EQUILIBRIUM DIALYSIS, MOVING BOUNDARY AND PAPER ELECTROPHORESIS STUDIES ON THE BINDING OF HUMAN SERUM PROTEINS WITH CALCIUM, MAGNESIUM, IRON, AND COPPER IONS

By

Henrietta Marie LeDuc

A THESIS

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

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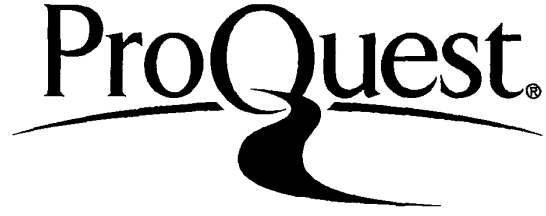

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VITA

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ABSTRACT

Human blood serum contains various proteins among which are albumin and the alpha, beta, and gamma globulins. Serum also contains various metal ion constituents. Among these ions are calcium, magnesium, iron, and copper, all believed to be protein-bound to some extent in serum.

The object of this investigation was to determine the human serum protein fraction (or fractions) associated with each of the four metal ions. The three approaches to the problem involved moving boundary electrophoresis and chemical analysis, paper electrophoresis and selective staining techniques, and equilibrium dialysis studies.

Moving boundary electrophoresis and chemical analyses for total protein, calcium, magnesium, iron, and copper were performed on the sera of 21 normal adult humans. When the results were tabulated and studied, it appeared that the following ion-protein associations occurred: (1) calcium with albumin and globulins, (2) magnesium with albumin, (3) iron with beta globulin, and (4) copper with gamma globulin.

Paper electrophoresis separations of the serum proteins were followed by selective staining procedures for protein and the various metal ions. Calcium was found to be present in all protein components. Strip elution techniques indicated that albumin (about 2/3 of the total protein) and gamma globulin (approximately 1/6 of the total protein) contributed equally in binding 2/3 of the total bound calcium. Magnesium was also found in all serum proteins. Iron appeared to be

present only in beta globulin, while copper and zinc seemed to occur in gamma globulin. Histochemical stains for potassium, phosphate, carbonate, chloride, and sulfate on serum proteins separated by paper electrophoresis failed to locate these ions.

Exhaustive dialysis of whole serum and isolated serum protein solutions in the same buffer medium as that used for electrophoretic investigations indicated that ion-protein binding did occur.

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I. INTRODUCTION

The chemical literature contains vast evidence that a protein may form: (a) a reversible metal complex, (b) an irreversible metal complex, or (c) both a reversible and an irreversible metal complex. The occurrence and extent of such binding have been demonstrated by various experimental methods such as migration in an electric field, changes in spectra, etc.

Human blood serum contains various proteins among which are albumin and the alpha, beta, and gamma globulins. Some of the metal ion constituents of this serum have been found to possess biochemical function. Among these are calcium, magnesium, iron, and copper. The four metal ions are all regarded to be protein-bound to some extent in human blood serum.

The object of this study was to determine and demonstrate the human serum protein fraction (or fractions) associated with calcium, magnesium, iron, and/or copper ions. The investigative approaches involved two types of electrophoretic analysis, chemical analysis, and equilibrium dialysis. One method of electrophoretic analysis utilized the moving boundary technique for serum protein composition together with chemical analysis for serum metal ion content. It was assumed that the correlation of results from these analyses would be indicative of specific protein-ion combinations.

The other method of electrophoretic analysis utilized serum protein separation on filter paper with subsequent selective staining to locate protein and metal. If protein and metal were found to migrate together, it was presumed that they were associated. Exhaustive dialysis of protein solutions together with chemical analysis were performed to substantiate information on protein-metal ion association.

II. HISTORICAL

A. Binding of Metal Ions to Serum Proteins

This review is limited chiefly to studies concerning the binding of calcium, magnesium, iron, and copper with human serum or plasma and isolated blood proteins.

1. Calcium. -- By the method of compensation dialysis (69), it was reported in 1911 that about 40% of the total serum calcium was non-diffusible. The observation was published by Rona and Takahashi (83), and they suggested that this amount of calcium was in some way irreversibly protein-bound. In 1931, Greene and Power (35) accomplished compensation dialysis in vivo by connecting the dialysis tubes to the femoral veins and arteries of dogs. They estimated the blood calcium to be 35-45% bound. A year later, Nicholas (71) reported 36% of the total serum calcium to be nondiffusible as determined by ultrafiltration, while Watchorn and McCance (101) found 43%. Todd (99) in 1941, stated it to be 53%. These variations in result might be attributed to different experimental conditions involving pressure and time.

Equilibrium studies of isolated human serum albumin and globulins with calcium were made by McLean and Hastings (68) in 1935 and by Weir and Hastings (103) in 1936, while Drinker, et al. (21) in 1939, used equine globulins. All suggested that the complex between calcium and the respective proteins can, as a first approximation, be described by

applying the mass action law. The expression as given by Alberty (3) to represent such a series of ion complexes PA_x (x varies from 1 to n) in equilibrium with free protein P and interacting ion A is:

$$PA_{n-1} + A \rightleftharpoons PA_n$$

$$K_n = PA_n/PA_{n-1} + A$$

In general, the calcium studies indicate that one-third to one-half of the total serum calcium is irreversibly protein-bound. Both albumin and globulin are capable of binding calcium reversibly.

2. Magnesium. -- Of the four metal ions herein considered, magnesium has been the least investigated. The reason for this may be due to the assumption that its properties are like those of calcium since the two have similar electronic configurations. Also the lack of interest in magnesium with respect to human pathologies and lack of sensitive analytical methods for its determination may be additional factors.

By in vivo compensation dialysis, Greene and Power (35) in 1931 ascertained the binding of magnesium to be 35-45% of the total, the same as that obtained for calcium. However, the next year by ultrafiltration techniques Watchorn and McCance (101) determined that only 25% of the total was bound, in contrast to 43% for calcium.

3. Iron. -- Barkan (7) observed in 1927 that less than one-fourth of the total iron in human plasma was non-dialyzable. Ten years later, the same investigator with Shales (8) demonstrated that when serum was one-half saturated with ammonium sulfate, the iron coprecipitated quantitatively with the globulins. In 1934, Fowweather (28) obtained

1.5-2.5 times more iron from ashed serum than from the protein-free filtrate. Kitzes, et al. (47), noticed in 1947 the equal distribution of iron between filtrate and precipitate upon trichloracetic acid treatment of serum. In the same year, Holmberg and Laurell (43) (in contrast to Barkan) reported that the iron-bonding serum component was not precipitated with ammonium sulfate. They concluded that iron was bound to albumin as did Eisler and co-workers (24). The latter, in 1936, found iron migrated with albumin during moving boundary electrophoresis of serum proteins.

Cohn (16) showed in 1948 that serum beta globulin combined with iron to form a pink complex. Four years later, Koechlin (53) demonstrated that the reaction involved one molecule of the globulin with one or two atoms of iron.

Many reports in the literature on this subject show contradiction or confusion. Although they agree that iron is partially protein-bound, it has not been established whether the protein involved is albumin or globulin. Serum beta globulin has been shown to combine with iron.

4. Copper. -- Warburg and Krebs (100) discovered in 1927 that copper is loosely bound in serum and that binding is altered by lowering pH. A year later, Abderhalden and Möller (1) stated that copper was not dialyzable. In 1937, Boyden and Potter (9), who concluded all plasma copper was bound, investigated the effect of changing pH with hydrochloric and sulfuric acids. They suggested that bound copper occurred in more than one form.

Eisler, et al. (24), investigated the migration of copper during electrophoresis of serum in 1936 and came to regard it as bound to

albumin. However, according to the 1947 report of Holmberg and Laurell (43), serum copper was coprecipitated with the globulins by one-half saturation of serum with ammonium sulfate.

Tanford (93) in 1952 analyzed bovine serum albumin polarographically in the presence of divalent copper and determined complex-formation. Klotz and co-workers (52) confirmed this finding in 1955 with bovine albumin by absorption spectra analysis, but could not detect a like reaction when human albumin was employed.

By radioactive isotope tracer techniques, Wolff, et al. (105), in the same year, also found species differences. They decided that copper transport in serum involved nonspecific loosely-bound complexes. An examination of human serum protein precipitated by heavy metal ions led Astrup and associates (5) to the discovery in 1954 that gamma globulin bound the cupric ion firmly and irreversibly.

In 1938, Mann and Keilin (66) isolated a metal protein complex from horse serum (haemocuprein) which had a 0.34% copper content. Ten years later, Holmberg and Laurell (44) isolated a similar protein (ceruloplasmin) from human serum and characterized it as a metallo-globulin.

Cohn (16) demonstrated in 1948 that a human serum beta globulin combined with copper to form a blue-green complex. In 1952, Koechlin (53) observed that the reaction seemed to involve one molecule of protein with one or two atoms of copper.

These studies show that all serum copper is bound in the species investigated. It may or may not be associated with albumin.

In contrast to bovine, human serum albumin does not react with copper whereas beta globulin does. A copper-globulin compound has been isolated from human and equine sources. Gamma globulin binds divalent copper irreversibly.

B. Electrophoresis

1. Moving boundary. -- The need for effective separation and analysis of serum proteins has been uniquely met in electrophoresis. A chemical method is inadequate to deal with such mixtures whose components are similar and whose distinctive properties may be destroyed by their chemical separation. The physical method, electrophoresis, uses the characteristic charge on each protein and the electrical forces applied are assumed to be harmless.

In 1937, Tiselius (95) described an apparatus which made possible the study of protein mixtures. Optical systems, utilizing the Schlieren lens, were soon adapted by Longsworth (62), Philpot (74), and Svensson (91) to simplify quantitative analysis. The extensive use of electrophoresis in biology and medicine is shown by a bibliography published late in 1955 which lists 3200 papers (42).

In moving boundary electrophoresis, a sharp initial boundary between protein and solvent is formed and observed as an electric field is applied. If the protein is homogeneous in mobility, the boundary remains single. If a mixture of proteins is present, each may move at a different speed depending on its charge. The initial boundary may separate into several boundaries, each representing a protein of

different mobility. The number and position of the boundaries are observed by change in refractive index produced by change in protein concentration.

In the quantitative Tiselius electrophoresis procedure, the proteins are separated and the pattern they form is photographed and enlarged. Ordinates are drawn from the lowest points between two peaks to the baseline (98) and the areas under each of the peaks are measured. If the specific refractive increment of each protein is known, the concentration of the components in the mixture can be determined.

Application to serum proteins. In 1937, Tiselius (96), using a basic phosphate buffer, found purified horse serum globulin contained three fractions which he named alpha, beta, and gamma in the order of their mobilities. The next year, Stenhagen (89) showed human serum contained albumin and three globulins similar to the equine. Longsworth (64), in 1942, employed a more basic barbiturate buffer to resolve alpha globulin into two fractions which he designated $alpha_1$ and $alpha_2$.

Cohn and associates (12,13,14,15) have used electrophoretic analysis as a control method in fractionation. They obtained purified fractions and information correlating chemical and biological properties with electrophoretic behavior. Tiselius (97) found antibody activity in gamma globulin and noted immune serum had more of this component than normal. By correlating chemical analysis with electrophoresis, Longsworth and MacInnes (63) showed that serum lipid was associated with

beta globulin. Seibert, et al. (86), reported concomitant increases in polysaccharides and alpha$_2$ globulin.

Serum protein-anion interaction studies. The mobilities of serum proteins and the number of fractions resolved electrophoretically depend upon ion species, pH, and ionic strength of the buffer. Thusly, Tiselius (96) fractionated alpha, beta, and gamma globulins in phosphate buffer of pH 8.0, while Longsworth (64) resolved alpha globulin into alpha$_1$ and alpha$_2$ in barbital buffer of pH 8.6. In 1951, Fischer, et al. (27), showed beta globulin consisted of two protein entities in a barbital-caprylate medium. These differences in mobility and resolution have been attributed to reactions of buffer anions with proteins (3).

Electrophoresis has been used to investigate reactions of proteins with other substances. Patterns and mobilities are obtained before and after equilibration with the reactant and changes indicate reaction. By these techniques, Rawson (78) in 1943 reported albumin was the only human plasma protein capable of reaction with diazo dyes. Two years later, Putnam and Neurath (76) showed the combination of isolated equine serum albumin with detergent anions. Also in 1945, Ballou and associates (6) concluded anion-human albumin association from studies with salts of the lower fatty acids. Alberty and Marvin (2) in 1951 found bovine albumin combined with chloride ion.

Their findings confirmed the equilibrium dialysis results of Scatchard, et al. (85), who studied bovine albumin combination with chloride ion in 1946. Klotz and co-workers investigated the combination of the same protein with azosulfonate dyes (49), sulfonate dyes (50),

and methyl orange (51). Spectral analysis demonstrated reaction in each case. In 1950, Karush (45), using dialysis, proposed that 22 binding sites per bovine albumin molecule were involved in reaction with an anionic azo dye.

2. Paper electrophoresis. The high cost of moving boundary Tiselius electrophoresis apparatus has precluded its use by many investigators. However, the development of simple and comparatively inexpensive equipment using paper saturated with buffer as a separation medium has made this technique more widely available.

In 1939, von Klobusitsky and König (48) observed the migration of a yellow chromoprotein from a snake venom on paper in a low-voltage direct current apparatus. Several years later, independent and almost simultaneous studies led to the development of several types of apparatus. Requisite to any type are: two vessels containing buffer and electrodes, paper which bridges the vessels and dips into the buffer, a source of potential, and an enclosure to decrease evaporation. To demonstrate the practicability of the procedure, in 1948 Wieland and Fischer (104) separated amino acid mixtures with a simple catenary paper suspension. Durrum (22) extended the technique two years later to peptides and serum proteins using a ridge-pole suspension. Also in 1950, Cremer and Tiselius (18) described a sandwich-type apparatus in which the paper was held between glass plates and immersed in chlorobenzene. This was soon modified to eliminate the chlorobenzene by clamping the paper between plates and sealing the edges (58). McDonald, et al. (67), designed an apparatus which held the paper taut and horizontal on a plastic frame.

Besides isolating the paper from the atmosphere to decrease chromatographic effects, the sample was applied to paper pre-wetted with buffer. Liquid levels in the buffer vessels were maintained equal by means of a connecting tube. Some investigators have advocated temperature control (18,56,57,67). In the electrophoresis procedure, a drop or streak of serum was placed on the edge of the paper, direct current was applied for a period of time after which the paper was removed from the apparatus and dried. Separated components were located by a specific protein dye and estimated by dye elution or direct densitometry of the paper.

Parallel studies of the paper and moving boundary electrophoresis methods have met with various degrees of success. Some investigators found it necessary to use derived factors to achieve comparability (18,33,56,58,88). Others reported that rigidly standardized techniques of sample application, drying, and dyeing gave comparable results directly (30,36,40,54,61,65).

Selective staining of parallel strips for protein and for lipid or carbohydrate content substantiated earlier correlation studies using moving boundary apparatus and chemical analysis. The concept of serum lipid transport by beta globulin (63) has been confirmed (23,25,59,72, 84,92). Similarly, the carbohydrate-alpha globulin association (86) has also been demonstrated (55).

Protein ion interaction studies. Isotope tracer techniques combined with paper electrophoresis have led to some interesting results. Thus in 1952, Köiw and Grönwall (55) corroborated the formation of the

iron-beta globulin complex as reported by Cohn (16) in 1948. Iodine transport by albumin and alpha globulin was reported (60,82), while gold was found to be associated with the alpha and beta globulins (87).

III. EXPERIMENTAL

A description of the apparatus, materials and reagents, procedures, and results will be presented in this section. All solutions were prepared with reagent grade or C. P. chemicals, unless otherwise specified. Each solid was weighed on an Ainsworth chain weight balance and dissolved in water distilled from all-glass apparatus. All photometric measurements were made with a Cenco-Sheard-Sanford Photelometer. Computations were made with the aid of a Fridén calculating machine.

A. Apparatus

1. Moving Boundary Electrophoresis

The Tiselius apparatus and accessories were purchased from the American Instrument Company, Inc., Silver Spring, Maryland. Included were the Aminco-Stern electrophoresis instrument (Figures 1 and 2), dialysis units, motor driven compensator with syringe, three-way stopcock provided with rubber tubing, standard clinical cell with holder, silver-silver chloride electrodes, film holder, enlarger-darkroom unit, and the Keuffel and Esser compensating polar planimeter.

2. Paper Electrophoresis

The LKB apparatus consisting of a power unit and horizontal strip equipment (Figure 3) was purchased from Ivan Sorvall, Inc., Norwalk, Connecticut. A wooden rack held the paper horizontal while it was dried

with the aid of an infrared lamp. A 10 x 16 inch Pyrex cake pan was used to contain the various staining solutions.

3. Exhaustive Dialysis

Dialysis units were the same as those used for the moving boundary procedure previously mentioned. Each dialyzer consisted of a Pyrex cylinder (4 inch diameter x 6 inch height) with a removable stainless steel cover. Details of design were given by Reiner and Fenichel (79).

B. Materials and Reagents

1. For Moving Boundary Experiments

a. Serum. -- Participating subjects were 21 normal adult humans, 15 female and 6 male, aged 20-54 years. Seventy-five ml. of blood was drawn from each fasting subject by venepuncture into three sterile syringes. The blood was transferred immediately to test tube, allowed to clot two to three hours, and centrifuged 15 minutes at 2,000 r.p.m. The supernatant serum was siphoned off, centrifuged, and decanted into sterile test tubes. If not to be used immediately, serum was deep-freeze stored at -5°. According to the data in Table I no significant change in protein or metal ion content occurred during such storage.

b. Materials for electrophoresis.

Dialysis membrane. -- Visking cellulose tubing 1 1/8 inch flat diameter was used for serum dialysis.

Buffer. -- Veronal buffer of pH 8.6 and μ 0.1 described by Longsworth (64) was prepared by dissolving 5.526 g. diethylbarbituric

acid (U.S.P.) and 61.85 g. sodium diethylbarbiturate (U.S.P.) in three liters of water.

Saturated potassium chloride. -- This solution of electrolyte, introduced into the electrode vessels, served as a salt bridge.

Film. -- Schlieren cylindrical lens patterns were recorded on 4 x 5 inch Eastman Contrast Process Panchromatic film.

Photographic solutions. -- Kodak D-8, SB-1, and F-5 were used in film processing.

c. Protein analysis. -- Reagents for the biuret method were prepared as directed by Kingsley (46) and Weichselbaum (102).

Standard protein solution containing 6.61 g. protein per 100 ml. was prepared by diluting two ml. Armour's Protein Standard Solution (9.91 g. protein per 100 ml. determined by micro-Kjeldahl analysis) with one ml. water.

Sodium sulfate, 23% (w/v), was prepared by dissolving 230 g. of anhydrous solid in water and diluting to one liter. This solution was stored at 37°.

Stock biuret reagent was made by dissolving 45 g. sodium potassium tartrate, 15 g. cupric sulfate pentahydrate, and 5 g. potassium iodide in 0.2 N sodium hydroxide made to one liter.

Working biuret reagent one fifth the concentration of the stock solution was prepared from 200 ml. by dilution to one liter with 0.2 N sodium hydroxide containing 5 g. potassium iodide per liter.

d. Metal ion analytical reagents.

Calcium. -- The reagents given in the Clark-Collip modification (11) of the Kramer-Tisdall method (56) were used. They are as follows:

Ammonium oxalate, 4% (w/v), was prepared by dissolving 4 g. in water and diluting to 100 ml.

Ammonium hydroxide, 1.2% (v/v) was obtained by diluting 2 ml. concentrated reagent to 100 ml.

Sulfuric acid, 2 N, was prepared by diluting 56.6 ml. concentrated reagent to one liter.

Potassium permanganate, 0.01 N, was prepared and standardized against a weighed amount of sodium oxalate.

Magnesium. -- Solutions described and used by Garner (31) were employed in this analysis.

A stock standard (1 mg. magnesium per ml.) was prepared by dissolving 10.13 g. magnesium sulfate heptahydrate in water, adding 0.5 ml. chloroform, and diluting to one liter.

The working standard (0.02 mg. magnesium per ml.) consisted of 2 ml. of stock diluted to 100 ml.

Trichloracetic acid, 10% (w/v), was obtained by dissolving 100 g. in water and diluting to one liter.

Gum ghatti, ca 0.1% (w/v), was prepared by suspending overnight 1 g. of solid tied in muslin in one liter of water. The residue was discarded and 2 ml. chloroform added.

Titan yellow (Allied Chemical and Dye Corporation), 0.05% (w/v), was made by dissolving 50 mg. in water, diluting to 100 ml., and filtering. This solution was freshly prepared for each determination.

Sodium hydroxide, 4 N, was furnished by dissolving 160 g. in water and diluting to one liter.

Iron. -- All reagent solutions except the standard were prepared according to directions given by Kitzes, et al. (47). The standard solution was made as described by Wong (106).

Iron stock standard (0.1 mg. iron per ml.) was prepared by dissolving 0.7020 g. crystalline ferrous ammonium sulfate in 50 ml. water, adding 20 ml. 10% (v/v) sulfuric acid and enough 0.1 N potassium permanganate to oxidize the ferrous ion. Dilution to one liter followed.

Working iron standard (5 µg. iron per ml.) was obtained by diluting 5 ml. of above stock solution to 100 ml.

Trichloracetic acid, 25% (w/v), was made by dissolving 50 g. in water and diluting to 200 ml.

Para-nitrophenol (Eastman Kodak Company), 0.1% (w/v), was prepared by dissolving 100 mg. in 100 ml. water.

Ammonium hydroxide, 6 N, was had by diluting 80 ml. of concentrated reagent to 200 ml.

Acetate buffer, 1.6 M, pH 4.58 (checked by pH meter), was prepared by dissolving 33.4 g. anhydrous sodium acetate in 27.2 ml. glacial acetic acid and water and diluting to 250 ml.

Thioglycolic acid (Eastman Practical Grade, "95 + %") was used without dilution.

Alpha, alpha'-bipyridine (Eastman Kodak Company), 0.2% (w/v), was produced by dissolving 0.2 g. in 5 ml. glacial acetic acid and diluting to 100 ml. with water.

Copper. -- The reagent solutions described by Gubler and associates (37) were used for this analysis.

Copper stock solution (100 µg. copper per ml.) was prepared by dissolving 0.3928 g. copper sulfate pentahydrate in water and diluting to one liter.

Two standard copper solutions were prepared by diluting 0.4 ml. stock to 50 ml. (0.8 µg. per ml.) and 1.0 ml. stock to 50 ml. (2.0 µg. per ml.).

Hydrochloric acid, 2 N, was obtained by dilution of 85.5 ml. concentrated reagent to 500 ml.

Trichloracetic acid, 20% (w/v), was prepared by dissolving 100 g. in water and diluting to 500 ml.

Sodium pyrophosphate, saturated, was obtained with 100 g. of solid and 200 ml. water.

Sodium citrate, saturated, was prepared by adding 350 g. to 200 ml. water.

Ammonium hydroxide, 39% (v/v), was obtained by diluting 200 ml. concentrated reagent with 100 ml. water.

Sodium diethydithiocarbamate (Fisher Scientific Company), 0.1%, was prepared by dissolving 100 mg. in water and diluting to 100 ml.

2. Materials and Reagents for Paper Electrophoresis

a. Serum. -- Portions of the same human blood serum as described for the preceding experiments were used.

b. Buffer. -- The Longsworth (64) pH 8.6, µ 0.1, veronal buffer was employed. It was used with Whatman No. 1 paper cut to 18 x 44 cm. rectangular sheets as the supporting medium.

c. Protein stain. -- The bromphenol blue staining bath and rinse solutions as described by Durrum and co-workers (23) were used.

The bath was prepared by dissolving 100 mg. bromphenol blue (Nutritional Biochemical Corporation) in 50 ml. glacial acetic acid, adding 50 g. mercuric chloride, and diluting to one liter with water.

Acetic acid, 2% (v/v), rinse solution, was obtained by diluting 20 ml. glacial acetic acid to one liter.

Sodium acetate, 0.5% (w/v) in acetic acid, prepared by dissolving 5 g. in one liter of above 2% acetic acid, was the final rinse.

Ethanol, 50% (v/v), was used to elute the fixed stain.

d. Metal ion staining reagents.

Calcium. -- The alizarin red S histochemical stain developed in 1952 by Dahl (19) was employed to locate calcium upon the filter paper. In 1955, Natelson and Penniall (70) demonstrated that the calcium-alizarin color in solution obeyed Beer's law.

Alizarin red S (Eberbach & Son Company), 1% (w/v) was prepared by dissolving 10 g. in a liter of water containing 1 ml. concentrated ammonium hydroxide.

Ammonium hydroxide, 29% (v/v), obtained by diluting 500 ml. concentrated reagent with 500 ml. water, was used for elution.

Magnesium. -- The staining solution for the identification of this metal ion upon filter paper was of the following composition:

56 ml. 0.1% (w/v) gum ghatti

84 ml. 0.05% (w/v) Titan yellow

112 ml. 4 N sodium hydroxide.

These three reagents were among those used in the Garner method (31) for the colorimetric determination of magnesium in serum. The volume proportions 1 : 1.5 : 2 in the above order were the same as those given in Garner's analytical procedure.

Iron. -- A 20% (w/v), potassium thiocyanate reagent used as a stain for iron was prepared by dissolving 50 g. in 240 ml. water and adding 10 ml. concentrated hydrochloric acid.

Copper. -- The stain for detecting this ion was obtained by mixing the following solutions:

50 ml. saturated sodium pyrophosphate

50 ml. saturated sodium citrate

100 ml. 39% (v/v) ammonium hydroxide

50 ml. 0.1% (w/v) sodium diethyldithiocarbamate

The above four reagents and the volume proportions of 1 : 1 : 2 : 1 were as used in the Gubler method (37) for serum copper determination.

3. Materials and Reagents for Dialysis

Dialysis membrane. -- Visking cellulose tubing 1 1/8 inch flat diameter was used in all experiments.

Buffer. -- The Longsworth (64) pH 8.6, μ 0.1, veronal buffer served as solvent for the solid protein fractions and as external liquid in dialysis.

Serum. -- Portions of the human sera as described for moving boundary experiments were pooled both for dialysis and for the preparation of protein solutions Albumin II, Albumin III, and Mixed Globulins which are described subsequently.

Albumin I. -- A lyophillized sample of 99% human albumin made from the Cohn (15) Fraction V and labeled as rework #127 was obtained from the Biological Products Section of the Division of Laboratories of the Michigan Department of Health. A 50 ml. solution, designated Albumin I, was prepared by dissolving 6.52 g. of the material in veronal buffer.

Albumin II. -- Forty-eight g. ammonium sulfate was slowly added with stirring to 96 ml. pooled human sera. After 30-minute centrifugation, the clear supernatant albumin solution was siphoned off and placed in a Visking dialysis sac. Pervaporation (94) for four days resulted in deposition of salt on the outside of the membrane which was removed by washing with water every 12 hours. When finished there remained 52 ml. clear protein solution within. This was labeled as Albumin II solution and found to consist of 83% albumin by electrophoretic analysis.

Albumin III. -- The protein solution referred to as Albumin III was obtained from 100 ml. of human serum by the low temperature methanol precipitation procedure of Pillemer and Hutchinson (75) to remove globulins. Following concentration by pervaporation of the supernate to 43 ml., electrophoretic analysis showed the preparation to contain 93% albumin.

Mixed Globulins. -- This preparation was made by dissolving the Pillemer-Hutchinson globulin precipitate (above) in a minimum amount of saline solution (0.9% sodium chloride) and diluting to 50 ml. with veronal buffer.

Gamma Globulin. — A rework sample marked as #104 made from the Cohn (15) Fractions II and III was also generously provided by the Biological Products Section of the Division of Laboratories of the Michigan Department of Health. Electrophoretic analysis showed 97% gamma globulin. A 50 ml. solution was prepared by dissolving 1.96 g. of the lyophillized material in 0.1 M veronal buffer of pH 8.6.

Paper strip analytical reagents. -- The same reagents as previously described for total protein and metal ion determinations in moving boundary experiments were used.

C. Experimental Procedures

Results of moving boundary electrophoretic analysis of proteins have been correlated with certain other serum constituents. Thus, in 1937 Tiselius (97) observed that serum high in gamma globulin also gave high values for immune bodies. This serum protein fraction has since been used for protection against poliomyelitis, measles, and hepatitis and from it several types of antibodies have been isolated (34). Longsworth and MacInnes (63) noted that serum with high beta globulin content possessed a large amount of lipid and proposed that a lipoprotein complex existed. Seibert and co-workers (86) observed that serum high in alpha$_2$ globulin also had a high polysaccharide content and regarded the two to be associated. The existence of these globulin-lipid and globulin-carbohydrate combinations has been demonstrated directly by paper electrophoresis (23,25,55,59,72,84,92).

1. Moving Boundary Methods

The experimental approach adopted for indication of protein-metal ion association involved correlations between amounts of protein components obtained by moving boundary electrophoresis and metal ions present in serum. In this connection, serum of 21 normal human subjects was used for investigation.

a. Electrophoresis techniques

Dialysis. -- Ten ml. serum was diluted with ten ml. of veronal buffer to produce an approximate protein concentration of 3.2-3.8 g. per 100 ml. The diluted serum was osmotically equilibrated by dialysis against 800 ml. of the same buffer solution for 24 hours at 2°.

Electrophoresis. -- Buffer, saturated potassium chloride, and dialyzed serum were introduced into the cell in the prescribed manner. By visual observation, initial boundaries were set two cm. from the lateral edges of the screen. Electrophoresis at 15 milliamperes and 360 volts was continued until maximum separation of protein components was achieved. The time varied from 90 to 120 minutes (5400 to 7200 seconds).

Photography. -- Negatives of ascending and descending patterns were obtained by four to five second exposures of the panchromatic film and proper processing. Examples of resulting patterns are given in Figure 4. Enlarged images, 3 X linear magnification, were traced on paper. (See Figure 5).

Pattern area measurement. -- Ordinates were drawn on the enlargement from the minima between adjacent peaks to the baseline as suggested

by Tiselius and Kabat (98). Each of these separated areas was measured planimetrically. Figure 5 shows an enlarged ascending pattern with ordinates drawn. The equation for calculation is given in Appendix I.

Both ascending and descending patterns were measured. The values obtained from the descending were discarded because some separations were not distinct. (See Figure 4) Ideally, ascending and descending patterns would be mirror images. In practice, however, the peaks in the descending patterns are not as steep. This may result in incomplete resolution of albumin (the largest component) and alpha$_1$ (the smallest) and of gamma globulin and the epsilon anomaly, a nonprotein boundary which represents a buffer gradient that remains near the initial descending boundary.

The average of three planimetric determinations on the ascending pattern of each subject is reported in Table IIa.

b. Total protein determination. -- The Kingsley method (46) with Weichselbaum's (102) biuret reagent was used for total serum protein determinations. The procedure is based on the color reaction between peptide linkages and basic copper sulfate. The determination involves photometric comparison of the protein content of unknown human serum with known bovine serum and assumes identical species response.

Suspensions of human and bovine serum were prepared for total protein analysis by mixing 0.5 ml. of each serum with 9.5 ml. 23% sodium sulfate. Four ml. of working biuret reagent was added to each of the following: first, 2 ml. water (blank); second, a 2 ml. portion of human serum suspension (unknown); and third, a 2 ml. portion of bovine serum

suspension (known). After 15 minutes, the photometer with green filter (central maximum 525 mμ) was set to 100 with the blank (I_0) and values were obtained for human (I_u) and standard bovine (I_k) sera. The values were converted to absorbancy units by the relationship: $A_s = \log_{10} \frac{I_0}{I}$. The equation for the calculation of total serum protein is given in Appendix I.

Figure 6a shows that the concentration-absorbancy relationship follows Beer's law. The results of serum protein analyses are reported in Table IIb.

c. Procedures for serum metal ion determinations

Calcium. -- The Clark-Collip modification (11) of the Kramer-Tisdall (56) method was used. In this analysis, calcium oxalate was precipitated directly from dilute serum, converted to oxalic acid, and titrated with 0.01 N potassium permanganate solution (41). Results are reported in Table III.

Magnesium. -- The method developed by Garner (31) for serum or plasma was employed. Basically, the procedure involves the release of magnesium associated with serum proteins by acid denaturation and precipitation. The formation of a colored complex between magnesium and Titan yellow in the filtrate allows the photometric comparison with standards.

Four ml. serum was mixed with 8 ml. water and 4 ml. 10% trichloracetic acid, allowed to stand five minutes; then the filtrate through Whatman No. 44 paper was collected. Two magnesium standards (corresponding to 1 and 2 mg. per 100 ml. serum) were also prepared; one contained 1 ml. working standard and 5 ml. water, the other 2 ml. working

standard and 4 ml. water. A blank consisted of 6 ml. water. Two ml. trichloracetic acid was added to each standard and the blank. One ml. 0.1% gum ghatti, 1.5 ml. of 0.05% Titan yellow, and 2 ml. 4 N sodium hydroxide were added to an 8 ml. portion of collected serum filtrate, standards, and blank. After setting the photometer (green filter) to 100 with the blank, readings were obtained for serum and standards. These results were converted to absorbancy units as previously described. A linear curve from the two standard solution values was constructed showing magnesium ion concentration in mg. per 100 ml. versus corresponding absorbancies. Since the standards were prepared as above, serum concentrations within this range were read directly from the curve. Figure 6b is a typical standard absorbancy curve for magnesium. The analytical results are given in Table III.

Iron. -- The colorimetric method for serum ferric ion analysis described by Kitses and associates (47) was utilized. In principle, the method involves the heat release of iron from protein, pH adjustment of the protein-free supernate, reduction to the ferrous state by thioglycolic acid, and development of a color by this form with alpha, alpha'-bipyridine.

Three ml. water was mixed with 4 ml. human serum in a centrifuge tube and heated to opacity in a boiling water bath. After cooling in a 5-10° water bath, 2 ml. 25% trichloracetic acid was added and contents mixed. The tube was next heated in a 90-95° water bath for 3 minutes and cooled again. After 5 minutes of centrifugation at 2,500 r.p.m., the supernate was decanted into a 75 ml. Nessler tube pre-calibrated to a

15 ml. volume. The protein residue in the centrifuge tube was washed with 4 ml. water and 1 ml. trichloracetic acid. Then the afore-described procedure was repeated. The washing was combined with the original supernate. At this point, standard ferric ion solutions were prepared in similar Nessler tubes by introducing 1, 2, and 4 ml. working standard (containing 5 µg. per ml.) and 3 ml. trichloracetic acid. A blank consisted of 3 ml. of the acid.

A drop of 0.1% para-nitrophenol indicator was added to the serum filtrate, the 3 standards, and the blank. Ammonium hydroxide (6 N) was added dropwise to each solution until yellow. After adding 1 ml. of pH 4.58 acetate buffer, water was supplied to make a thoroughly mixed total volume of 15 ml. Finally, two drops of thioglycolic acid and 1 ml. 0.2% alpha, alpha'-bipyridine reagents were added to each tube. The photometer with green filter was adjusted to 100 for the blank, and readings for serum and the 3 standards were recorded. These were converted to absorbancy units and a standard curve was drawn as is presented in Figure 7a. In order to express serum iron in µg. per 100 ml. serum, the interpolated concentrations were multiplied by 25. Analytical findings for serum iron content are presented in Table III.

Copper. -- The procedure described by Gubler, et al. (37), was adapted for the determination of serum copper. Essentially, it involves the release of copper from protein by acid and subsequent protein precipitation. Pyrophosphate and citrate remove interfering iron and a colored copper-carbamate complex is produced.

Two ml. serum was mixed with 2 ml. 2 N hydrochloric acid and allowed to stand 10 minutes. Upon the addition of 2 ml. 20% trichloracetic acid, mixing and standing time were repeated. Following 45 minutes of centrifugation at 3,000 r.p.m., the supernate was decanted into a 1 x 9 cm. test tube. Two ml. of 2 N hydrochloric acid and 2 ml. 20% trichloracetic acid were also added to 2 ml. of each of two standard copper solutions (0.8 and 2.0 µg. per ml.) and to a 2 ml. water blank. All tubes contained a volume of 6 ml. Four ml. portions of above serum supernate, standards, and blank were transferred to photometer cuvettes. Four-tenths ml. saturated sodium pyrophosphate, 0.4 ml. saturated sodium citrate, and 0.8 ml. 39% ammonium hydroxide were added to each cuvette. Following mixing and setting the photometer with blue filter (central maximum 410 mµ) to 100 with the blank, readings in the absence of color reagent were noted for serum and the standards (I_1) to correct for the innate color of the serum filtrate.

Four-tenths ml. 0.1% sodium diethyldithiocarbamate was added to all cuvettes and the second set of photometer readings was obtained (I_2). Results were converted to absorbancy units and tabulated as A_{S1} and A_{S2} values for before and after color development of serum and standards. The calculation of the quantity ($A_{S2}-A_{S1}f$), the color absorbancy of the copper-carbamate complex corrected for volume change, is shown in Appendix I. From a plot of ($A_{S2}-A_{S1}f$) versus µg. copper/100 ml. which results in the standard curve, the serum copper content was read directly. Figure 7b illustrates such a standard copper curve. Results of determinations of serum copper values in µg./100 ml. are given in Table III.

2. Procedures for Paper Electrophoresis and Staining

Since direct demonstration by isolation of human serum lipo- or glyco-protein fractions upon paper had been accomplished, it was deemed feasible to extend this technique to metal ion-proteins. Filter paper electrophoresis was used to separate the serum proteins. Serum samples from the aforementioned 21 normal subjects were used in 38 paper runs. From each sheet a strip was stained to locate proteins and other strips were stained to locate metal ions.

a. Electrophoresis. -- Six hundred ml. of pH 8.6, u 0.1 veronal buffer was placed in each electrode vessel of the paper electrophoresis apparatus. A liquid junction, established between vessels by siphon, maintained liquid levels. Paper was inserted and wetted by the buffer solution due to capillarity. A 0.2 ml. sample of undiluted serum was streaked across the end of the paper 8 cm. from the horizontal edge adjacent to the cathode. (See Figure 8). The variable resistance was adjusted to apply 200 volts and 2.4 milliamperes. In all cases, electrophoresis was continued for 14 hours at 5°. The paper, removed from the apparatus, was dried in air with the aid of an infra-red lamp. Of the original 18 x 44 cm. rectangular sheet, the 10 x 30 cm. mid-section was retained and cut longitudinally into five strips, each 2 cm. wide and 30 cm. long. (See Figure 9a).

b. Protein staining. -- One 2 x 30 cm. strip from a run on human serum was placed in 250 ml. of bromphenol blue staining bath for six hours with occasional agitation. Rinsing as described by Durrum, et al. (23) was accomplished by three 5-minute immersions in 250 ml. 2% acetic

acid and a final 10-minute immersion in 250 ml. 0.5% sodium acetate in 2% acetic acid. The paper was again dried. The strip showed five blue areas on a white background. Figure 10 indicates the locations of these colored areas.

On the same strip, as shown in Figure 9b, the line of sample application and 0.25 cm. either way was designated as zero migration distance. From this 0.5 x 2 cm. area, transverse segments (cross-pieces) 0.5 cm. wide were marked, assigned migration distances, and cut off for elution. The stain from each segment was eluted by a 30-minute immersion in two ml. of 50% (v/v) ethanol. After photometry (green filter) of the segment eluates, a plot of migration distance versus eluate absorbancy was made. A resulting representative plot from the paper electrophoresed human serum proteins is shown in Figure 11. The curve is similar to a moving boundary pattern.

Quantitative analysis of the paper electrophoresis pattern for protein distribution followed the same procedure as that used in moving boundary electrophoresis. Ordinates were drawn from the minima between peaks to the baseline (zero absorbancy), separated areas were measured with the planimeter, and the relative amounts of the serum proteins were calculated.

c. Detection of metal ions on paper. -- Staining techniques upon paper were attempted for numerous of the serum ions. In addition to the four metal ions initially reported in this investigation, zinc was also successfully located. No colors were obtained when stains for potassium, phosphate, carbonate, chloride, and sulfate were tried.

Calcium. -- For qualitative work, a strip 2 cm. wide was immersed two minutes in the 1% alizarin red S staining bath, rinsed briefly (15 seconds) six times with water, and dried. Five orange-pink areas on a pale pink background resulted. Figure 10 shows the positions of these calcium-alizarin areas.

For the quantitative distribution of calcium among the serum proteins, a 6 x 30 cm. strip was stained for calcium as just described. Migration distances (in 0.5 cm. units) were marked as in the protein procedure. The stain from each 0.5 x 6 cm. segment was eluted by 30-minute immersion in three ml. of 29% ammonium hydroxide. Photometry (green filter) of eluates and graphing of results with migration distance were done as previously described for serum proteins. The findings are represented in Figure 12.

Magnesium. -- Several 2 x 30 cm. strips were immersed in the Titan yellow staining bath for periods of time ranging from two minutes to four hours. No matter how long the immersion, red spots on a yellow background were visible only when the paper was wet with staining solution but disappeared during drying. Figure 10 shows the locations of the colored areas on the wet paper strips.

Iron. -- Another strip from a serum paper electrophoresis run was stained in 20% thiocyanate solution for 15 minutes, rinsed with five changes of water, and dried. The position of the single pink area of iron-thiocyanate complex that resulted is shown in Figure 10.

Copper. -- A 2 x 30 cm. strip was immersed for 15 minutes in the diethyldithiocarbamate copper staining bath and dried. The position of the one resulting pale yellow area is represented in Figure 10.

Zinc

Historical. In 1924, Pauli and Schön (73) demonstrated an association of equine albumin with zinc ion by conductivity measurements. In 1950, Cohn and co-workers (17) reported that the addition of zinc ion to plasma effected protein precipitation with less alcohol and that the zinc could be removed completely from the proteins. Gurd and Goodman (39) in 1952 showed a totally reversible reaction when human serum albumin was equilibrated with zinc chloride solutions. The first precipitate formed in the reaction of bivalent zinc with human serum was found to contain albumin and gamma globulin by Ressler, et al. (81), in 1954. Thus, it has been demonstrated that albumin and globulins react reversibly with zinc ions.

Staining reagents. The solutions to locate this ion on paper were prepared from directions given by Glick (32).

Sodium nitroprusside (Eastman Kodak Company, Practical Grade), 10% (w/v), was furnished by dissolving 25 g. in water and diluting to 250 ml.

Potassium sulfide "N.F.", 2.5% (w/v), was prepared by dissolving 6.25 g. in 250 ml. water.

Procedure. A paper strip was stained 15 minutes with 10% sodium nitroprusside solution at 50°. Excess reagent was removed by washing 15 minutes with running distilled water. A one-minute immersion in 2.5% potassium sulfide yielded a single lavender area. Its location is indicated in Figure 10.

Attempted spectrographic analysis for metal ions on paper.

Attempts were made to identify spectrographically the metal ion residues in serum proteins separated by paper electrophoresis. A 0.2 ml. serum sample was separated on Whatman No. 1 filter paper, and a 2 cm. strip was stained for protein. The remainder of the paper was cut into five transverse segments each known (from the protein stain) to contain a single protein component. A blank run (with the same conditions of buffer, voltage, time, etc.) using no serum was made and segments were cut. All papers were dry-ashed and residues transferred to cupped carbon electrodes. Emission arc spectra of the carbon electrodes (to correct for electrode impurities), paper blanks (to correct for paper impurities), and proteins were obtained with a Bausch and Lomb medium quartz spectrograph. Examination of the spectrographic plate showed no significant differences between the protein, paper, and electrodes. The metal ions of interest were present as impurities in paper and electrodes.

Presuming that failure was due to the minute quantities of metals present in 0.2 ml. of serum, one ml. of serum was separated on heavy 3M Whatman paper. Protein isolation, paper segmentation, and spectrographic analysis were repeated. Line intensities obtained from blank paper and proteins were again of the same magnitude. Hence this type of analysis was abandoned.

3. Procedure for Exhaustive Dialysis

To determine the time necessary for exhaustive dialysis, the following procedure was used. Two identical 30 ml. pools of undiluted serum

(labeled 1 and 2) were equilibrated for seven days at 2° against 650 ml. of veronal buffer. Every 24 hours, the volume of pool 1 was measured and a two ml. portion removed for analysis and replaced by two ml. from pool 2. The outside buffer liquid of each was changed daily. Analyses for residual calcium, corrected for volume changes, compared with dialysis time showed by plot that after five days no further removal of calcium occurred. (Figure 13).

The course of exhaustive removal of the other ions, magnesium, iron, and copper, was presumed to be the same. Other studies showed that they, too, were removed as completely as possible by five day dialysis.

After these preliminary conditions were established, five-day exhaustive dialysis was performed upon the following six labeled protein solutions: Albumin I (99%, Cohn Fraction V), Albumin II (83%, from ammonium sulfate precipitation of globulins), Albumin III (93%, from methanol precipitation of globulins), Mixed Globulins (methanol precipitate), Gamma Globulin (97%, Cohn Fractions II and III), and whole serum. A 30 ml. portion of each of the above listed solutions contained within a cellophane membrane was equilibrated at 2° against 650 ml. of veronal buffer for five days by changing the outside buffer daily. Determinations of total protein and magnesium, calcium, copper, and iron metal ion concentrations were made on dialyzed and undialyzed portions of each protein solution. The results of these analyses are given in Table VI.

TABLE I

EFFECT OF DEEP-FREEZE STORAGE ON TOTAL PROTEIN AND METAL ION CONTENT OF HUMAN SERUM

Days of Storage	Total Protein[1]	Calcium[2]	Magnesium[2]	Iron[3]	Copper[3]
0	7.01	10.7	1.4	69	140
12	--	--	--	69	140
35	--	--	--	69	144
60	7.01	--	--	--	--
80	--	10.5	1.4	--	--

[1]g./100 ml.
[2]mg./100 ml.
[3]µg./100 ml.

TABLE IIa

PLANIMETRIC ANALYSIS OF SERUM ELECTROPHORETIC PATTERNS FOR EACH OF 21 HUMAN SUBJECTS

Subject Number	Relative Fractional Composition of Serum Protein Components				
		Globulins			
	Albumin	$Alpha_1$	$Alpha_2$	Beta	Gamma
1	0.555	0.039*	0.085	0.173**	0.148
2	0.575	0.053	0.109	0.116	0.147
3	0.527	0.087**	0.117	0.123	0.145
4	0.609	0.053	0.105	0.113	0.119
5	0.591	0.054	0.124**	0.128	0.103*
6	0.477*	0.073**	0.096	0.116	0.238**
7	0.618**	0.042	0.084	0.101*	0.155
8	0.622**	0.050	0.111	0.093*	0.124
9	0.601	0.051	0.083	0.134	0.141
10	0.595	0.038*	0.067*	0.107*	0.193**
11	0.506*	0.065	0.119**	0.159	0.152
12	0.473*	0.063	0.113	0.175**	0.175
13	0.540	0.058	0.107	0.125	0.170
14	0.528	0.060	0.113	0.149	0.152
15	0.553	0.043	0.092	0.148	0.164
16	0.603	0.048	0.090	0.151	0.108*
17	0.578	0.047	0.094	0.170**	0.111*
18	0.466*	0.064	0.114	0.167**	0.180**
19	0.655**	0.032*	0.053*	0.137	0.125
20	0.548	0.076**	0.095	0.155	0.127
21	0.565	0.033*	0.098	0.122	0.183
$\bar{X}$	0.561	0.054	0.099	0.136	0.151
s	0.052	0.014	0.018	0.025	0.033
$\bar{X} \pm s$	0.509-	0.040-	0.081-	0.111-	0.118-
	0.613	0.068	0.117	0.161	0.184

*Values less than $\bar{X} - s$
**Values more than $\bar{X} + s$

TABLE IIb

TOTAL PROTEIN CONTENT AND SERUM PROTEIN DISTRIBUTION
BY MOVING BOUNDARY ELECTROPHORETIC ANALYSIS

Subject Number	Total Protein g./100 ml.	Composition of Serum Components in g./100 ml.				
		Albumin	Globulins			
			$Alpha_1$	$Alpha_2$	Beta	Gamma
1	6.61	3.67	0.26*	0.56	1.14**	0.98
2	6.70	3.85	0.36	0.73	0.78	0.98
3	6.70	3.53	0.58**	0.78	0.82	0.97
4	6.80	4.14	0.36	0.71	0.77	0.81
5	6.97	4.12	0.38	0.86**	0.89	0.72
6	6.88	3.28	0.50	0.66	0.80	1.64**
7	6.43*	3.97	0.27*	0.54*	0.65*	1.00
8	6.97	4.34**	0.35	0.77	0.65*	0.86
9	7.17	4.31	0.37	0.60	0.88	1.01
10	7.56**	4.50**	0.29	0.51*	0.81	1.46**
11	7.01	3.55	0.46	0.83**	1.11	1.07
12	6.61	3.13*	0.42	0.75	1.16**	1.16
13	6.61	3.57	0.38	0.71	0.83	1.12
14	6.70	3.54	0.40	0.76	1.00	1.02
15	7.37**	4.08	0.32	0.68	1.09	1.21
16	6.98	4.21	0.34	0.63	1.05	0.75*
17	6.70	3.87	0.31	0.63	1.14**	0.74*
18	6.61	3.08*	0.42	0.75	1.10	1.24
19	7.07	4.63**	0.23*	0.37*	0.97	0.88
20	7.26**	3.98	0.55**	0.69	1.13**	0.92
21	7.46**	4.21	0.25*	0.73	0.91	1.37**
$\bar{X}$	6.91	3.88	0.37	0.68	0.94	1.04
s	0.31	0.43	0.09	0.12	0.17	0.24
$\bar{X} \pm s$	6.60-7.22	3.45-4.31	0.28-0.46	0.56-0.80	0.74-1.11	0.80-1.28

*Values less than $\bar{X} - s$
**Values more than $\bar{X} + s$

TABLE III

RESULTS OF METAL ION ANALYSES

Subject Number	Calcium mg./100 ml.	Magnesium mg./100 ml.	Iron µg./100 ml.	Copper µg./100 ml.
1	10.7	1.3	144**	94
2	10.2	1.3	138	140
3	11.1	1.4	94	128
4	10.4	1.5	94	70
5	11.8	1.3	125	80
6	10.8	1.2	128	184**
7	10.5	1.3	47*	70
8	12.1**	1.6**	56*	140
9	10.9	1.4	138	132
10	11.9	1.4	69	204**
11	10.3	1.6**	94	88
12	9.2*	1.2*	144	76
13	9.7	1.4	106	76
14	10.3	1.4	69	76
15	12.2**	1.3	119	76
16	10.8	1.4	62	44*
17	10.9	1.5	188**	106
18	9.7*	1.1*	72	132
19	11.2	1.4	125	68
20	11.7	1.4	156**	76
21	11.6	1.4	56*	220**
$\bar{X}$	10.9	1.4	101	109
s	0.8	0.1	39	49
$\bar{X} \pm s$	10.1-	1.3-	62-	60-
	11.7	1.5	140	158

*Values less than $\bar{X} - s$
**Values more than $\bar{X} + s$

TABLE IV

VALUES FOR NORMAL HUMAN SERUM

Electrophoresis

Relative Fractional Composition of Serum Protein Components

	Globulins				
Albumin	Alpha$_1$	Alpha$_2$	Beta	Gamma	Reference
0.533	0.080	0.104	0.138	0.142	86
0.603	0.040	0.097	0.128	0.132	90
0.568	0.072	0.087	0.128	0.144	77
0.560	0.072	0.088	0.131	0.147	80
0.560	0.045	0.115	0.160	0.120	10
0.601	0.050	0.093	0.115	0.141	29
0.561	0.054	0.099	0.136	0.151	This work

Total Protein and Metal Ions

Total Protein g.[1]	Calcium mg.[2]	Magnesium mg.[2]	Iron µg.[3]	Copper µg.[3]	Reference
5.5-8.4	9-11	1-3			41
			110 f.[4]		38
			129 m.[5]		38
			60-200		26
				84-143 f.[4]	37
				68-134 m.[5]	37
6.91	10.9	1.4	101	109	This work

[1]g. per 100 ml. serum
[2]mg. per 100 ml. serum
[3]µg. per 100 ml. serum
[4]for adult females
[5]for adult males

TABLE V

COMPARISON OF MOVING BOUNDARY AND PAPER ELECTROPHORETIC ANALYSIS ON THE SAME SERUM

Protein	Moving Boundary	Paper
Albumin	0.601	0.602
$Alpha_1$ Globulin	0.051	0.037
$Alpha_2$ Globulin	0.083	0.083
Beta Globulin	0.134	0.128
Gamma Globulin	0.141	0.150

TABLE VI

ANALYSES OF PROTEIN SOLUTIONS BEFORE AND AFTER DIALYSIS

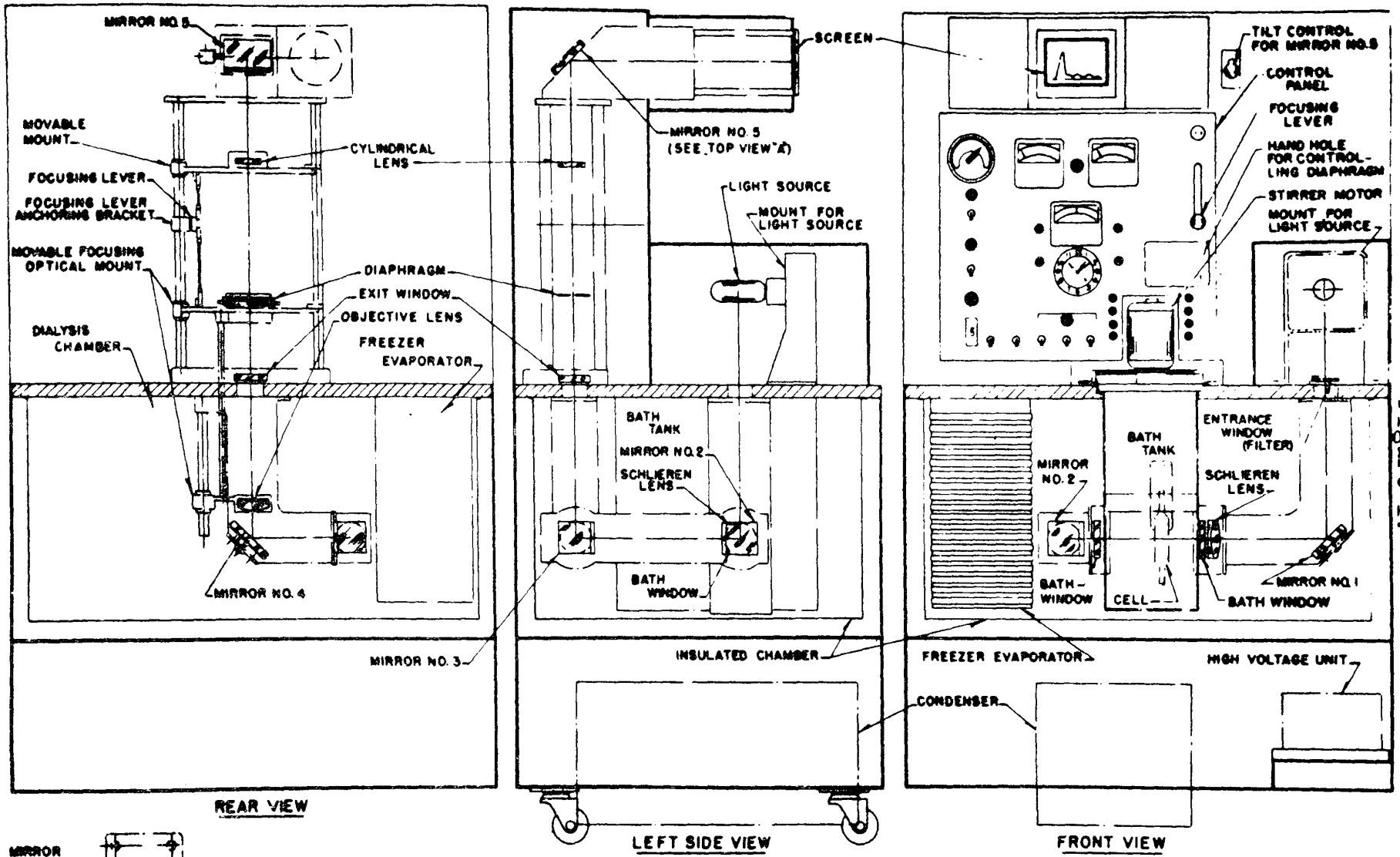
Protein Solution	Dialysis Period (Days)	Volume (ml.)	Total Protein g./100 ml.	Calcium mg./100 ml.	Magnesium mg./100 ml.	Iron µg./100 ml.	Copper µg./100 ml.
A I	0	30	13.52	4.73	0	--	--
	5	63	6.44	0.46	0		
A II	0	30	3.65	12.10	1.5	70	82
	5	75	1.46	0.48	0.2	12	0
A III	0	30	4.24	13.00	1.6	82	96
	5	54	2.36	1.02	0.3	14	0
MG	0	30	4.10	4.97	0	87	96
	5	33	3.75	3.27	0	35	83
GG	0	30	3.92	4.64	0	--	--
	5	32	3.68	4.37	0		
Serum	0	30	6.61	10.65	1.3	152	160
	5	54	3.70	2.71	0	47	98

-- Extremely high values obtained. (Cohn fractions were prepared in stainless steel apparatus.)

TABLE VII

TOTAL AND BOUND METAL IONS

Protein Solution		Calcium mg./g.Protein	%	Magnesium mg./g.Protein	%	Iron µg./g.Protein	%	Copper µg./g.Protein	%
A I	Total	0.35		0		--		--	
	Bound	0.07	20	0	--	--	--	--	--
A II	Total	3.32		0.4		19		22	
	Bound	0.33	10	0.1	25	8	42	0	0
A III	Total	3.07		0.4		19		23	
	Bound	0.43	14	0.1	25	6	32	0	0
MG	Total	1.22		0		21		23	
	Bound	0.87	71	0	--	9	43	22	96
GG	Total	1.18		0		--		--	
	Bound	1.24	105	0	--	--	--	--	--
Serum	Total	1.61		0.2		23		24	
	Bound	0.73	45	0	0	10	43	26	108

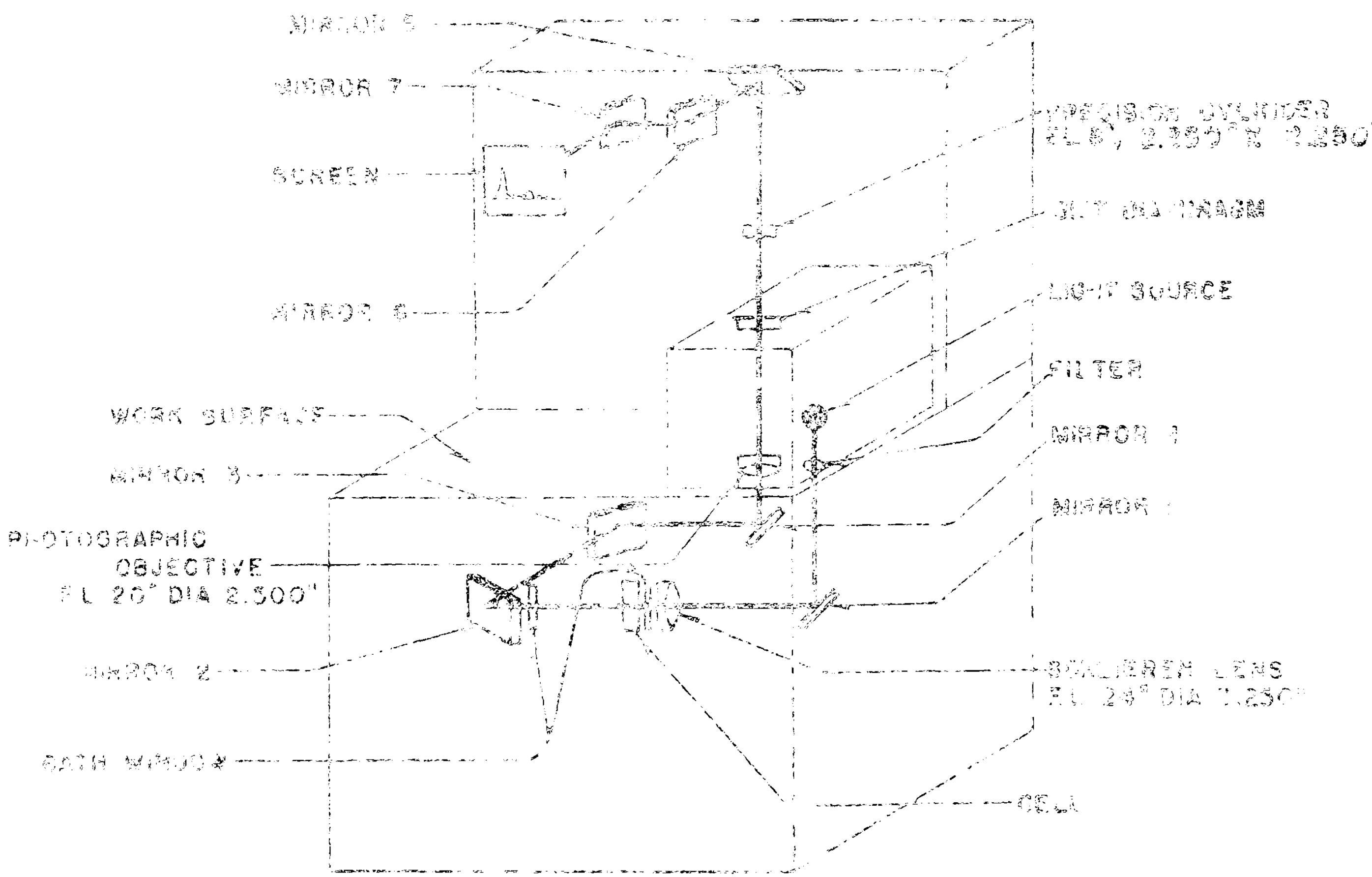


INCIDENCE ORDER OF LIGHT BEAM:

ENTRANCE WINDOW, MIRROR NO. 1, SCHLIEREN LENS, BATH WINDOW, CELL, BATH WINDOW, MIRRORS NOS. 2, 3 & 4, OBJECTIVE LENS, EXIT WINDOW, DIAPHRAGM, CYLINDRICAL LENS, MIRRORS NOS. 5, 6 & 7, SCREEN.

LOCATION OF COMPONENT SYSTEMS IN THE AMINCO–STERN ELECTROPHORESIS APPARATUS

Figure 1.



OPTICAL DIAGRAM
OF
THE AMINCO-STERN
ELECTROPHORESIS APPARATUS

Figure 3. LKB Paper Electrophoresis Apparatus

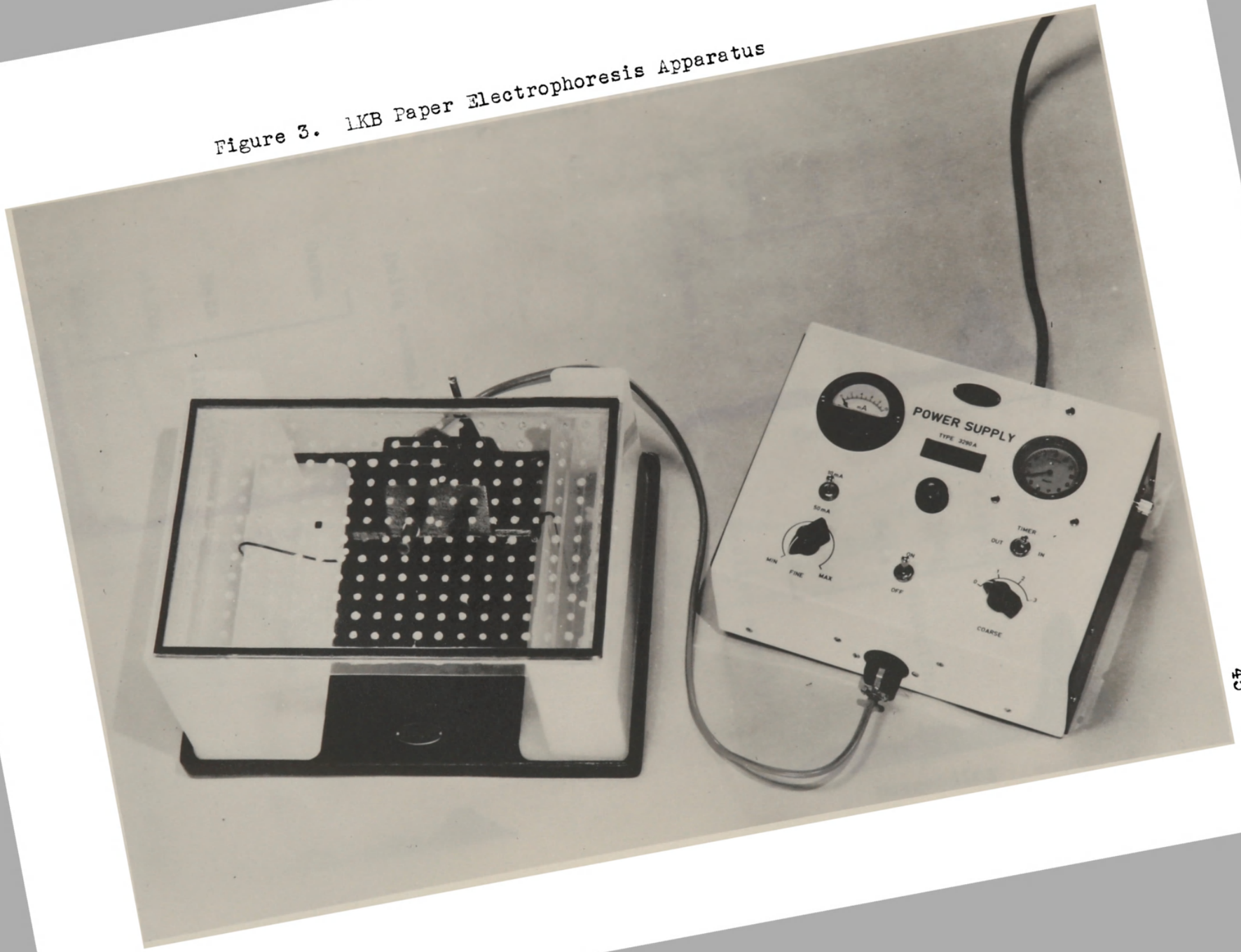


Figure 4. Examples of Moving Boundary Patterns of Human Serum

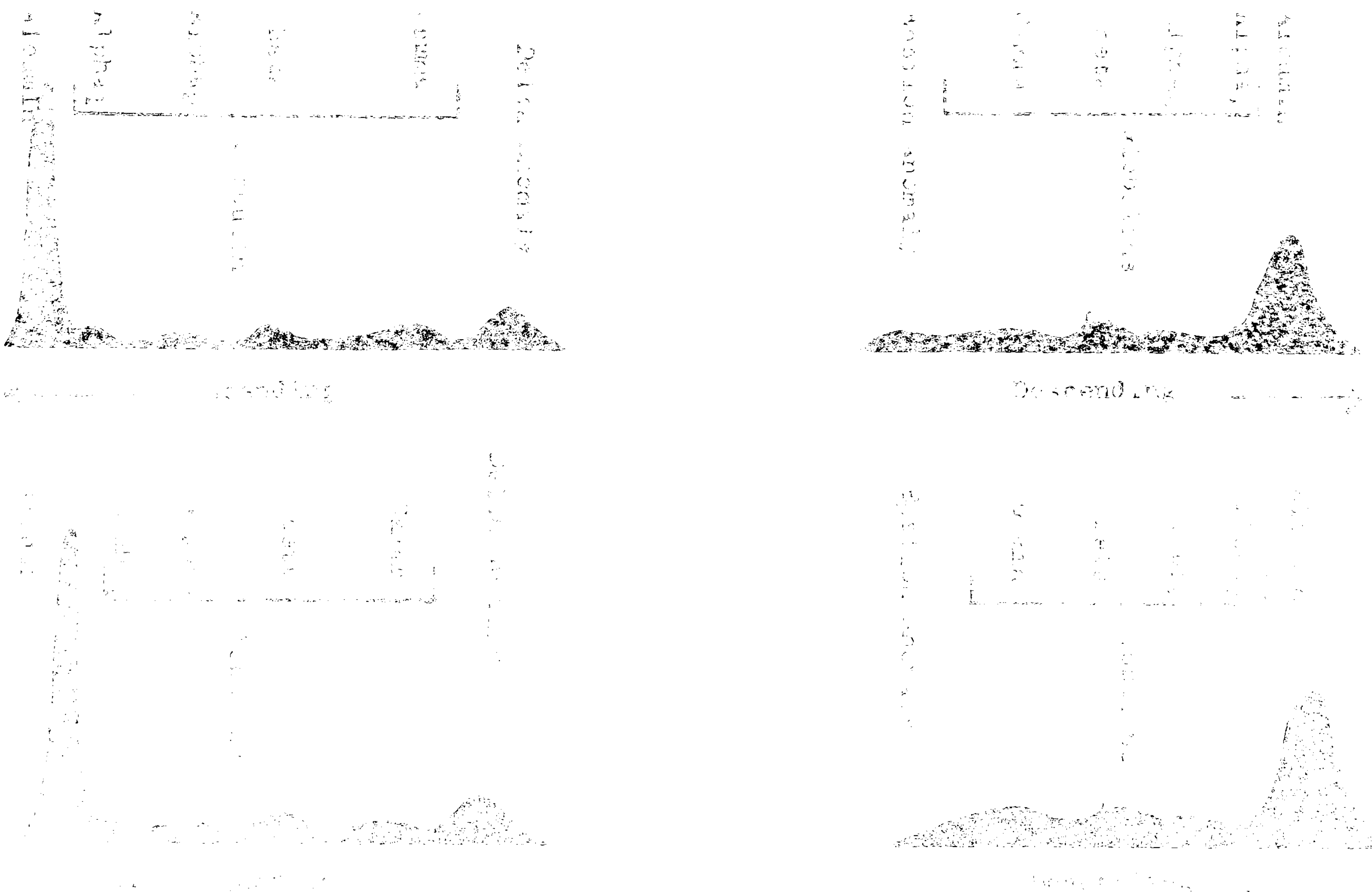


Figure 5. Enlarged Ascending Moving Boundary Pattern with Ordinates Drawn

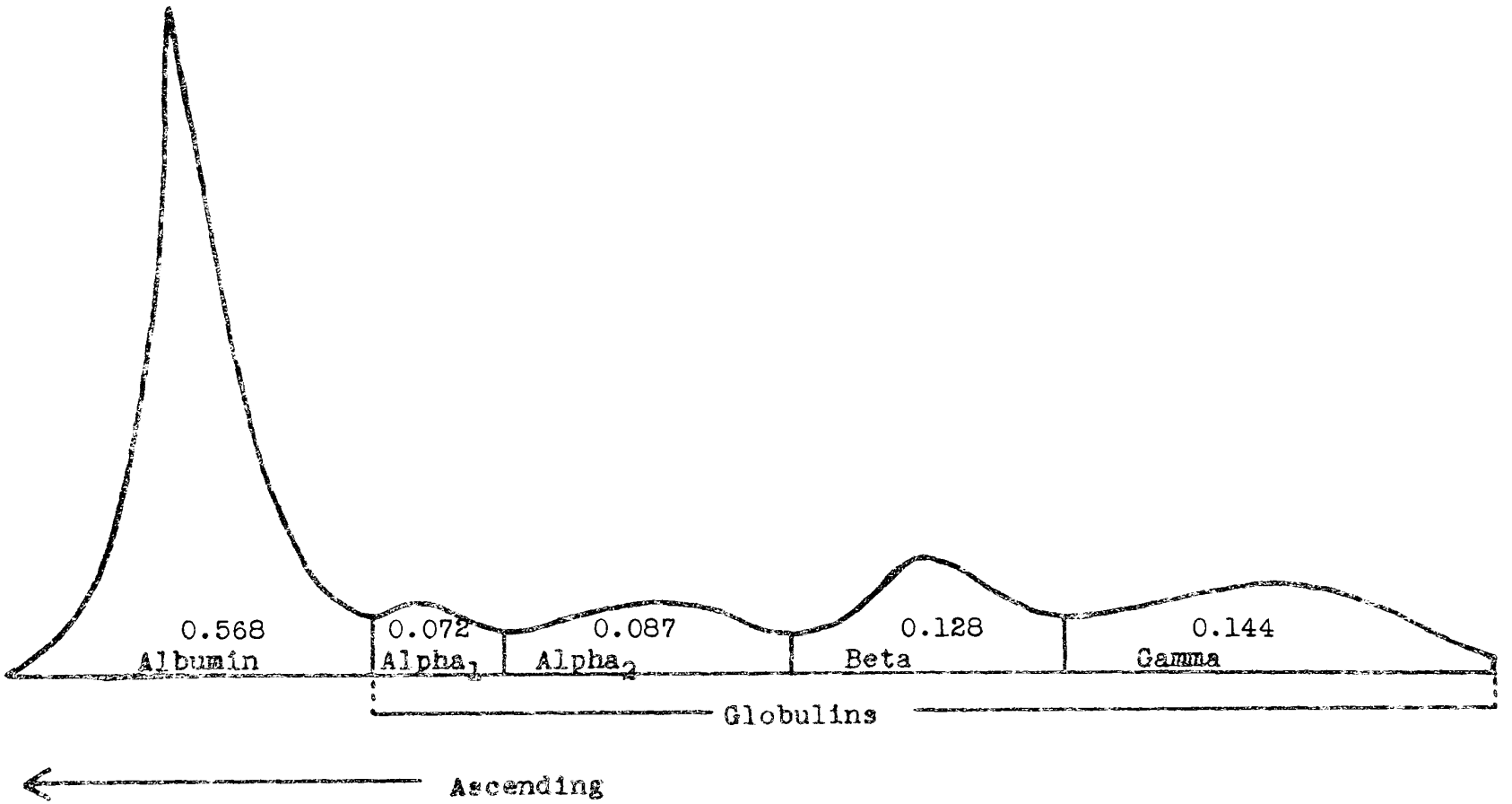
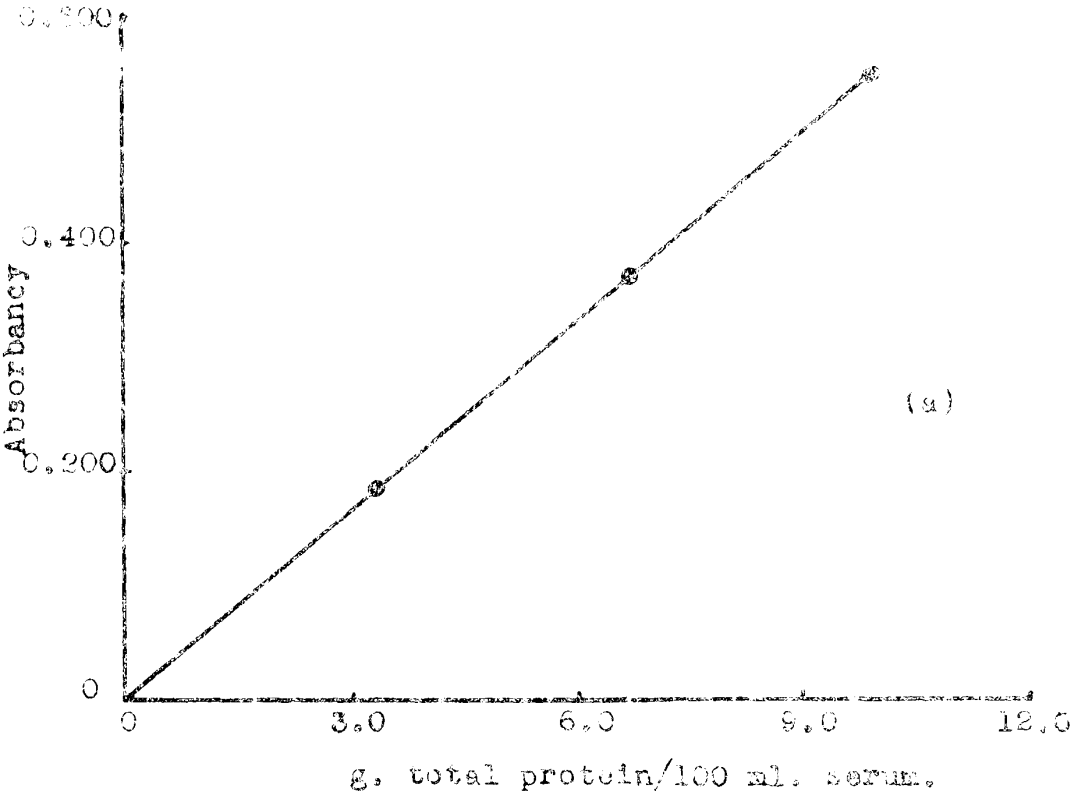


Figure 6. Standard Curves for (a) Total Protein and (b) Magnesium in Human Serum



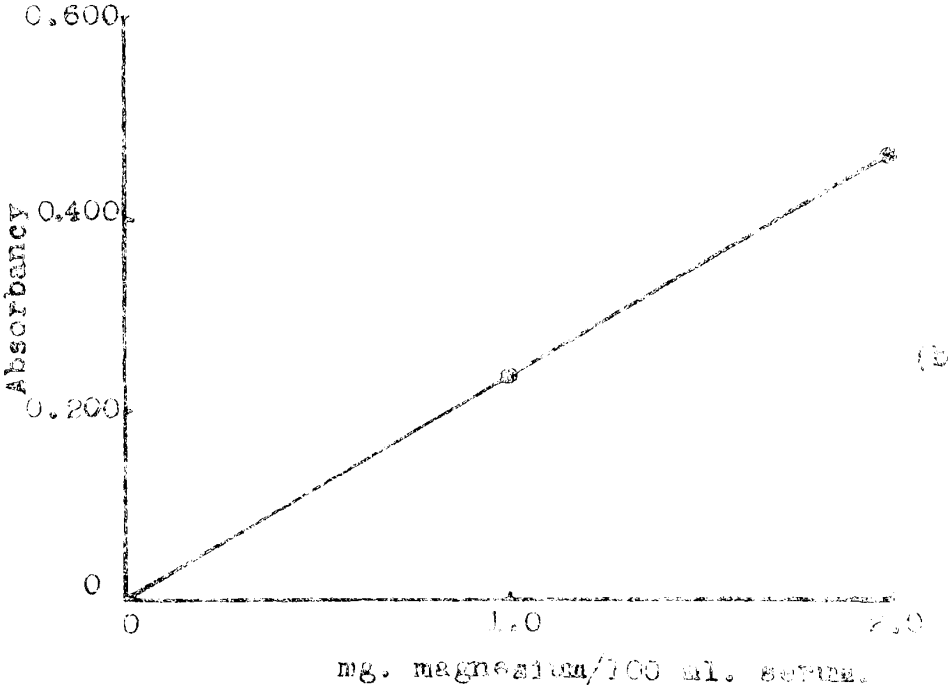
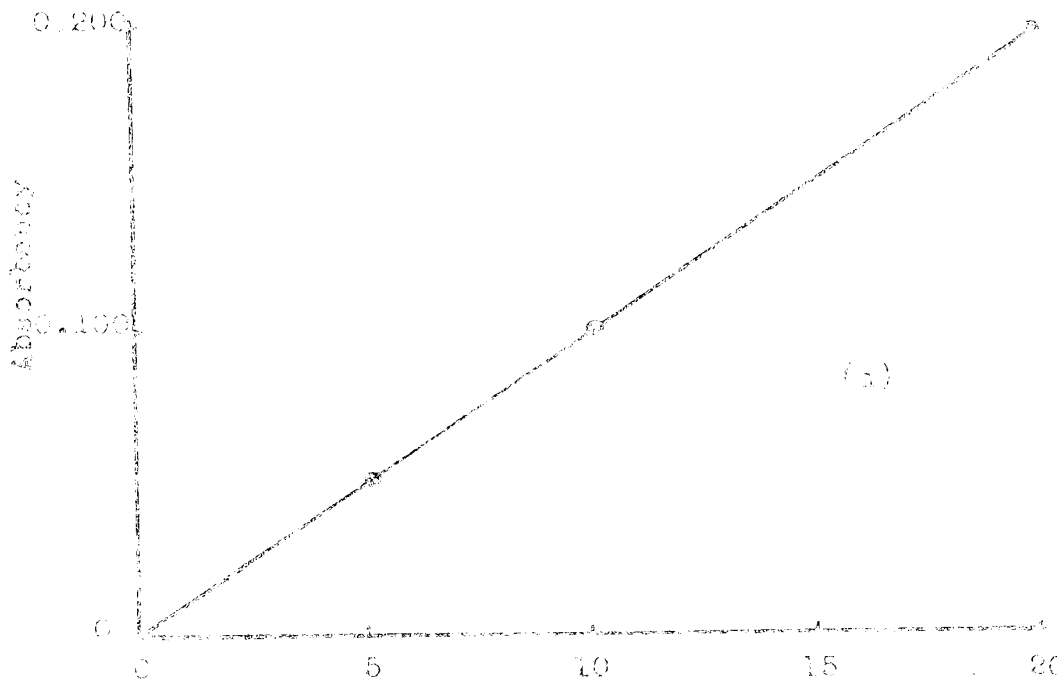


Figure 7. Standard Curves for (a) Iron and (b) Copper in Human Sera



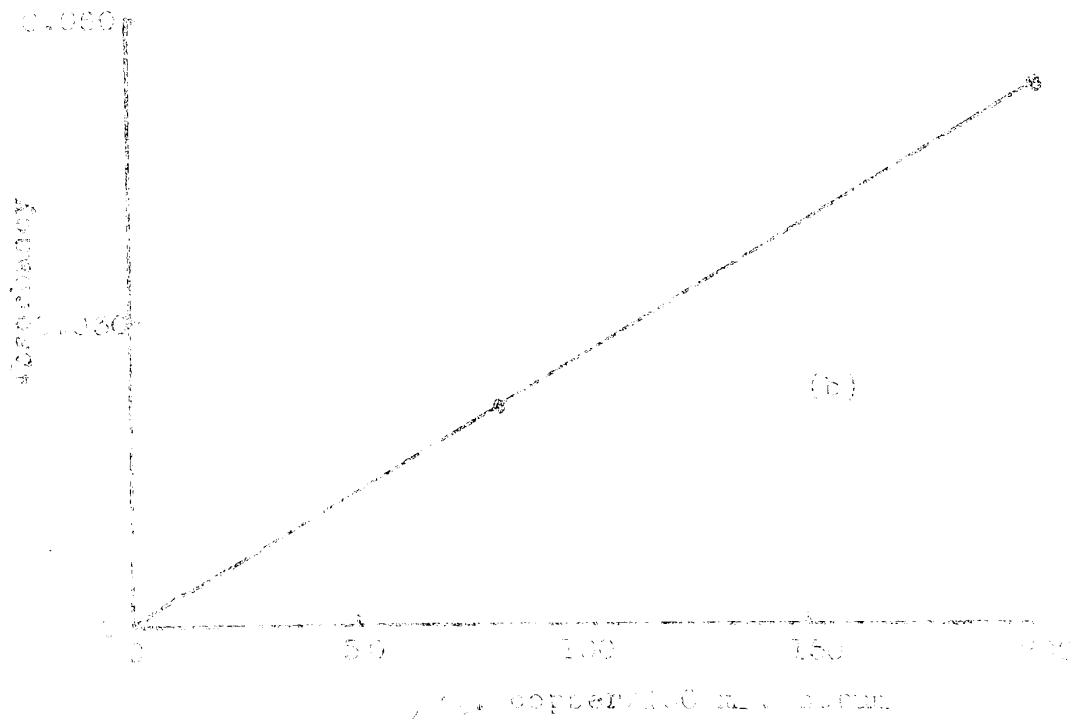
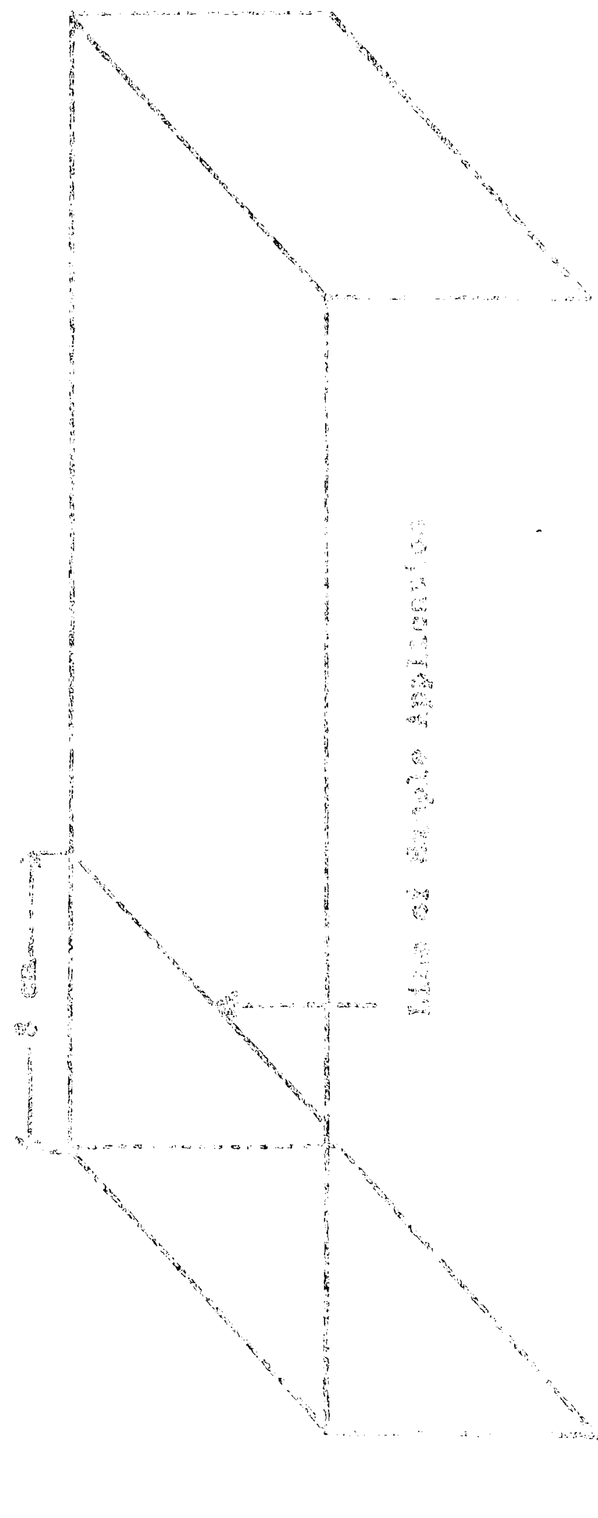


Figure 4. Position of Line of Sample Application on Paper



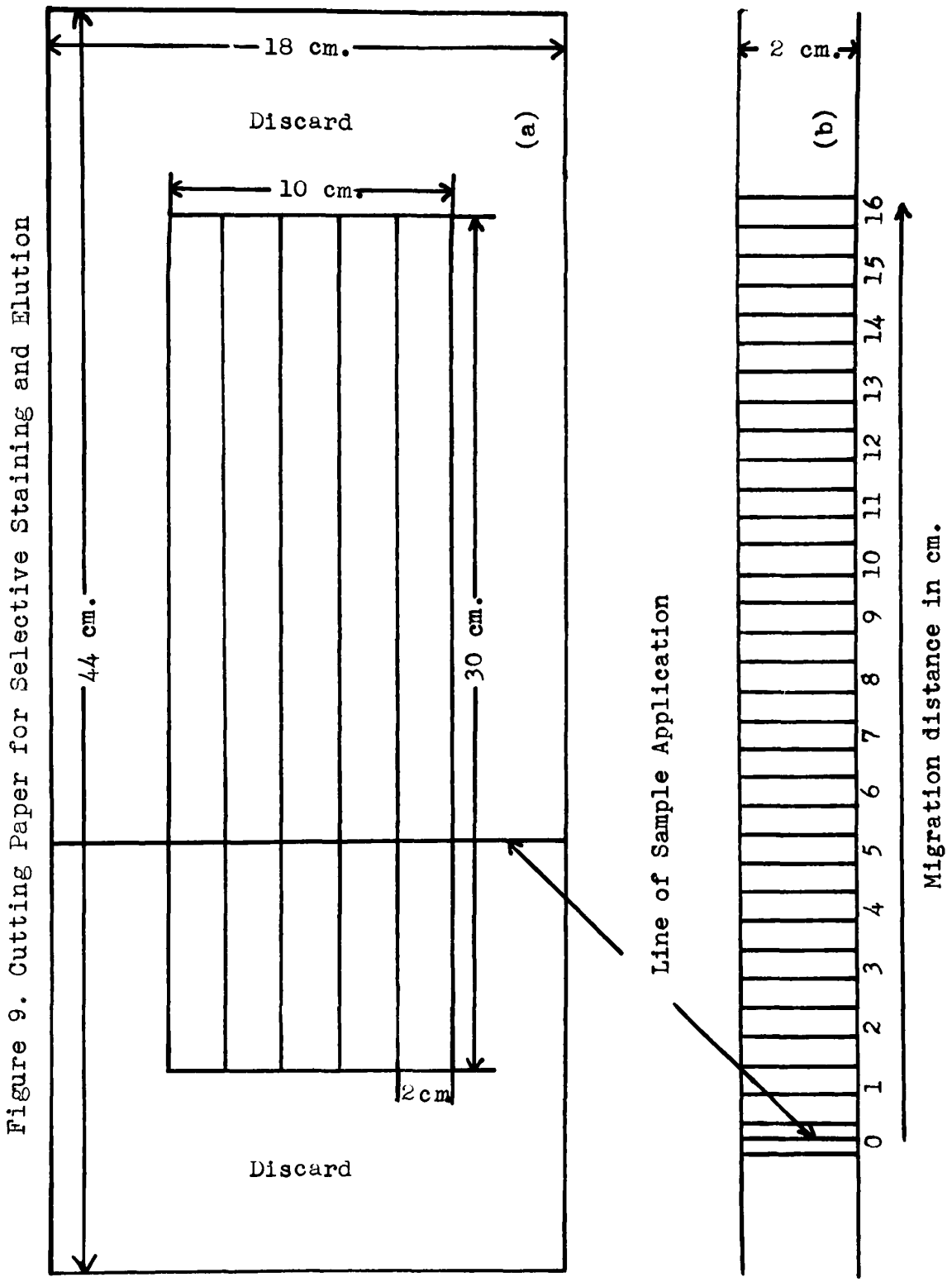
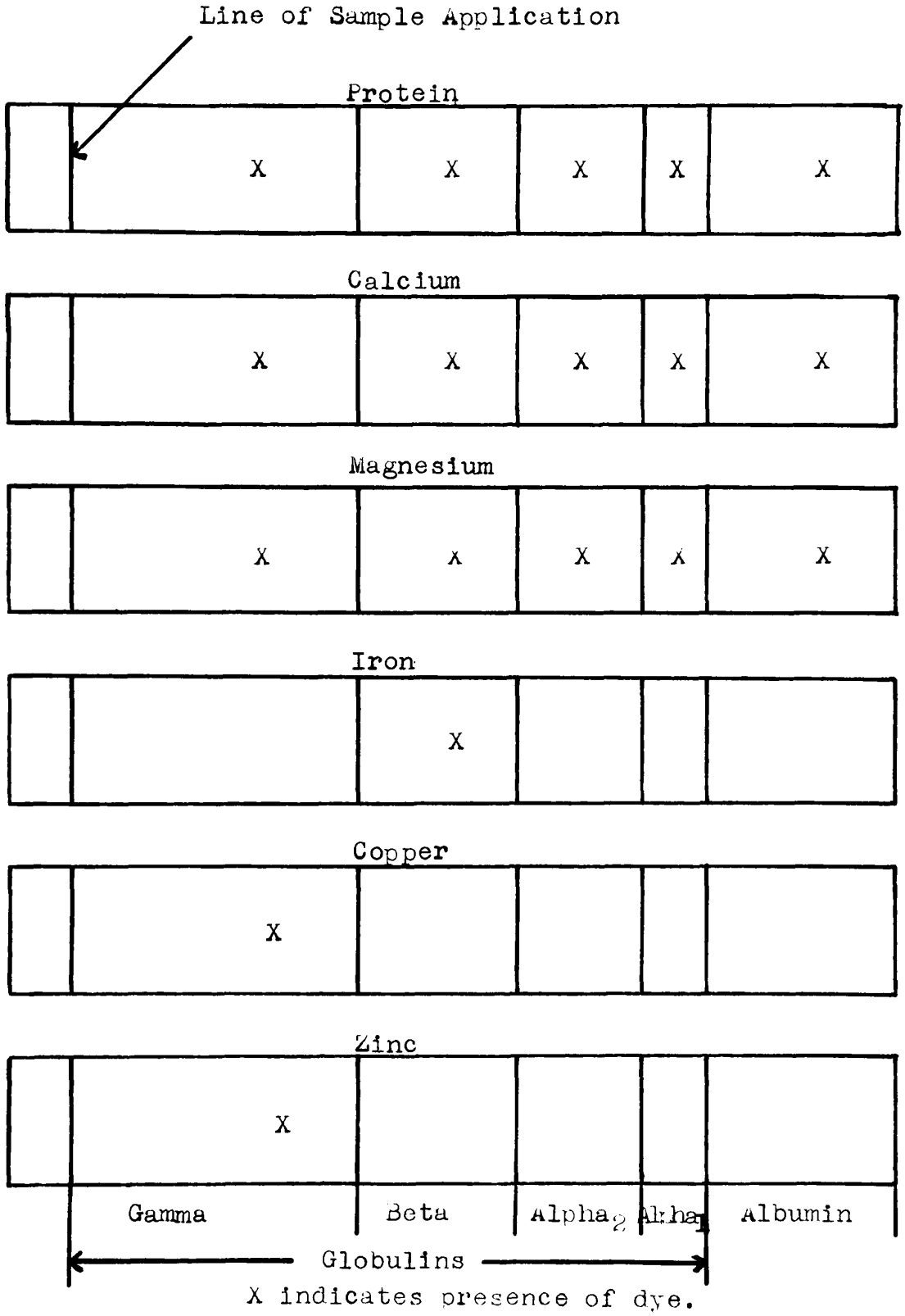


Figure 9. Cutting Paper for Selective Staining and Elution

Figure 10. Qualitative Results of Selective Staining



X indicates presence of dye.

Figure 11. Example of Serum Protein Separation by Paper Electrophoresis

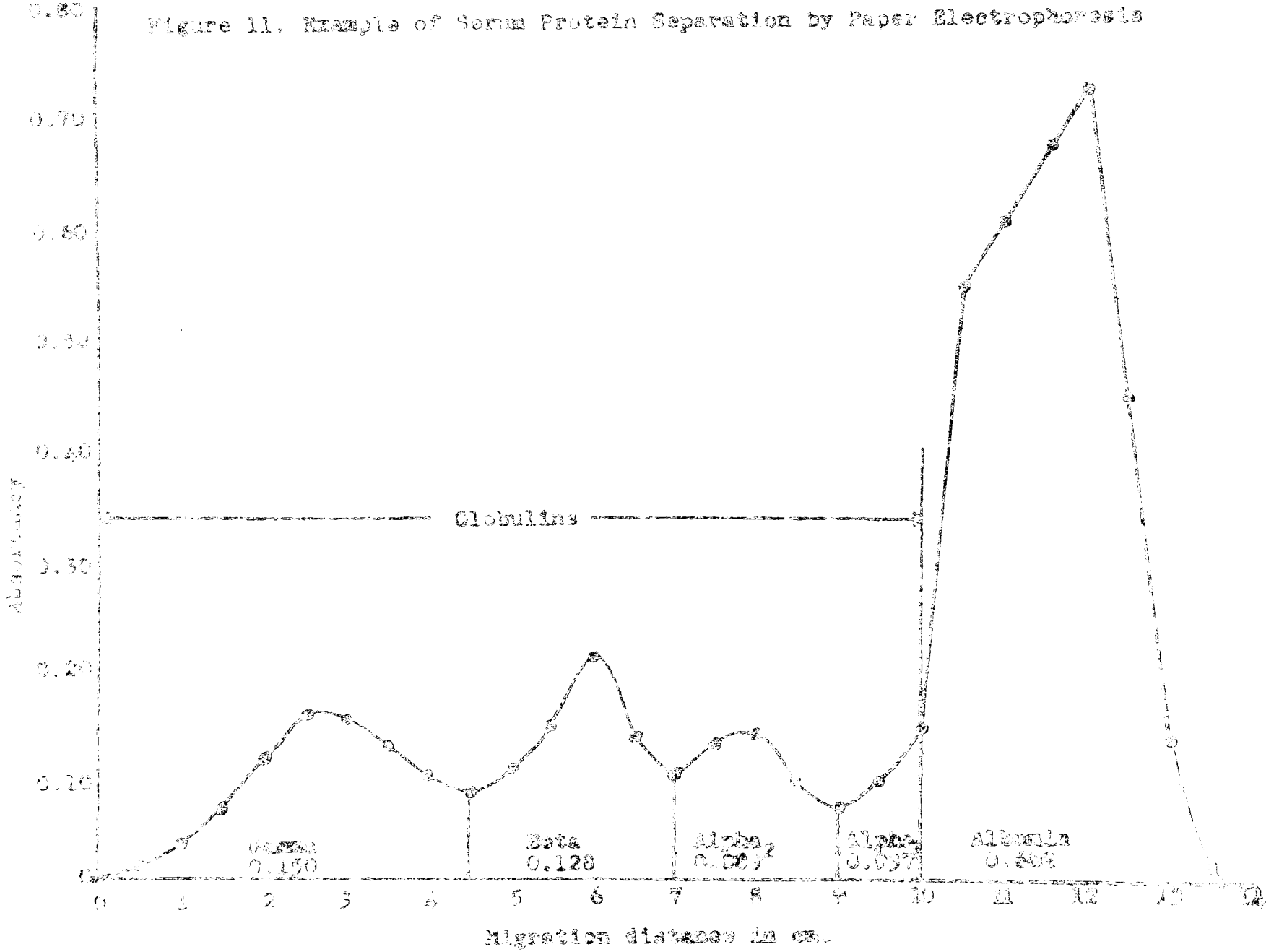


Figure 12. Comparative Distribution of Calcium and Serum Proteins

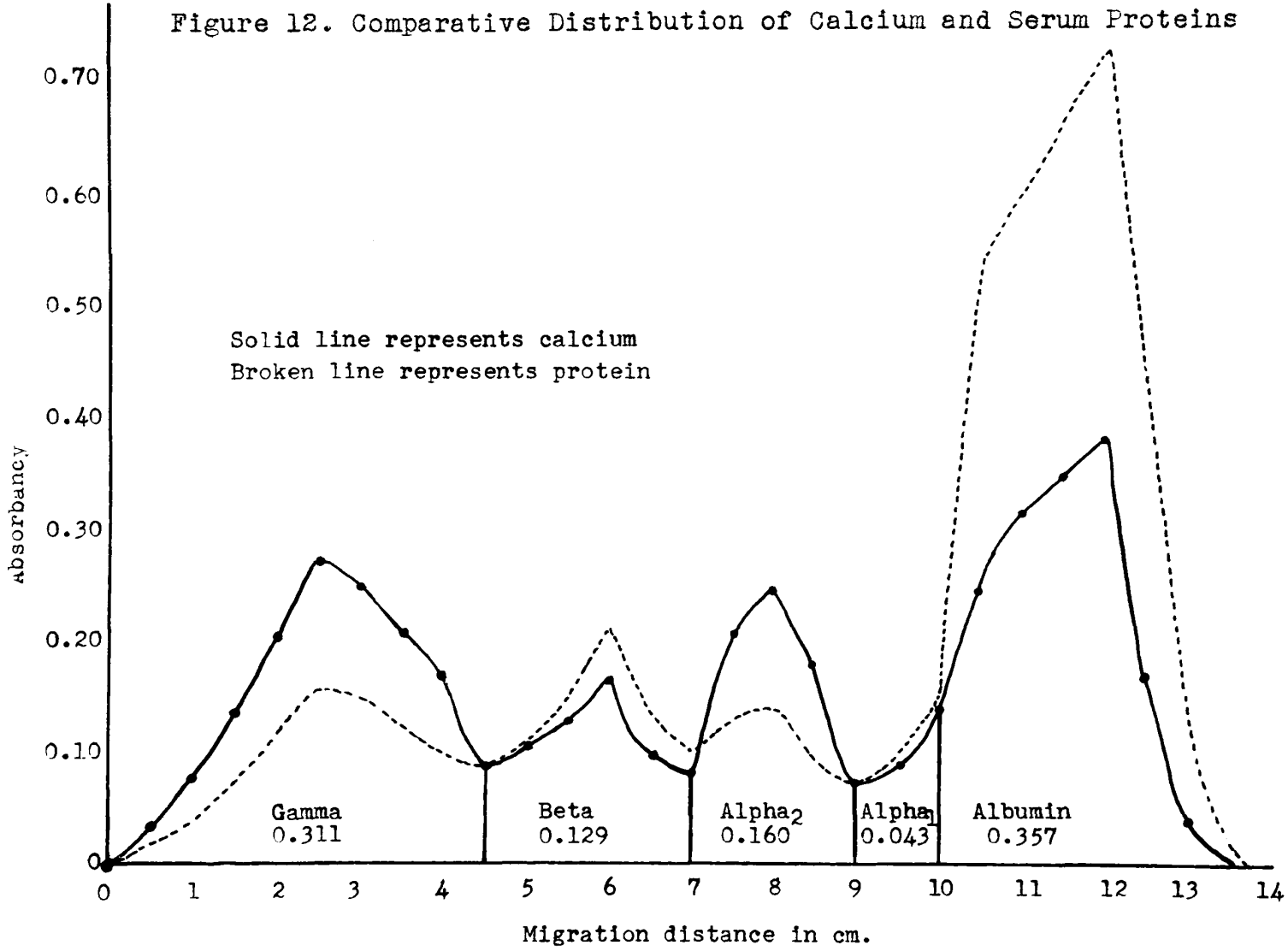
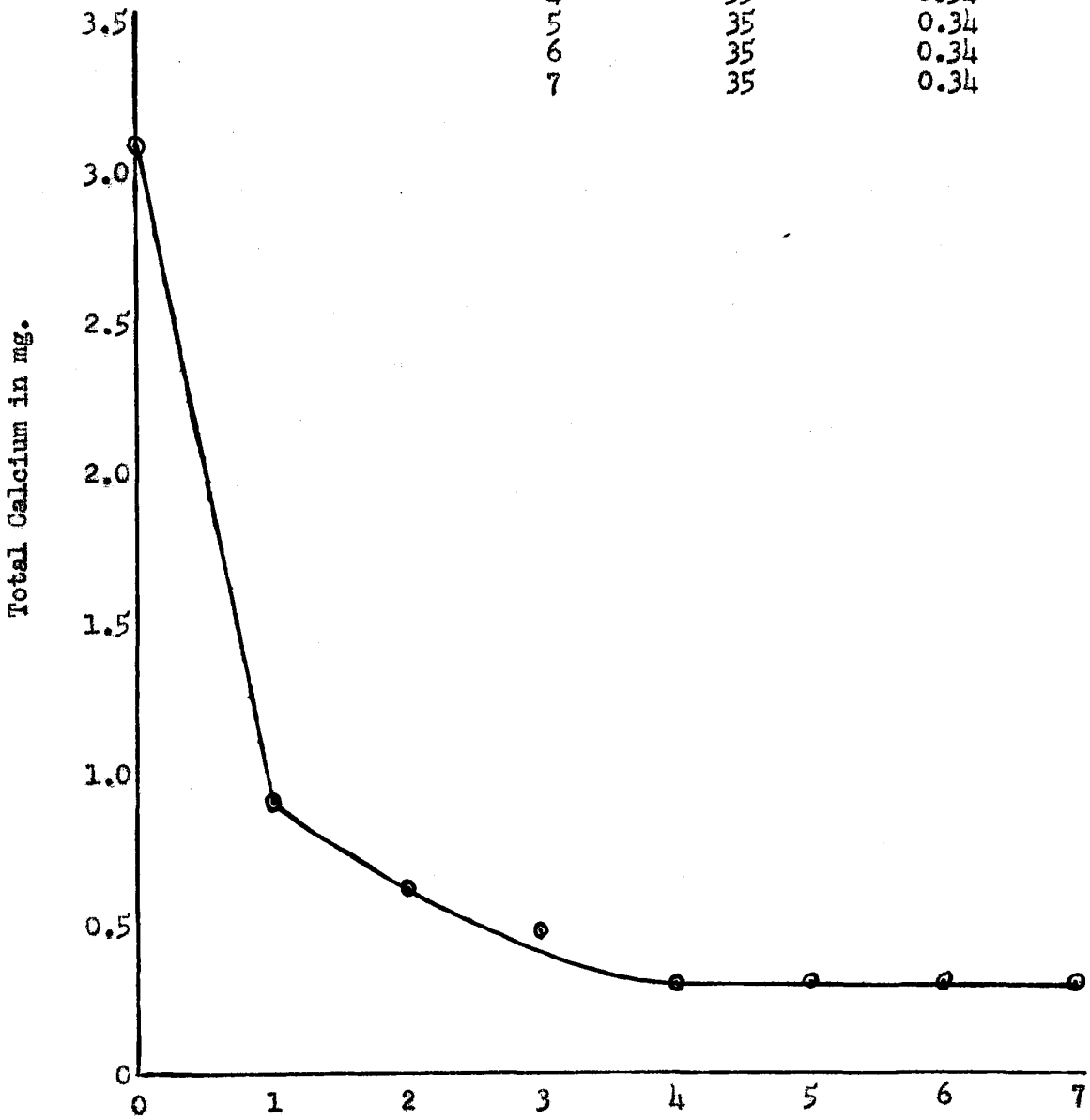


Figure 13. Exhaustive Dialysis

Days of Dialysis	Volume	Total Mg. Calcium
0	30	3.10
1	32	0.91
2	33.5	0.66
3	34	0.50
4	35	0.34
5	35	0.34
6	35	0.34
7	35	0.34



IV. DISCUSSION

This section will attempt to evaluate and interpret experimental results on metal ion binding by human serum proteins.

A. Moving Boundary Experiments

1. Statistical considerations. -- Electrophoretic analysis yielded the relative fractional amounts of each serum protein (e.g., Table IIa, for subject number 1, showed albumin was 0.555 of the total serum protein). The total protein in serum was determined to enable the calculation of absolute amounts (in g. per 100 ml.) of protein components. A sample calculation is given in Appendix I.

The relative amounts of serum proteins are given in Table IIa. The results of total protein determinations together with the absolute amounts of the serum protein components obtained by calculation are presented in Table IIb.

The arithmetic mean ($\bar{X}$) and standard deviation (s) for total protein and for the relative and absolute amounts of serum proteins were calculated by the statistical equations (20):

$$\bar{X} = \frac{\Sigma X}{N} \qquad s = \sqrt{\frac{\Sigma (X - \bar{X})^2}{N - 1}}$$

in which X = a single observation and N = number of observations. The means, standard deviations, and ranges ($\bar{X} \pm s$) for each value are included in Table II. Values less than $\bar{X}$-s are designated *, while values

more than $\bar{X}+s$ are designated **. This was done to facilitate correlation observations.

Table III presents the results of serum determinations of calcium, magnesium, iron, and copper for the same subjects as Tables IIa and IIb. Here again, means and standard deviations were calculated and values exceeding the $\bar{X}\pm s$ range are indicated * and **.

To demonstrate the validity of results of serum protein distribution obtained by electrophoresis and the determinations of total protein and metal ions in human blood serum, Table IV gives the mean values resulting from this experimental work together with values from other literature sources (10,26,29,38,41,77,80,86,90). Examination of the table shows acceptable agreement of results.

To obtain the data presented in Table I, determinations of total protein and metal ions were made on human serum before freezing and checked after 35-80 days of deep-freeze storage. The table shows that no significant changes occurred with cold-storage treatment of serum.

2. Serum metal ion contents and correlation with serum protein fractions. -- Data of Tables II and III indicate the following:

Calcium. -- The serum concentration of this metal ion exceeded the range 10.1-11.7 mg. per 100 ml. for subjects 8, 10, 12, 15, and 18. In all cases, total protein exceeded the range 6.60-7.22 and/or albumin exceeded the range 3.45-4.31 g. per 100 ml. The coincident increases (or decreases) in calcium and total protein and/or albumin suggest that a relatively large amount of bound calcium may be associated with albumin and that the globulins may also participate in binding.

Magnesium. -- Serum magnesium contents for subject 6, 8, 11, 12, and 18 were outside the $\bar{X} \pm s$ range of 1.3-1.5 mg. per 100 ml. The serum albumin was low for the three subjects with low magnesium values (6, 12, and 18) and high for one subject with a high magnesium value (8). These findings suggest that albumin would be the serum protein primarily involved in magnesium association.

Iron. -- Values less than 62 or more than 140 µg. per 100 ml. serum were obtained for subjects 1, 7, 8, 17, 20, and 21. For all except the last subject, a concomitant change in the same direction occurred in the beta globulin fraction. These observations tend to indicate an iron-beta globulin association.

Copper. -- Copper values greater than 158 µg. per 100 ml. serum were obtained for subjects 6, 10, and 21. Of the serum proteins, gamma globulin exceeded 1.28 g. per 100 ml. in each case. A copper value below 60 and a gamma globulin content below 0.80 occurred in subject 16. These parallel changes suggest the possibility of copper-gamma globulin binding.

B. Paper Electrophoresis Experiments

1. General considerations. -- Figure 11 is a sample electrophoretic pattern obtained by the horizontal strip procedures for human serum protein separation, staining, and strip elution. Alpha$_1$ globulin was found visible on paper as a separate and distinct blue colored area. However, when elution data (migration distance versus absorbancy) were plotted, no separate peak attributable to this fraction appeared.

Distortion of the albumin peak suggested that alpha$_1$ globulin was included in the albumin. A calculation of mobility relative to that of albumin indicated this was true.

Alpha$_1$ globulin has been shown to migrate at 0.82 the rate of albumin under the same experimental conditions. This value may be calculated from the absolute mobilities of the serum proteins (at pH 8.6, μ 0.1 veronal buffer) given by Armstrong, et al. (4), in the following manner:

4.85 ± 0.23 (mobility of alpha$_1$ globulin)/5.92 ± 0.21 (mobility of albumin) = 0.82 ± 0.07 (relative mobility of alpha$_1$ globulin).

The literature reveals that some investigators (30,36,40,54,61,65) have achieved similar results from both moving boundary and paper electrophoretic analysis. Other workers (18,33,56,58,88), failing to get agreement, attribute this to albumin tailing and the unequal staining of albumin and globulins. One object in the development of the paper electrophoresis procedures in this investigation was to obtain comparable results with the two methods. To compare paper with moving boundary results, portions of the same sera were examined by both electrophoretic methods. A sample result is given in Table V. The agreement obtained between the two methods tends to minimize albumin tailing (albumin low, globulins high) and unequal dye affinities (albumin high, globulins low). Apparently, comparability of results can be achieved with the proper paper electrophoretic techniques.

2. Metals found in proteins separated on filter paper. -- The qualitative results of paper electrophoretic separation of human

serum proteins and subsequent selective staining are presented in Figure 10.

Calcium. -- The orange-pink areas of the calcium-alizarin complex on a pale pink background corresponded to the five positions of the serum proteins as shown in Figure 10. Thus, calcium appeared to be present in albumin and all the globulins.

Visual comparison of color intensity on parallel strips stained for protein and calcium suggested an unequal distribution of metal ion on the proteins. Figure 12 presents elution data (migration distance versus absorbancy) for calcium and protein plotted on the same coordinate system. Measurement of areas of the two curves showed that: (a) albumin (almost two-thirds of the total serum protein) was associated with about one-third of the total bound calcium and (b) gamma globulin (approximately one-sixth of the total serum protein) was associated with almost one-third of the total bound calcium.

Magnesium. -- The five red areas given by the magnesium-Titan yellow complex were in the same positions as the areas stained for all of the separated serum proteins. Magnesium, therefore, seemed to occur in each of the serum proteins. Due to color instability of the complex and the strongly basic medium required for its formation, attempts to obtain magnesium distribution among the five protein components were unsuccessful.

Iron. -- The single pink iron-thiocyanate area corresponded with the beta globulin zone on paper strips. This observation suggested an association of iron with only this one serum protein.

Copper. -- The yellow area of the copper-carbamate complex was in the gamma globulin position, indicating bound serum copper occurred principally in that particular globulin.

Zinc. -- The lavender spot attributable to zinc was also discovered in the gamma globulin region. Consequently, zinc also seemed to be present only in this one globulin fraction.

C. Exhaustive Dialysis Studies

1. The change in protein and metal ion contents. -- The results of total protein and metal ion analysis before and after exhaustive dialysis are given in Table VI. The decrease in metal ion content per 100 ml. as shown was the result of both dilution of the protein solutions and actual metal ion transfer to the outside liquid. To eliminate dilution effects the weight of metal ion per g. of protein was determined for calcium, magnesium, iron, and copper. These results permit the calculation of percent retention (bound ion) as given in Appendix I. The binding of metal ion per g. protein and percent retention values are reported in Table VII.

As presented in the historical section, it is apparent that dialysis has been a widely used investigative method for the study of metal ion-protein binding. In this set of experiments, the dialysis conditions were limited to pH 8.6 in 0.1 M veronal buffer. The purpose of these exhaustive dialyses was to demonstrate that protein-metal ion binding was actually present under the conditions of the preceding electrophoresis experiments carried out in the same medium.

2. The retention of metal ions by serum proteins.

Calcium. -- Albumin I (99%, Cohn Fraction V), Albumin II (83%, ammonium sulfate removal of globulins), and Albumin III (93%, methanol precipitation of globulins) all showed 10-20% retentions of calcium. Mixed Globulins (methanol precipitate) and Gamma Globulin (97%, Cohn Fraction II and III) showed 71 and 100+% retentions, respectively. These data were consistent with the findings of paper electrophoresis in that albumin was associated with less calcium than expected from an equal distribution of bound calcium on the serum proteins, while gamma globulin was associated with more.

Magnesium. -- Results regarding the amount of this metal ion associated with the serum proteins were inconclusive. In the determination of serum magnesium, the bound metal was released merely by the acid precipitation of protein. Because of their mode of preparation, this loose binding behavior could account for the initial absence of magnesium in Albumin I, the Mixed Globulins, or Gamma Globulin. When Albumin II and Albumin III were exhaustively dialyzed, they showed 25% retentions as could be anticipated since they had no acid treatment and were the supernatants from serum. The lack of any retention of magnesium by whole serum is a discrepancy that may be attributed to an H-ion concentration effect (3).

Iron. -- After exhaustive dialysis, Albumin II (83%) and Albumin III (93%) both demonstrated the presence of bound iron. Since Cohn (12) reported the general order of precipitation of the serum proteins to be gamma, alpha plus beta, and albumin, the residual iron in these albumin

preparations might be associated with beta globulin. The 43% retention shown for both the Mixed Globulins and serum suggests that all bound serum iron was affiliated with the globulins.

Copper. -- Substantiation of earlier findings that copper was associated with globulin was obtained from the results of exhaustive dialysis. It was determined that zero per cent copper retentions were had by Albumin II and Albumin III whereas 96% retention was given by the Mixed Globulins. The complete retention of the metal ion by both Mixed Globulins and whole serum pointed out that practically all serum copper was protein bound.

D. Comparison of Findings with Other Work

Calcium. -- Correlation studies involving moving boundary and chemical analyses suggested calcium association with both albumin and globulins. Investigations of serum proteins separated electrophoretically on paper and selective staining demonstrated that: (a) calcium was bound to albumin and globulins, and (b) albumin and gamma globulin contributed equally in binding two-thirds of the total bound ion. Dialysis studies showed that albumin solutions retained calcium to the extent of 10-20%, Mixed Globulins 71%, Gamma Globulin 100+%, and serum 45% as protein bound.

Irreversibly protein bound calcium in serum has been reported to vary from 30-50% of the total (35,71,83,99,101). No studies were included to determine if albumin and/or globulins were specifically involved. But this investigation suggests the following with regard to serum protein-calcium interaction:

1. All five serum proteins examined contain bound calcium,
2. Albumin can form both a reversible complex with calcium as others indicate (21,68,103) and an irreversible complex with calcium, and
3. Gamma globulin tends to bind almost all of its calcium irreversibly, under the conditions studied.

Magnesium. -- Magnesium was found to be associated primarily with albumin from the moving boundary correlation studies. Paper electrophoresis indicated this ion to be present in all five serum proteins. After dialysis, albumin solutions showed 25% of their total magnesium content to be retained.

Reports in the literature state that 25-50% of the total serum magnesium may be bound (35,101). These findings contained herein with regard to serum protein-magnesium association are that:

1. All observed serum proteins contribute to the irreversible binding of the ion, and
2. Compared to calcium, magnesium binding at pH 8.6 is much less. Consequently, conclusions regarding calcium-protein interaction are not necessarily valid for magnesium.

Iron. -- The results of all experiments indicated iron was bound only to the beta globulin fraction of human serum. In correlation studies, high and low serum iron content was found whenever high and low beta globulin was observed electrophoretically. Selective staining for iron after paper electrophoresis indicated its presence apparently all in beta globulin. Dialysis showed that albumin solutions not completely

globulin-free, mixed globulins, and whole serum had retentions of 32-43% of the total serum iron.

In the literature, there is agreement that iron in serum is partially protein bound. Two reports stated that albumin was responsible for the iron ion binding (24,43), whereas another reported that the globulins were involved in this association (8). Cohn (16) and Koechlin (53) demonstrated complex formation between preparations of beta globulin and iron. The results of the present electrophoretic and dialysis investigations show an irreversible binding of iron and beta globulin.

Copper. -- The binding of serum copper principally by gamma globulin, as suggested by moving boundary studies, was substantiated by the paper electrophoresis technique. Exhaustive dialysis demonstrated no copper retention by albumin, but complete retention by the mixed globulins and whole serum, which lead to the conclusion that a globulin was the copper-binding protein.

All serum copper has been known to be protein bound (1,9,100) without clearly indicating the specific serum protein involved. One study indicated albumin binding (24), while another reported globulin binding of serum copper (43). More definitely, by using isolated protein preparations and metal ion addition, beta globulin was shown to complex copper (16,53) and gamma globulin to bind the cupric ion irreversibly (5).

The results of the present investigation demonstrate that a single serum protein fraction, gamma globulin, is mainly involved in binding copper.

Zinc. -- Zinc, located by histochemical staining after paper electrophoretic separation of serum proteins, appeared to be present only in gamma globulin. Previous workers reported that albumin and globulins of human serum reacted with zinc ion to produce reversible complexes (17,39,81). Prior to this study it was not known if zinc in human serum was entirely ionic or partially or totally protein bound. The occurrence of a colored spot attributable to this metal ion in the gamma globulin zone suggests that zinc is bound to some extent to this particular globulin.

E. The Significance of Protein-Metal Ion Complexes

The metal ions which occur in serum activate certain enzyme reactions. An explanation of this phenomenon is that the associated metal complex represents the active form of the enzyme with the metal serving as an activating proshtetic group. The metal proteinates also take part, to some extent, in the regulation of the metal ion concentration of the body fluids.

Specifically, protein bound calcium is believed to function in the mechanism of blood coagulation, the iron-binding globulin is thought to be responsible for iron transport, and the copper protein seems to function in the formation of hemoglobin. The high concentration of zinc normally found in leucocytes together with the observation of zinc in gamma globulin suggest a relationship between these two blood constituents that is concerned with infection.

V. SUMMARY

Moving boundary electrophoresis together with chemical determinations of total protein, calcium, magnesium, iron, and copper on normal human serum were undertaken to obtain information about the specific protein components concerned with metal ion binding. The results indicated the following:

1. Albumin and globulins were associated with calcium,

2. Albumin was primarily associated with magnesium,

3. Beta globulin was involved in binding iron, and

4. Gamma globulin appeared to bind copper.

Paper electrophoretic separation of serum proteins and selective metal ion staining demonstrated:

1. Albumin and the four globulins all contained calcium. Although albumin represented two-thirds of the total protein, this fraction had only one-third of the total bound calcium. Gamma globulin, about one-sixth of the total protein, also had one-third of the bound metal ion.

2. Magnesium appeared to be present in the five serum proteins.

3. Beta globulin was the only serum protein which contained iron.

4. Gamma globulin showed the presence of both copper and zinc.

Exhaustive dialysis studies of various protein solutions in the same buffer medium as that used for electrophoresis showed protein-metal ion binding was present.

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APPENDIX

Calculations

Relative fraction of total serum protein attributable to any single protein component = Area of pattern attributed to that component/Total area of pattern (exclusive of boundary anomaly).

g. total protein per 100 ml. serum = Absorbancy of unknown serum/ Absorbancy of known serum x 6.61 g. per 100 ml. (concentration of known serum).

($A_{S2}-A_{S1}f$) is the absorbancy due to the formation of the colored copper carbamate complex, corrected for the volume change on the addition of the color reagent. A_{S1} and A_{S2} are, respectively, the absorbancies of the solution before and after the addition of 0.4 ml. of sodium diethyldithiocarbamate, the color reagent. The factor for volume correction f is 5.6/6.0 since the volume of solution is 5.6 ml. before the addition of the reagent and 6.0 ml. after.

Absolute amount of a single protein fraction (g. per 100 ml. serum) = relative fractional amount of that fraction x g. total protein per 100 ml. serum.

Percent retention of any metal ion = mg. (or µg.) of that metal ion per g. protein after dialysis/mg. (or µg.) of that metal ion per g. protein before dialysis x 100.