken from the work of Bearn and Kunkel (6), who found that orally ingested copper was rapidly absorbed and held for some time by serum albumin.

Table XVII-D shows the copper-protein findings for some examples of selected pathologic states. The results are highly variable from one disease to another. It is suggested that copper binding is dependent upon the specific proteins present in the serum. For example, while large amounts of gamma globulin were present in the serum of multiple myeloma patients, no copper was found in this fraction for the three cases studied. Furthermore, a slight increase in the alpha-1 fraction of patient 4 (liver cirrhosis) was associated with an increased per cent of copper. On the other hand, patient 5 (severe burns) had a significantly elevated alpha-1 globulin fraction, yet there was no appreciable increase in the per cent of copper bound.

5. General Observations.- Figure 7 represents a summation of the data contained in the individual tables of results regarding metal ion distribution among serum protein fractions of normal subjects and M.S. patients. The shaded portions represent the mean values for M.S. patients, while the clear areas designate the controls. The "copper shift" is demonstrated by the increased shaded area in the alpha-2 copper portion as compared to the decreased shaded area in the gamma globulin fraction.

C. Dialysis Experiments

In the methods applied for the histochemical detection of calcium, magnesium-zinc and copper, it was assumed that the dye-reagent would be bound only at the metal-protein zones on the paper electrophoretograms. For the location of iron, the technique involved the elution of the metal from the paper electrophoretogram followed by addition of the color producing reagents. No interference due to the presence of protein was presumed to have occurred in either instance. However, during critical evaluation of the analytical results this assumption became uncertain. Therefore dialysis experiments were performed to see if the proportion of metal to protein had any effect upon the color development.

1. Pooled Serum Dialysis.- A sufficient quantity of serum for both "before" and "after" analyses upon any of the control sera was not available. Consequently a serum pool was made and used for study in order to become acquainted with the appropriate conditions for individual control serum dialysis.

The data in Table XVIII reveals that both calcium and magnesium

were completely removed from the serum by dialysis, whereas, iron was not. The diminished protein concentration observed after dialysis can be accounted for by dilution since a volume increase of about 10 per cent occurred.

It may be assumed that similar effects would occur in the case of individual control serum dialysis. Subsequent considerations are presented with these findings taken into account.

2. Protein.- The information in Table XIX indicates that no significant differences were found for the distribution of proteins before or after dialysis of control sera.

3. Calcium.- The data in Table XX show that less calcium-combining dye was taken up by the various serum protein fractions after dialysis. This would be true even if the "after" absorbancy figures were corrected for dilution. Since calcium was not found in any dialyzed control serum it may be assumed that both the calcium-protein complex and the metal-free protein bind alizarin red dye. Apparently the decrease in absorbancy of the eluted dye represents a loss of calcium from the protein.

The per cent distribution of calcium among the serum protein fractions was calculated from the difference in mean absorbancies of Table XX. The distribution found in this manner differs significantly from that given in Table XII. Although calcium is found in all protein fractions, the beta and gamma globulins are found to bind more calcium than previously concluded. Consequently, with correction for protein interference albumin and the alpha globulins are associated with less calcium than heretofore observed.

4. Iron.- The absorbancy values for iron determined among the protein fractions of serum before and after dialysis are given in Table XXI. The net results were, that even after dialysis, iron may be bound to some extent by all protein fractions and, in particular, to the alpha and beta globulins. No influence upon the iron-dye absorbancies due to the presence of protein in the paper electrophoretograms was seen.

5. Magnesium-Zinc.- According to the results in Table XXII more dye was taken up by the paper electrophoretograms after dialysis than before. Any dilution correction would make this difference even greater.

Spectral transmission curves were obtained for dye eluates from paper electrophoretograms of both dialyzed and non-dialyzed sera. The principal absorbance peak was at 515 mu for both samples.

Qualitative tests with dithizone upon paper electrophoretograms of dialyzed-serum as well as qualitative tests with 1-(1-hydroxy, 2-napthylazo) 5-nitro, 2-napthol, 4-sulfonic acid, sodium salt upon the dialyzed and non-dialyzed sera clearly indicated that zinc had been removed from the sera by dialysis.

In view of the above facts it might be surmised that the proteins of human serum interfere with the use of this latter dye for the histochemical detection of magnesium and/or zinc upon paper electrophoretograms.

6. Copper.- Table XXIII gives the absorbancies for section eluates of alizarin blue-stained paper electrophoretograms before and after serum dialysis. Dithizone staining of paper electrophoretograms after serum dialysis gave the characteristic brown color for copper in the

albumin area. Copper also appears to be firmly held by all the other protein fractions. No direct relationship between the eluted-dye absorbencies and protein presence was seen. It is suggested that alizarin blue dye may be quite useful in copper-protein binding studies.

D. Cholesterol Studies

The results of total cholesterol analyses upon sera of 26 control subjects and 20 M.S. patients appear in Table XXIV. Statistical treatment reveals that M.S. patients have higher cholesterol levels than normal subjects. This agrees with the findings of Dobin and Switzer (24), Pichler and Reisner (73) and Frisch (37). No correlation of serum cholesterol values with such factors as sex, age or stage of the disease was seen.

V. SUMMARY AND CONCLUSIONS

Paper electrophoretic separation of human serum proteins in conjunction with histochemical staining for several metals demonstrated that:

1. There were no significant differences between normal subjects and multiple sclerosis (M.S.) patients with respect to the distribution of protein fractions in their sera. Other pathological states gave protein distribution results comparable to previous findings for the specific disease.

2. Calcium was found similarily distributed in all protein fractions of the sera from both control subjects and M.S. patients. Albumin in both groups was found to bind from 45 to 55 per cent of the calcium. The presence of calcium among the protein fractions of other pathological sera appeared dependent upon the types and amounts of protein present.

3. Iron was found mainly in the alpha and beta globulins of control subjects as well as in M.S. patients. Iron binding by these fractions was not affected by the presence of abnormal proteins in the sera of other pathological cases.

4. The application of sodium 1-(1-hydroxy, 2-napthylazo), 5-nitro, 2-napthol, 4-sulfonate in the histochemical detection of magnesium-zinc upon paper electrophoretograms indicated that these metals were bound by all fractions of human serum proteins. No differences were seen between normal controls and M.S. patients in this regard. It appeared that magnesium and/or zinc distribution in the serum of other pathological states was dependent upon the varied types and amounts of protein fractions present.

5. The histochemical use of alizarin blue for copper estimations on paper electrophoretograms of normal and M.S. sera showed that the metal was bound by all protein fractions. The albumin and beta globulin fractions contributed most to copper binding. The binding of copper by proteins in other pathological sera appeared dependent upon the amount and type of "abnormal" protein present.

6. Analyses performed upon dialysed human serum showed that:

a) Although dilution occurred, no change in protein distribution resulted.

b) Calcium, magnesium and zinc were completely removed from normal serum by dialysis, while iron and copper were not.

c) Use of alizarin red dye for calcium serum-protein binding studies was suitable when correction for protein interference was made. Calcium was found principally in albumin and the beta and gamma globulins.

d) No interference by protein on the determination of iron in human serum paper electrophoretograms was seen. Iron was bound to some extent by all serum protein fractions, especially by the alpha and beta globulins.

e) The presence of protein seriously interfered with the use of sodium 1-(1-hydroxy, 2-napthylazo), 5-nitro, 2-napthol, 4-sulfonate for magnesium-zinc detection on paper electrophoretograms.

f) Alizarin blue staining of serum paper electrophoretograms for copper detection was not influenced by the presence of protein. Copper appeared to be firmly bound by all protein fractions, especially albumin.

7. Although no correlation was observed between age, sex or stage of the disease, cholesterol levels were higher in the sera of M.S. patients than in normal control sera.

8. This investigation revealed that histochemical staining methods for metal detection upon serum protein electrophoretograms appear useful. In addition, the refinement of these techniques should allow the study of metal-protein relationships to be made under more nearly physiological conditions. However, more sensitive and specific reagents are desirable. Future studies should consider further the problem of protein effects upon metal-dye complexes.

BIBLIOGRAPHY

1. Abderhalden, E. and Moller, P., Untersuchungen uber den Gehalt des Bluterum en Eisen, Kupfer und Mangan. Z. physiol. Chem., 176, 95-100 (1928).

2. Bachra, B. N., Dauer, A. and Sobel, A. E., The Complexometric Titration of Micro and Ultramicro Quantities of Calcium in Blood Serum, Urine and Inorganic Salt Solutions. Clin. Chem. 4, 107-119 (1958).

3. Bancroft, H., Introduction to Biostatistics, New York, Hoeber-Harper, 210 pp. 1957.

4. Barkan, G., Die Verteilung des leicht abspaltbaren Eisen zwischen Blutkorperchen und Plasma und sein Verhalten unter experimentellen Bedingungen. Z. physiol. Chem., 171, 194-221 (1927)

5. Barkan, G. and Schales, O., Chemischer Aufbau und physiologiche Bedeutung des "laicht abspaltbaron" Bluteisens. 13. Mitteilung in der Reihe der Eisenstudion. Z. physiol. Chem., 248, 96-116 (1937).

6. Bearn, A. G. and Kunkel, H. G., Localization of Cu^{64} in Serum Fractions Following Oral Adminstration: An Alteration in Wilson's Disease. Proc. Soc. Exper. Biol. and Med., 85, 44-47 (1954).

7. Bennetts, H. W. and Beck, A. B., Bull. Coun. Sci. Industr. Res. Aust. No. 147 (1942) "Seen in Abstract Only". Thompson, R. H. S. and King, E. J., Editors, Biochemical Disorders in Human Disease New York, The Academic Press, 1957, 843 pp.

8. Bennetts, H. W. and Chapman, F. E. Copper Deficiency in Sheep in Western Australia. A Preliminary Account of the Etiology of Enzootic Ataxia of Lambs and an Anemia of Ewes. Australian Vet. J. 13, 138-149 (1937).

9. Bernsohn, J. and Cochrane, Leila S. Electrophoretic Fractionation of Serum Protein in Multiple Sclerosis. Proc. Soc. Exp. Biol. Med., 86, 540-542 (1954).

10. Block, R. J., Durrum, E. L. and Zweig, G., A Manual of Paper Chromatography and Paper Electrophoresis, page 359, Academic Press, Inc., New York. (1955).

11. Boyden, R. and Potter, V. R., On the Form of Copper in Blood Plasma, J. Biol. Chem., 122, 285-290 (1937).

12. Braunsteiner, H. Gisinger, E. and Pakesch, F., Ferritin, Transferrin und Serumeisen, Klin. Wochschr., 30, 394-401 (1952).

13. Cantarow, A. and Schepartz, B., Chapter 22, Inorganic Metabolism, Philadelphia, W. B. Saunders Co., 1954, 848 pp.

14. Chiavacci, L. V. and Sperry, W. M. Concentration of Cholesterol and of Lipid Phosphorous in Blood Serum in Multiple Sclerosis. Arch. Neurol. Psychiat., 68, 37-42 (1952).

15. Cohn, E. J. Chemical Specificity of Interaction of Diverse Human Plasma Proteins, Blood, 3, 471-485 (1948).

16. Cohn, E. J., Gurd, F. R. N., Surgenor, D. M., Barnes, B. A., Brown, R. K., Deronaux, G., Gillespie, J. M., Kalirt, F. W., Lever, W. F., Lin, C. H., Mittleman, D., Moulton, R. F., Schmid, K. and Uronia, E., A System for the Separation of the Components of Human Blood: Quantitative Procedures for the Separation of the Protein Components of Human Plasma. J. Am. Chem. Soc., 72, 465-474 (1950).

17. Cohn, E. J., Chapter 3, Interaction of Proteins with Each Other and with Heavy Metals, J. L. Tullis, Editor, Blood Cells and Plasma Proteins--Their State in Nature, New York, The Academic Press, 1953, 436 pp.

18. Consden, R. and Stainer, W. M., A Simple Electrophoresis Apparatus Nature, 170, 1069-1070 (1952).

19. Coolidge, T. B., A Simple Cataphoresis Apparatus, J. Biol. Chem, 127, 551-553 (1939).

20. Copeland, B. E. and Sunderman, F. W., The Magnesium Binding Property of the Serum Proteins, J. Biol. Chem., 197, 331-341 (1952).

21. Cremer, H. D. and Tiselius, A., Electrophoresis of Egg Albumin on Filter Paper. Biochem. Z., 320, 273-283 (1950).

22. Cumings, J. N., Goodwin, H. J. and Earl, C. J., Blood Copper and its Relationship to the Globulins, J. Clin. Pathol., 8, 69-72 (1955).

23. Dahl, L. K., A Simple and Sensitive Histochemical Method for Calcium, Proc. Soc. Exper. Biol. Med., 80, 474-497 (1952).

24. Dobin, N. B. and Switzer, J. L., Liver Function and Other Blood Chemistry Tests in Multiple Sclerosis. Arch. Neurol. Psychiat., 71, 405-424 (1954).

25. Drinker, H., Greem, A. A. and Hastings, A. B., Equilibria Between Calcium and Purified Globulins. J. Biol. Chem., 131, 641-647 (1939).

26. Durrum, E. L., A Microelectrophoretic and Microionophoretic Technique. J. Am. Chem. Soc., 72, 2943-2948 (1950).

27. Eisler, B. and Resdahl, K. G., Untersuchungen uber die Zustandenform des Kupfers in Blutserum mit Kilfe der Kataphorese. Biochem. Z., 286, 435-438 (1936).

28. Ehrmantraut, H. C., The Clinical Significance of Paper Electrophoresis. Presented at a Joint Meeting of Pacific Northwest Society of Pathologists and the Northwest Region, College of American Pathologists, May 2, 1958.

29. Faulkner, W. R., Normal Paper Electrophoresis Values of Serum Proteins as Obtained by Spinco Procedures, Clin. Chem., 5, 375 (1959).

30. Feigel, F., Spot Tests in Inorganic Analysis, New York, Elsevier Publishing Co., 1958, 600 pp.

31. Ferro, P. V. and Ham, Anna B., Lab-Trol Bulletin No. 101, Nov. 1955, Dade Reagents, Inc. Miami, Florida.

32. Flynn, F. V. and DeMayo, P., Microelectrophoresis of Protein on Filter Paper. Lancet, 261, 235-239 (1951).

33. Fog, M., On the Pathogensis of Multiple Sclerosis. Acta Psychiat. et neurol. Scandinav, 74, 22 (1951).

34. Fontes, G. and Thivolle, L., Surla teneur du serum en fer non hemoglobinque et sur sa dimunition as cours de l'anemie experimentale. Compt. rend. soc. biol., 93, 687-689 (1925.

35. Fowweather, F. S., The Determination of Iron in Blood-Plasma. Bio. Chem. J., 28, 1160-1164 (1934).

36. Fraser, R. and King, E. J., Chapter IX, Diseases of Bone and the Parathyroid Gland, Thompson, R. H. S. and King, E. J., Editors, Biochemical Disorders in Human Disease, New York, The Academic Press, (1957) 843 pp.

37. Frisch, C. Cholesterinstoffwechsel bei der multiplen sklerose. Wein. klin. Wochschr., 50, 596-598 (1937).

38. Greenberg, D. N., Amino Acids and Proteins, Springfield, Illinois, C. C. Thomas, (1951) 950 pp.

39. Greene, C. H. and Power, N. H., The Distribution of Electrolytes Between Serum and the In-Vivo dialysate. J. Biol. Chem., 91, 183-202 (1931).

40. Gurd, F. R. N. and Goodman, D. W., Preparation and Properties of Serum and Plasma Proteins. XXXII. The Interaction of Human Serum Albumin with Zincoins., J. Am. Chem. Soc., 74, 670-675 (1952).

41. Holmberg, C. G. and Laurell, C. B., Studies on the Capacity of Serum to Bind Iron. The Regulation of Mechanism of Serum Iron. Acta Physiol. Scand., 10, 307-319 (1945).

42. Holmberg, C. G. and Laurell, C. B., Investigations on Serum Copper. I. Nature of Serum and its Relation to the Iron-Binding Protein in Human Serum. Acta Chem. Scand., I, 944-950 (1947).

43. _____ and _____, Investigations on Serum Copper. II. Isolation of the Copper-Containing Protein and a Description of some of its properties. Acta Chem. Scand., 2, 550-556 (1948).

44. Horst, W. Transport und Bindung im Serum. Klin. Wochschr., 32, 961-968 (1954).

45. Innes, J. R. M. and Shearer, G. D., L. "Swayback": A Demelinating Disease of Lambs with Affinities to Schilder's Encephalitis in Man. J. Comp. Path., 53, 1-41 (1940).

46. Jencks, W. P., Smith E. R. B., and Durrum, E. L., The Clinical Significance of the Analysis of Serum Protein Distribution by Filter Paper Electrophoresis. Am. J. Med., 21, 387-405 (1956).

47. Jones, H. H., Jones, Jr., H. H., Howard, R. R. and Bucch, L. D., Metabolic Characteristics of Multiple Sclerosis: Chemical Abnormalities in the Blood and Urine of a Large Series of Patients and Metabolic Balance in Two Patients. Ann. N. Y. Acad. Sci., 58, 656-667 (1954).

48. Kitzes, G., Elvehjem, C. A. and Schuette, H. A. The Determination of Blood Plasma Iron. J. Biol. Chem., 155, 653-660 (1944).

49. Von Klobusitsky, D. and Konig, P. Venom of the Genus Bothrops VI, Arch. Expt. Path. Pharmakol., 192, 271 (1939), "Seen in Abstract Only" C. A., 34, 4081 (1940).

50. Klotz, I M., pp. 257-290, Thermodynamic and Molecular Properties of Some Metal-Protein Complexes, McElroy, W. D. and Glass, B., Editors, The Mechanism of Enzyme Action, Baltimore, The Johns Hopkins Press, 1954, 819 pp.

51. Klotz, I. M., Urquhart, Jean and Fiess, H. A., Interactions of Metal Ions with the Sulfhydryl Group of Serum Albumin, J. Am. Chem. Soc., 74, 5537-5538 (1952).

52. Klotz, I. M., Urquhart, Jean, Klotz, T. A. and Ayers, J., Slow Intramolecular Changes in Copper Complexes of Serum Albumin. The Role of Neighboring Groups in Protein Reactions. J. Am. Chem. Soc., 77, 1919-1925 (1955).

53. Koechlin, B. A., Preparation and Properties of Serum and Plasma Proteins XXVIII. The Beta Metal-Combining Protein of Human Plasma. J. Am. Chem. Soc., 74, 2649-2653 (1952).

54. Konig, P., Actas e Trabalhos do Terceiro Congresso Sud-Americano de Chimica, Rio de Janeior e San Paulo, 2, 334 (1937) "Seen in Abstract Only", Block, R. J., Durrum, E. L. and Zweig, G., A Manual of Paper Chromatography and Paper Electrophoresis, New York Academic Press, Inc., (1955) 484 pp.

55. Kunkel, H. G. and Tiselius, A., Electrophoresis of Proteins on Filter Paper. J. Gen. Physiol., 35, 89 (1951).

56. Lahey, M. E., Gubler, C. J., Brown, D. M., Smith, E. L., Jager, B. V., Cartwright, G. E. and Wintrobe, M. M., Copper Metabolism VIII. Correlation Between the Serum-Copper Level and Various Serum-Protein Fractions. J. Lab. Clin. Med., 41, 829-835 (1953).

57. Lederer, E. and Lederer, M., Chapter I, Chromatography, New York, Elsevier Publishing Co., 1953.

58. LeDuc, Henrietta, M., "Equilibrium Dialysis, Moving Boundary and Paper Electrophoresis Studies on the Binding of Human Serum Proteins with Calcium, Magnesium, Iron and the Copper Ions." A Ph.D. Thesis, Michigan State University, 1956, 77 numbered leaves.

59. Liddell, H. F. and Williams, Sylvia, Derivatives of 1-Amino, 2-Napthol 4-Sulfonic Acid as Reagents for the Colorimetric Determination of Zinc. Analyst, 83, 111-113 (1958).

60. Lillevik, H. A., LeDuc, Henrietta M., Ewing, D. T. and Morse, P. F., Moving Boundary and Paper Electrophoresis Studies on the Binding of Human Serum Proteins with Metal Ions, Fed. Proc., 16, No. 910 (1957).

61. Mandel, E. E., Serum Iron and Iron-Binding Capacity in Clinical Diagnosis, Clin. Chem., 5, 1-12 (1959).

62. Mandelbrote, B. M., Stainer, M. W., Thompson, R. H. S. and Thuston, M. N., Studies on Copper Metabolism in Demyelinating Diseases of the Central Nervous System. Brain, 71, 212-228 (1948).

63. Markowitz, H., Cartwright, G. E. and Wintrobe, M. M., Copper Metabolism XXVII. Isolation and Properties of an Erythrocyte Copper Protein, J. Biol. Chem., 234, 40-45 (1959).

64. McDonald, H. J., Iongraphy: A New Frontier in Electrophoresis, J. Chem. Educ., 29, 428-436 (1952).

65. McLean, F. C. and Hastings, A. B., The State of Calcium in the Fluids of the Body, I. The Conditions Affecting the Ionization of Calcium. J. Biol. Chem., 108, 285-322 (1935).

66. Natelson, S., Microtechniques of Clinical Chemistry, Springfield, Illinois, C. C. Thomas, (1957) 484 pp.

67. Neale, F. C., The Demonstration of The Iron-Binding Globulin (Transferrin) in Serum and Urine Proteins by Use of Iron-59 Combined with Paper Electrophoresis, J. Clin. Pathol., 8, 334-337 (1955).

68. Nicholas, H. C., Diffusible Serum Calcium by High Pressure Ultrafiltration. J. Biol. Chem., 97, 457-463 (1932).

69. Ogryzlo, M. A., Maclachlan, M., Dauphinee, J. A. and Fletcher, A. A., The Serum Proteins in Health and Disease, Am. J. Med., 27, 596-616 (1959).

70. Pauli, W. and Schon, M., Untersuchungen an Electrolyt frein Wasserloseichen Proteinen, Biochem. Z., 153, 253 284 (1924).

71. Persson, I., Concerning the Serum Lipids in Multiple Sclerosis. Acta Psychiat. Neurol. Scand., 33, 103-114 (1958).

72. Peters, T., Giovanniello, T. J., Apt, L. and Ross, J. F., A Simple Improved Method for the Determination of Serum Iron, J. Lab. Clin. Med., 48, 280-288 (1956).

73. Pichler, E. and Reisner, H., Schwankungen des Cholesterinspiegels bei Multipler Sklerose. Wein. klin. Wschr., 51, 1304-1309 (1938).

74. Prasad, A. S. and Flink, E. B., Effect of Carbon Dioxide on Concentration of Calcium in an ultrafiltrate of Serum obtained by Centrifugation, J. Appl. Physiol., 10, 103-107 (1957).

75. _____ and _____, Base-Binding Property of the Serum Proteins, with Respect to Calcium, J. Lab. Clin. Med., 51, 345-364 (1958).

76. Prasad, A. S., Flink, E. B. and Zinneman, H. H., Base-Binding Property of the Serum Proteins with Respect to Magnesium, J. Lab. Clin. Med., 54, 357-364 (1959).

77. Reichstein, T. and Euw, J., Über Bestandteile der Nebennierenrinde. Isolierung der Substanzen Q (Desocy-corticosteron) und R sowie weiterer Stoffe. Helv. Chim. Acta., 21, 1197-1210 (1938).

78. Ressler, N., Uhl, H. and Boyle, A. J., The Relation Between the Second Ionization Potential of a Metal to its Interaction with Serum, J. Colloid Sci., 9, 112-131 (1954).

79. Rona, P., and Takahashi, D., Uber das Verhalten des Calciums in Serum und uber den Gehalt der Blutkorperchen an Calcium. Biochem. Z., 31, 336-334 (1911).

80. Saifer, A., Rabiner, A. M., Oreskes, I. and Volk, B. W., Electrophoretic and Chemical Serum Protein Fractions in Multiple Sclerosis, Am. J. Med. Sci., 225, 287-290 (1953).

81. Schade, A. L. and Caroline, L., An Iron-Binding Component in Human Plasma, Science, 104, 340-341 (1946).

82. Scheinker, I. M., Circulatory Distrubances and Management of Multiple Sclerosis. Ann. N.Y. Acad. Sci., 58, 582-594 (1954).

83. Schoenheimer, R. and Sperry, W. M., A Micro Method for the Determination of Free and Combined Cholesterol, J. Biol. Chem., 106, 745-760 (1934).

84. Shearer, G. D. and McDougall, E. I., Some Observations on Sway-back Disease of Lambs, J. Agr. Sci., 34, 207-212 (1944) "Seen in Abstract Only," C. A., 39, 1670-4 (1945).

85. Shibata, S. and Hasegawa, K., A Simple Method for the Determination of Serum Cholesterol. Igaku to Seibutsugaku, 39, 21-25 (1956) "Seen in Abstract Only", C. A., 52, 6473g (1958).

86. Strain, H. L., On the Combination of Electrophoretic and Chromatographic Adsorption Methods, J. Am. Chem. Soc., 61, 1292-1293 (1939).

87. _____, Chromatographic Adsorption Analysis, Chapter I, New York, Interscience (1941).

88. Surgenor, D. M., Koechlin, B. A. and Strong, L. A., Chemical and Immunological Studies on the Products of Human Plasma Fractionation XXXVII. The Metal-Combining Globulin of Human Plasma. J. Clin. Invest., 28, 73-78 (1949).

89. Surgenor, D. M., Hunter, M. J. and Brown, R., Chapter I, The Nature and Properties of the Enzymes of Normal Human Plasma, J. L. Tullis, Editor, Blood Cells and Plasma Proteins - Their State in Nature, New York, The Academic Press, 1953, 436 pp.

90. Tanford, C., The Effect of pH on the Combination of Serum Albumin with Metals, J. Am. Chem. Soc., 74, 211-215 (1952).

91. Thompson, R. H. S. and Watson, D., Serum Copper Levels in Pregnancy and Pre-Eclampsia, J. Clin. Pathol., 2, 193-196 (1949).

92. Thompson, R. H. S. and Cumings, J. N., Chapter X, Diseases of the Nervous System, Thompson, R. H. S. and King, E. J., Editors, Biochemical Disorders in Human Disease, New York, The Academic Press, 1957, 843 pp.

93. Tiselius, A., A New Apparatus for Electrophoretic Analysis of Colloidal Mixtures. Trans. Faraday Soc., 33, 524-531 (1937).

94. Todd, W. R., The Diffusible Serum Calcium in Normal Individuals and Hypoparathyroid Patients Treated with Parathormone, Dihydrotachysterol and Vitamin D. J. Biol. Chem., 140, cxxxiii (1941).

95. Valle, B. L., The Metabolic Role of Zinc, J. Am. Med. Assn., 162, 1053-1057 (1956).

96. Vlad, A., Gaba, Em. and Gugianu, S., Orientation Studies on the Modifications of Blood Proteins in Multiple Sclerosis. Acad. Rep. Populare Romine, Inst. Neurol. "I.P. Pavov," Studies Cercteri neurol. 3, 217-226 (1958) "Seen in Abstract Only", C. A. 53, 6515d (1959).

97. Volk, B. W., Saifer, A. and Rabiner, A. M., Protein Fractions in Serum and Cerebrospinal Fluid in Multiple Sclerosis. Ann. N. Y. Acad. Sci., 58, 602-612 (1954).

98. Wallenius, G., A Note on Serum Iron Transportation. Scand. J. Clin. and Lab. Invest., 4, 24-26 (1952).

99. Warburg, O. and Krebs, H. A., Uber locker gebundenes Kupfer und Eisen im Blutserum. Biochem. Z., 190, 143-149 (1927).

100. Watchorn, E. and McCance, R. A., Inorganic Constituents of Cerebrospinal Fluid. II. The Ultrafiltration of Calcium and Magnesium from Human Serum. Biochem. J., 26, 54-64 (1932).

101. Weir, E. G. and Hastings, A. B., The Ionization Constants of Calcium Proteinate Determined by the Solubility of Calcium Carbonate, J. Biol. Chem., 114, 397-406 (1936).

102. Wilmont, V. A. and Swank, R. L., The Influence of Low-Fat Diet on Blood Lipid Levels in Health and in Multiple Sclerosis, Am. J. Med. Sci., 223, 25 (1952).

103. Wolff, H. P., Lang, N. and Knedl, M., Untersuchungen mit Cu^{64} uber die Bindung des Kupfer an Serumeiweisskorper. Klin. Wochschr., 33, 186-187 (1955).

104. _____, _____ and _____, Hemopoietically Active Metals as Bound by Different Serum Protein Fractions. Electrophoretic Studies With Iron-59, Copper-64, Zinc-65 and Cobaltin Animals and Normal and Anemic Subjects. Rev. Belge Pathol. et Med. Exptl., 24, 98-100 (1955).

APPENDIX I

APPENDIX I

1) The calculation of results for total serum protein, calcium, cholesterol, magnesium and iron follow the same general formula;

$$\text{Concentration of Unknown} = \frac{\text{Concentration of Standard} \times \text{Titration or Absorbancy Value of Unknown}}{\text{Titration or Absorbancy value of Standard}}$$

Example: Concentration of protein standard = 7.2 g./100 ml.
Absorbancy reading of Standard = 0.325
Absorbancy reading of Unknown = 0.312

$$\text{Total serum protein} = \frac{7.2 \times 0.312}{0.325} = 6.9 \text{ g./100 ml.}$$

2) Protein distribution in g./100 ml. serum is obtained by multiplying the relative portion of each fraction by its total protein value. For example, Table VI, subject 1 shows albumin as 65.8 per cent, Table VII, subject 1 shows a total protein of 6.9 g./100 ml.

0.658 x 6.9 = 4.54 grams albumin/100 ml. serum.

3) Per cent Metal Ion Distribution.- The following data were obtained from an electropheretogram stained with alizarin red dye for the detection of calcium:

Width of Paper Segment(cm.)	Corresponding to Protein Fraction	Absorbancy Reading of Eluate
15	Albumin	0.340
10	alpha-1 globulin	0.083
14	alpha-2 globulin	0.108
14	beta globulin	0.118
27	gamma globulin	0.210
30	Blank	0.122

Corrected albumin absorbancy = $0.340 - \frac{0.122 \times 15}{30} = 0.279$

Sum of all corrected absorbancies = 0.533

Per cent Calcium in albumin = $\frac{0.279 \times 100}{0.533} = 52.4$