COMPARISON OF THE ELECTROPHORETIC PATTERNS OF NORMAL CANINE SERUM AND PLASMA AND CHANGES IN THE SERUM AND PLASMA OF HEMOLYZED SPECIMENS

Dissertation for the Degree of M. S.

MICHIGAN STATE UNIVERSITY
VIDA M. AMOG

1973

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ABSTRACT

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By

Vida M. Amog

Comparison of electrophoretic patterns of normal canine plasma and serum revealed a greater concentration of the beta-3 fraction in plasma due to the presence of fibrinogen. When serum or plasma of hemolyzed canine blood was analyzed electrophoretically, there was slurring of the beta globulin peaks due to the presence of free hemoglobin and there were increases in alpha-2 globulins due to the formation of the haptoglobin-hemoglobin complex.

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Vida M. Amog

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Pathology

1973

To my mother,

Mrs. Florentina M. Amog,

and the loving memory of my father,

Mr. Benjamin M. Amog

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INTRODUCTION

Electrophoresis may be defined as the separation of components in a mixture based on their differing rates of migration in an electric field. The method is particularly valuable for separating labile macromolecules because it can be carried out in a supported aqueous medium under mild conditions of pH and ionic strength.

Electrophoresis was first used only as a means for measuring ionic mobilities and isoelectric points. After Tiselius' important work in 1937, and with later improvements in the optical technique, it became possible to use the method for quantitative analysis of complex mixtures, as well as for the separation of different substances. The analysis of mixtures soon became the most important application of electrophoresis.

There have been many reports based on electrophoretic studies of protein distribution in both human beings and animals. However, results have differed because of the differences among the various techniques and species. Hence, it is desirable that each laboratory establish its own standard reference electrophoretic patterns for the species of interest.

At times, it is necessary to use plasma for electrophoretic analysis when serum is not available. However, plasma contains fibrinogen which, in human plasma, migrates between the beta and gamma globulins. Thus, a comparison of the electrophoretic patterns of serum and plasma of animals is useful.

Hemolysis of blood specimens is a common problem in clinical laboratories. In many cases, it is impossible to obtain specimens completely free of hemolysis. This may be the result of increased erythrocyte fragility or improper handling of specimens. If the patient is not available for collection of another sample, it may be necessary to perform the electrophoretic analysis on the hemolyzed specimen. Hemolysis produces certain characteristic changes in serum and plasma reflecting the presence of free hemoglobin and the formation of the haptoglobin-hemoglobin complex.

This investigation was designed to compare the electrophoretic patterns of a) serum and plasma, b) serum of partially hemolyzed specimens with serum of specimens free of hemolysis, and c) plasma of partially hemolyzed specimens with plasma of unhemolyzed specimens.

LITERATURE REVIEW

Historical Background

Experiments in electrophoresis started in the early part of the nineteenth century. These studies increased in number into the twentieth century, culminating in Arne Tiselius' important work in 1937 concerning the moving boundary for which he earned the Nobel Prize in Chemistry in 1948. Since then, many investigators have contributed to the development of these techniques.

The books *Serum Proteins and Dysproteinemias* (1964), edited by F. W. Sunderman and F. W. Sunderman, Jr., and *Electrophoresis of Proteins and the Chemistry of Cell Surfaces* (1964), by H. A. Abramson *et al.* offer a fairly complete historical background of electrophoresis and topics related to protein chemistry.

One of the earliest reports concerning protein electrophoresis in dogs was by Munro and Avery (1946) and concerned the effects of hepatectomy on the relative concentrations of plasma proteins.

Vesselinovitch (1959) gives an excellent review of paper electrophoresis in domestic animals, including dogs. He stated that the first extensive study of serum proteins of domestic animals was by Boguth (1953), and the first results of paper electrophoresis of canine serum were published by de Wael and Teunissen (1954). However, de Wael's and Teunissen's studies were restricted to animals with hepatic disorders.

Due to differences in techniques and instruments used by various laboratories, the number of bands resolved may range from 5 to as many

as 90 as reported by Farrow (1972) for human serum. However, only a small number of these proteins have known biological functions. It is probable that the plasma proteins of animals are equally numerous.

Principle of Electrophoresis

The principle of electrophoresis is simple. An ion or group will migrate towards the positive or negative electrode, depending on its charge, when placed in an electric field. The charged species moves in the electric field at a rate which is a function of its size, shape and charge. After some time, the different charged species in the mixture separate into zones detected by suitable techniques (Smith, 1968; Shaw, 1969).

The movement of ions is influenced by four factors: 1) Electric field strength. When a charged species is dissolved or suspended in a buffer solution and subjected to a uniform field, the particles will migrate at a constant rate determined by their physical shape, size, and charge. Positively charged species move towards the cathode (-) and negatively charged species towards the anode (+) (Longsworth, 1959; Wieme, 1965). 2) Ion mobility, which is the distance travelled by the migrating species in relation to the support medium in a given time in a field of unit potential gradient (Longsworth, 1959; Smith, 1968). 3) Buffer pH and ionic strength. The pH of the electrolyte has an influence on electrophoretic behavior because the net charge of most species is dependent on pH. The ionic strength is usually adjusted to 0.05 to 0.1, which is optimal for a compromise between high mobilities and sharpness of zones. Since conductivity and power consumption are functions of ionic strength, it is desirable to keep the ionic strength low. This minimizes heat production and electrode products formed

(Shaw, 1969). 4) Supporting medium. This usually consists of paper, starch gel, agar gel, cellulose acetate or other porous material that is saturated with buffer. Each has its own advantages and disadvantages (Williams and Nixon, 1964; Smith, 1968; Shaw, 1969).

Supporting Media

Historically, the most commonly used supporting medium for electrophoresis has been filter paper. Although excellent reproducibility, low cost and convenience can be achieved with paper electrophoresis, the disadvantages of tailing, blurring and reduced resolution are seen in the electrophoretic pattern. These are due to the fibrous structure of paper and the interaction of ionic sites on the paper and the charged species being separated (Jencks *et al.*, 1955; Peeters, 1959; Yeoman, 1959).

Thin, porous cellulose acetate membrane was introduced as a medium for electrophoresis by Kohn in 1957. Due to its regular and fine-textured pores, there is little or no adsorption to the serum. The time required for electrophoretic determination is greatly reduced while accuracy and reproducibility are improved.

The use of gels has been shown to be a rapid means of producing high resolution separation of proteins. Among them is the agar gel which is still widely used today. It forms a firm colloidal gel in concentrations as low as 1%. Compared to other gels, it possesses some outstanding advantages, including ease of handling, excellent transparency, stability in the gel state, reusability after re-melting and re-pouring, low cost and rapid separation. R. J. Wieme (1965) has published detailed information on the use of agar gel as a medium for electrophoresis.

Starch (Kunkel and Slater, 1952) and polyacrylamide gels[1] have also been used for electrophoretic analysis of serum (Raymond and Weintraub, 1959; Smith, 1968). The use of these gels permits the resolution of 20 bands or more (Henry, 1965; Smith, 1968). Polyacrylamide gel is especially useful in molecular weight determinations (Shapiro, 1967). However, for routine clinical purposes, the use of these gels is not generally practical.

Preservation and Storage of Samples

Fresh specimens are to be preferred (Coles, 1967). Henry (1965) reported that samples can be stored at room temperature for 3 days without any significant change or at refrigerator temperatures (5 to 10 C) for as long as 1 month, although long storage in the refrigerator may result in diminished resolution in the globulin fraction. Damm and King (1965), on the other hand, reported the stability of samples at refrigerator temperatures for at least a week.

There are conflicting reports regarding the effects of freezing. Oberman *et al.* (1956) suggested freezing samples no longer than 2 days. Engel *et al.* (1961) generally observed that freezing does not produce notable alterations in starch gel electrophoresis. He noted, however, one instance of diminished resolution and slight alteration in mobility. Henry (1965) reported serum stability for at least 6 months in the frozen state. Repeated freezing and thawing should be avoided, for inactivation and denaturation may occur in some labile proteins (lipoproteins, purified antibodies and pure ovalbumin).

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[1] Cyanogum 41, a synthetic gel.

Principal Fractions of Serum Proteins

The number of serum protein fractions which can be identified varies considerably depending on the method used for analysis. However, there are 4 major fractions routinely isolated by electrophoretic methods: albumin, alpha (α) globulin, beta (β) globulin, and gamma (γ) globulin (Tiselius, 1937). Each one has its own characteristic rate of migration.

Albumin. This is primarily synthesized in the liver. One of its main functions is the maintenance of intravascular osmotic pressure (Coles, 1967; Tietz, 1970; Farrow, 1972). It also acts as a transport agent for drugs, pigments and other substances (Coles, 1967; Farrow, 1972). Albumin comprises approximately one-half of the serum protein concentration in both normal man and animals. However, the mean concentration in animals varies from species to species (Coles, 1967; Haurowitz, 1963).

Albumin is the fastest moving fraction on electrophoresis at pH 8.6. Human albumin has a molecular weight of approximately 69,000, a sedimentation coefficient of 4.7 S and is isoelectric at pH 4.7 (Sibley and Hendrickson, 1970; Haurowitz, 1963). It is internally crosslinked by many disulfide bonds. Aspartic acid (ASP) is its N-terminal residue and leucine (LEU) is its C-terminal residue. Its amino acid sequence is not well known and only incomplete data are available (Murayama, 1964).

The albumin level is seldom increased above the normal range except in severe dehydration and shock (Coles, 1967). A relative decrease in this fraction is significant and may be due to reduced synthesis, more rapid catabolism or increased globulin concentration (e.g., malnutrition, hepatic disease) (Kaneko and Cornelius, 1970).

Alpha (α) Globulins. This comprises a group of proteins which bind a number of substances for transport in the plasma. Included are haptoglobin, α-lipoprotein, transcortin, and ceruloplasmin which bind, respectively, hemoglobin, lipids, corticosteroids, and copper (Farrow, 1972).

On electrophoretic patterns at least two α-globulin bands, identified as α_1 and α_2, are seen. The α_1 globulins form a narrow, often barely discernible, band adjacent to albumin. An ionic strength of the buffer of 0.05 or higher permits a good separation of this band from the albumin zone (Wieme, 1965). The α_2 globulins appear as a discrete, compact band. It is in this area where haptoglobin migrates.

Haptoglobin, from the Greek word *haptein* (to fix, seize, or hold fast), is a serum glycoprotein which has a high affinity for globin, whether free or in the form of hemoglobin, with which haptoglobin forms a very stable complex. Haptoglobin is synthesized mainly in the liver and, in complex with hemoglobin, is removed from the circulation by the reticuloendothelial system at the rate of 13 mg. of hemoglobin/100 ml. of plasma/hour (Louderback and Shanbrom, 1968; Blumberg, 1964).

Giblett (1961) gives an excellent review of the physiology, chemistry and genetics of haptoglobin. There are 3 main types of haptoglobin in human beings, namely 1-1, 1-2, and 2-2. These are genetically determined by a pair of autosomal genes Hp^1 and Hp^2, which are responsible for the 3 types Hp^1/Hp^1, Hp^2/Hp^2 and Hp^2/Hp^1. A fourth haptoglobin type, 0-0 (ahaptoglobinemia), has been shown to occur in 30% of Nigerian Negroes, 5% of American Negroes, 2% of British Caucasians, and 1% of Danish Caucasians. It has also been noted that 90% of all newborn infants lack haptoglobin, but that it appears within 4 to 6 months (Smith, 1968; Miale, 1962, 1972).

Although there has been a great deal of investigative work on human haptoglobin, there are relatively few reports on this subject in animals. Only one form (corresponding to human type 1-1) has been detected in animals, including dogs. Shim *et al.* (1971) investigated canine serum haptoglobins and found them similar to human type 1-1 with respect to subunit structure, hemoglobin-binding mechanism and binding sites. However, free canine haptoglobin (molecular weight 81,000) had a slightly faster mobility in starch gel electrophoresis than type 1-1 human haptoglobin (molecular weight 85,000 to 100,000). This was attributable mainly to the slightly lower molecular weight of canine haptoglobin which enables faster migration in the commonly employed molecular sieving starch gel medium.

Reduced α-globulin concentrations are seldom seen in domestic animals except in severe, chronic liver disease (Coles, 1967; Farrow, 1972). Haptoglobin concentrations are decreased in conditions characterized by intravascular hemolysis. Elevated concentrations of α-globulins (particularly α_2) are seen in inflammatory reactions (e.g., bacterial and viral infections, trauma, fever) (Coles, 1967; Kaneko and Cornelius, 1970; Farrow, 1972).

Beta (β) Globulins. The main site of biosynthesis of beta (β) globulins is the liver (Farrow, 1972). Included in this group are transferrin (an iron-binding protein), hemopexin (a heme-binding protein) and beta-lipoprotein, which is concerned with the transport of lipids (Wieme, 1965; Smith, 1968; Farrow, 1972).

A varying number of beta fractions occur in electrophoretic patterns depending on the method used. Oftentimes, especially in man, only a single β peak is identified. In dogs, there may be a single

band (Vesselinovitch, 1958; Irfan, 1967), 2 bands (de Wael, 1956; Bulgin *et al.*, 1971), or 3 bands (Kozma *et al.*, 1967). Detailed characterization of these bands requires sophisticated techniques such as starch gel electrophoresis or immunoelectrophoresis. It is in this fraction that free hemoglobin migrates electrophoretically as shown by several studies (Allison and Rees, 1957; Nyman, 1960; Dacie and Lewis, 1963; Louderback and Shanbrom, 1968).

Hemoglobin. Hemoglobin (molecular weight 69,000) is a combination of protein, globin, with heme. Heme is a protoporphyrin consisting of iron chelated with 4 pyrrole groups. In man, there are several known normal and abnormal hemoglobins, each of which has a distinct electrophoretic mobility (Bromberg *et al.*, 1972).

Sydenstricker *et al.* (1956) reported that canine hemoglobin has an electrophoretic mobility faster than that of Hb S, and that it appears as a homogeneous compound. However, they did not mention the number of dogs used. This agrees well with LeCrone's (1970) observations on 21 adult dogs. It is possible that there are more than one hemoglobin found in dogs. This awaits further research.

Fibrinogen. Previous workers have reported that fibrinogen (ϕ) which is present in plasma but not in serum, migrates between the β and γ globulins (Abramson, 1964). It is a glycoprotein which is converted to fibrin in the presence of Ca^{++} by thrombin. It is also known as Factor I, playing an important role in blood coagulation. It is synthesized in the liver. Fibrinogen is easily precipitated by inorganic salts at high concentrations e.g., 1/2 saturated solution of NaCl, 1/4 saturated solution of $(NH_4)_2SO_4$, and is the only protein reversibly precipitated at 56 to 58 C (Hawk, 1965; Henry, 1965; Schalm, 1972).

On electrophoresis on agar gel, fibrinogen does not migrate. This is due to its precipitation by the agar (Wieme, 1965). Increased fibrinogen concentrations may occur in connection with hepatic diseases, acute infections and septicemias. Plasma fibrinogen concentration is decreased in afibrinogenemia, shock, severe burns, and hepatic insufficiency (Hawk, 1965; Coles, 1965; Schalm, 1972).

Gamma (γ) Globulins. These are normally produced by the cells of the reticuloendothelial tissues (spleen, bone marrow, lymph nodes, and intestinal mucosa) (Tietz, 1970; Farrow, 1972). Immunoglobulins are antibodies produced in response to antigenic stimulation. There are 5 principal types recognized in man: IgG, IgA, IgM, IgD and IgE. Each molecule consists of 2 heavy (H) and 2 light (L) polypeptide chains (Waldenstrom, 1968; Terry and Fahey, 1964; Farrow, 1972). Increased gamma globulin concentrations may be seen in cases of myeloma, severe, chronic infection, chronic, active hepatitis, or advanced neoplasia. Reduced concentrations may result either from congenital conditions (e.g., hypogammaglobulinemia) or acquired conditions such as reticuloendothelial neoplasia (Coles, 1967; Farrow, 1972; Kaneko and Cornelius, 1970). Electrophoretically, the gamma globulins migrate towards the cathode, behind the application point. This is due to electro-osmosis (Smith, 1968).

MATERIALS AND METHODS

The experimental studies were divided into 3 parts: 1) comparison of normal canine serum and plasma, 2) comparison of serum from partially hemolyzed blood and unhemolyzed blood, and 3) comparison of plasma from partially hemolyzed blood and unhemolyzed blood.

Electrophoresis was carried out by the method of Cawley and Eberhardt (1962) with some modifications.

Method: Two electrophoresis strips were prepared for each animal in the following manner: 4 ml. of 1% Ionagar^{R} in 1/2 strength Veronal buffer[1] were pipetted onto 35mm. film-strips[2] 6-1/2 inches in length. Serum or plasma (0.006 ml.) was applied to the center of the strips using a Spinco applicator. Eight strips (from 4 dogs) were placed in a Durrum cell containing the Veronal buffer. Electrophoretic fractionation was accomplished using 150 volts and 60 to 80 milliamperes for 1 hour 15 minutes. At the end of the run the strips were fixed in 90% methanol. The strips were then dried in a 95 to 100 C oven for 30 minutes and stained with 0.2% thiazine red[3] in 10% acetic acid. The strips were then rinsed in distilled water, decolorized in several washes of 5%

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[1]Barbital-Sodium Barbital Mixture, pH 8.6, ionic strength 0.075, Harleco, 60th and Woodland Avenue, Philadelphia, Pennsylvania 19143.

[2]E. I. duPont de Nemours, Photo Products Dept., 7415 Melvina, Niles, Illinois 60648. Safety Motion Picture Film, 0.004" thick, unperforated.

[3]Thiazine Red R, Color Index No. 14780, Harleco, 60th and Woodland Avenue, Philadelphia, Pennsylvania 19143.

acetic acid, air dried, and scanned at 505 nm with a densitometer-integrator.[1] The densitometer converts the color density pattern into a concentration curve and the integrator measures the relative area under each peak.

Total protein concentration was determined by the biuret method of Gornall *et al.* (1949).

Experiment 1
Comparison of the Electrophoretic Patterns of Serum and Plasma

Source of Specimens. Blood samples were collected in evacuated glass tubes[2] from 24 clinically normal appearing adult dogs. Serum was obtained from clotted blood after centrifugation and plasma from samples with EDTA[3] in the ratio of 9 mg./7 ml. of blood. Hematocrit values and hemoglobin concentrations were immediately determined from EDTA samples. Packed cell volumes were determined using the microhematocrit method with centrifugation at approximately 10,000 g for 5 minutes in a micro-capillary centrifuge.[4] Hemoglobin was determined by the cyanmethemoglobin method.

The degree of hemolysis of hemolyzed samples was measured by determination of the hemoglobin concentration of serum and plasma using the method of Fielding and Langley (1958), except that a commercial hemoglobin control[5] was used in the preparation of the standard calibration curve. This method depends on the peroxidase activity of hemoglobin

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[1]Photovolt Densitometer, Model 425, Photovolt Corp., New York, N.Y.

[2]Vacutainer, Becton, Dickinson & Company, Rutherford, N.J.

[3]Tri-Potassium Ethylene Diamine Tetra-Acetate.

[4]International Equipment Company, Needham Heights, Massachusetts.

[5]Hycel Hemoglobin Control, Hycel, Inc., Houston, Texas.

which causes oxygen to be released from hydrogen peroxide. The free oxygen then oxidizes orthotolidine to a blue reaction product which is measured photometrically. The reagents required for preparation of the hemoglobin reagent are available commercially combined in tablet form.[1]

Determination of Fibrinogen Concentration. Fibrinogen was determined by the method of Loeb and Mackey (1972). The method is based on the heat precipitation of fibrinogen and quantitation of the precipitate by the biuret method. The concentration was derived from a standard curve prepared by using crystallized bovine albumin.

Isolation and Purification of Fibrinogen. To determine in which fraction fibrinogen migrates, it was isolated and purified. Electrophoresis was then performed on the concentrated, purified fibrinogen obtained.

Method. Fibrinogen was isolated by the method of Atencio *et al.* (1965). This method involves salt fractionation coupled with a cold insoluble protein precipitation. The protein fraction thus isolated, after being redissolved in 0.005 M citrate solution, was verified as relatively pure fibrinogen by demonstration of the fact that it was 94.4% clottable by addition of bovine thrombin.[2]

Total Protein. Total protein concentrations of all serum and plasma samples were determined by the method of Gornall *et al.* (1949).

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[1]Hematest Tablet^{R}, Ames Company, Inc., Elkhart, Indiana.

[2]Thrombin, Topical (bovine origin), Parke, Davis & Company, Detroit, Michigan.

Experiment 2
Comparison of Normal Canine Serum and Serum from Partially Hemolyzed Samples

Source of Hemolyzed Specimens. Blood was taken from 24 normal-appearing, adult dogs. A portion of the serum was pipetted off from the tube after centrifugation and the remainder of the specimen subjected to mechanical agitation to obtain hemolyzed samples. The degree of hemolysis of each sample was determined by determination of serum hemoglobin concentrations as previously described. Electrophoresis and total protein determinations were then carried out on each of the 24 samples and compared. The following determinations were carried out.

Serum Haptoglobin. Serum haptoglobin was measured using the method of Owen *et al.* (1960). This is a simple colorimetric method based on the peroxidase activity of haptoglobin-methemoglobin complexes.

The concentration of haptoglobin was obtained from a calibration curve and was expressed in terms of bound methemoglobin. All tests were done at room temperature (24 to 25 C).

Haptoglobin Isolation and Purification. To determine in which fraction haptoglobin migrates, it was isolated and purified according to the method of Connell and Smithies (1959). Dowex 2x-10 (200-400 mesh) anionic exchange resin, as the chloride, was used to adsorb the haptoglobins from dialyzed serum at pH 4.2. The resin was set up in a column after the adsorption and was washed with water to remove the soluble proteins. The haptoglobins were eluted with 0.05 M NaCl. All steps were carried out at room temperature except the dialysis, which was done in the cold room. The isolated haptoglobin was concentrated by vacuum dialysis and concentration determined by the method of Owen *et al.*

(1959). Electrophoresis was then carried out on the isolated, concentrated solution to determine the migratory characteristics of haptoglobin.

Hemoglobin Isolation. Hemoglobin electrophoresis was performed on the hemolysates of each sample to determine in which fraction pure hemoglobin migrates.

Method. Hemolysates were prepared according to the method of Dacie and Lewis (1963) with the following modifications: use of 1.5 ml. instead of 1.0 ml. of distilled water per milliliter of packed cells to make a concentration of approximately 10 gm./100 ml. hemoglobin solution; use of chloroform instead of carbon tetrachloride; and freezing and thawing of red cells to insure complete hemolysis before the addition of chloroform.

Experiment 3
Comparison of Normal Canine Plasma and Plasma from Partially Hemolyzed Samples

Source of Specimens. Blood was taken from 24 normal-appearing, adult dogs. A portion of the plasma was pipetted off from the tube after centrifugation and the remainder of the specimen subjected to mechanical agitation to obtain hemolyzed samples. The degree of hemolysis of each sample was determined by determination of plasma hemoglobin concentrations as previously described. Electrophoresis and the determination of total protein concentration were then carried out on each of the 24 samples and compared. The following determinations were performed: 1) fibrinogen determination on each of the 24 samples, 2) fibrinogen isolation as previously described, 3) electrophoresis of purified fibrinogen to determine migratory characteristics of fibrinogen,

4) hemoglobin isolation as previously described, and 5) electrophoresis of hemolysate to determine migratory characteristics of hemoglobin.

RESULTS

Experiment 1
Comparison of the Electrophoretic Patterns of Serum and Plasma

Representative electrophoretic tracings are shown in Figures 1 and 2. The concentration of the β_3 fraction of plasma was higher than that of serum due to the presence of fibrinogen ($P<0.01$). In Figures 3 and 4 it can be seen that isolated, concentrated fibrinogen migrated with the β_3 fraction. Fibrinogen determinations were done on plasma samples of each of the 24 dogs, and the concentrations were found to be in the range of 297-695 mg./100 ml., with a mean of 438 mg./100 ml. and a standard deviation of $\pm$ 22.2. The means, standard deviations and ranges of the concentrations of serum and plasma proteins as determined electrophoretically are graphically represented in Figure 5 and are shown in Table 1. In Table 1 it can be seen that there were also significant differences in the α_2 and β_1 fractions, these being in higher concentrations in plasma than in serum ($P<0.01$). In the case of the electrophoretic tracing shown in Figures 1 and 2, there were also higher concentrations of the α_1 and β_2 fractions in plasma. However, as can be seen in Table 1, these differences were not consistent throughout the group.

Experiment 2
Comparison of the Electrophoretic Patterns of Normal Serum and Serum from Partially Hemolyzed Specimens

Figures 6 and 7 compare representative electrophoretic analyses of normal serum and serum from partially hemolyzed blood in 24 dogs. After hemolysis of the samples, the concentration of the total protein was

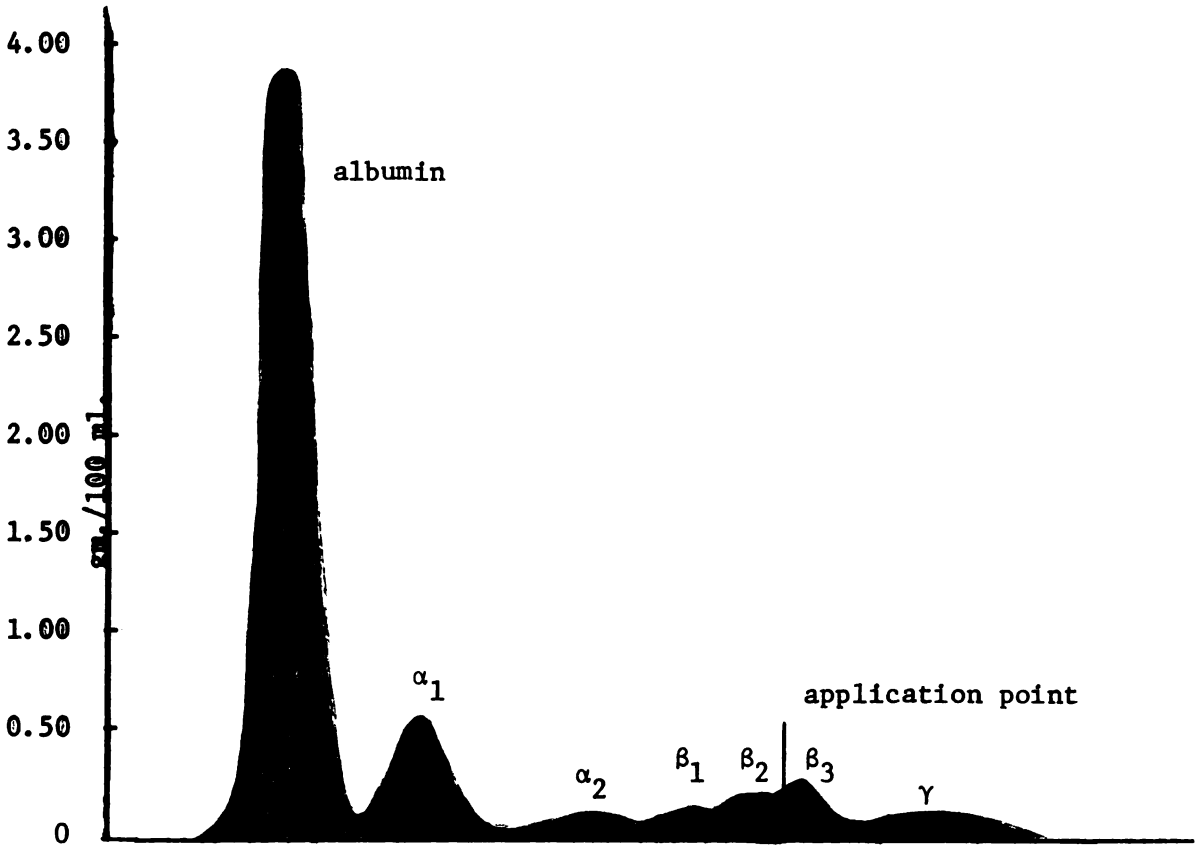

Figure 1. Tracing of electrophoretic analysis of serum of a representative normal dog (Dog 12).

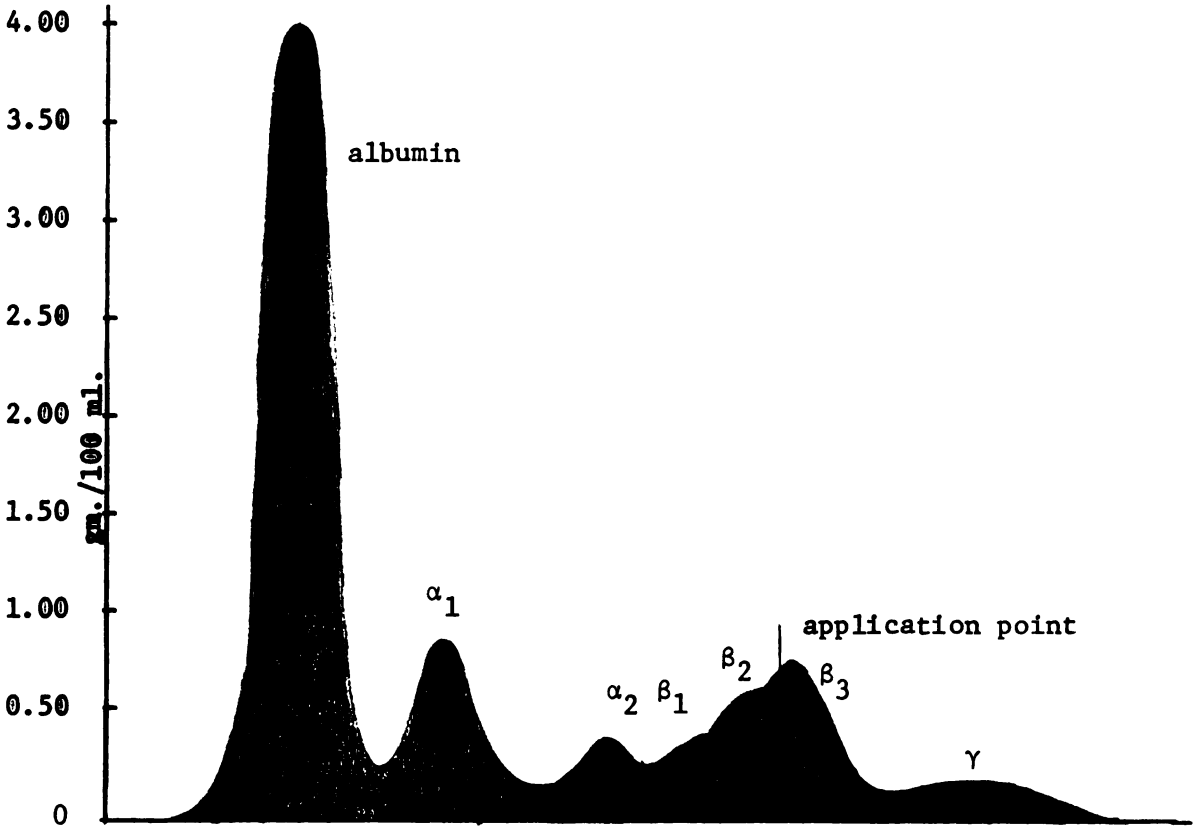

Figure 2. Tracing of electrophoretic analysis of plasma of Dog 12.

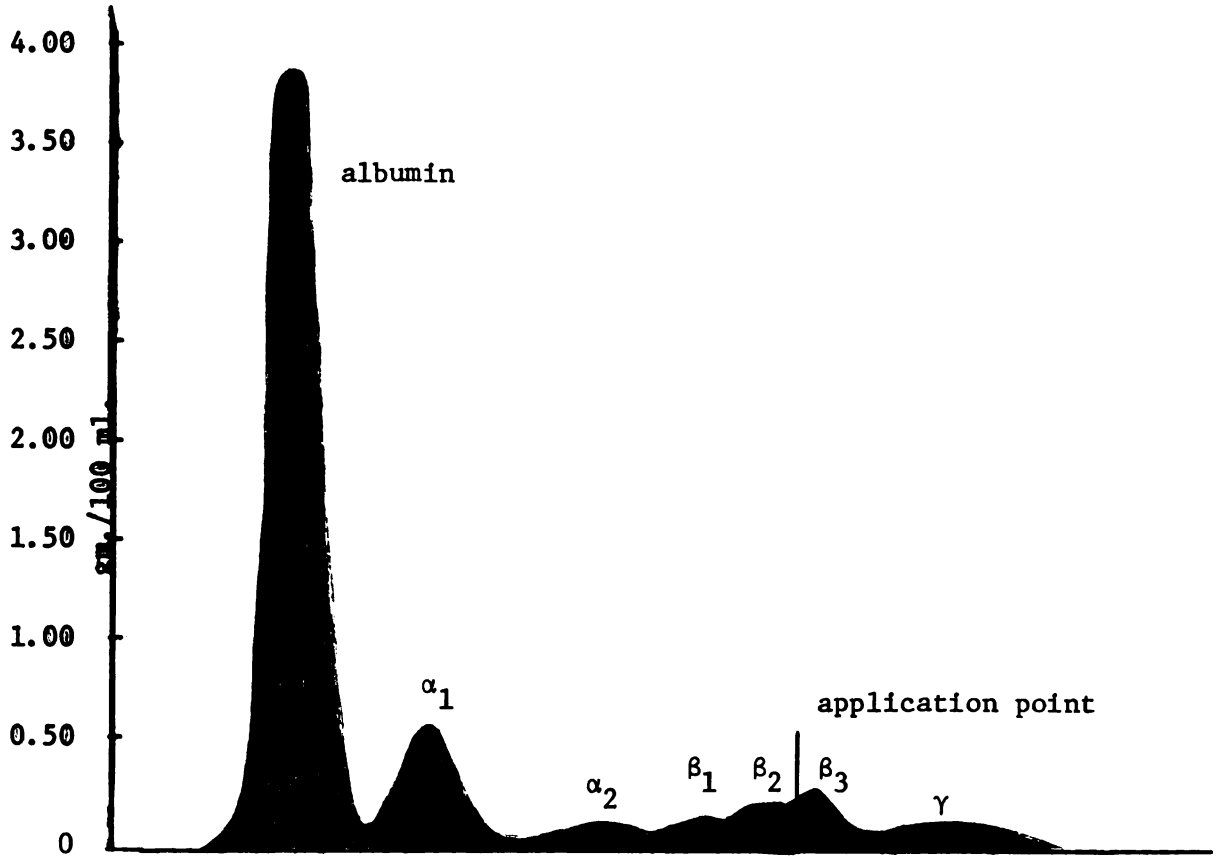

Figure 1. Tracing of electrophoretic analysis of serum of a representative normal dog (Dog 12).

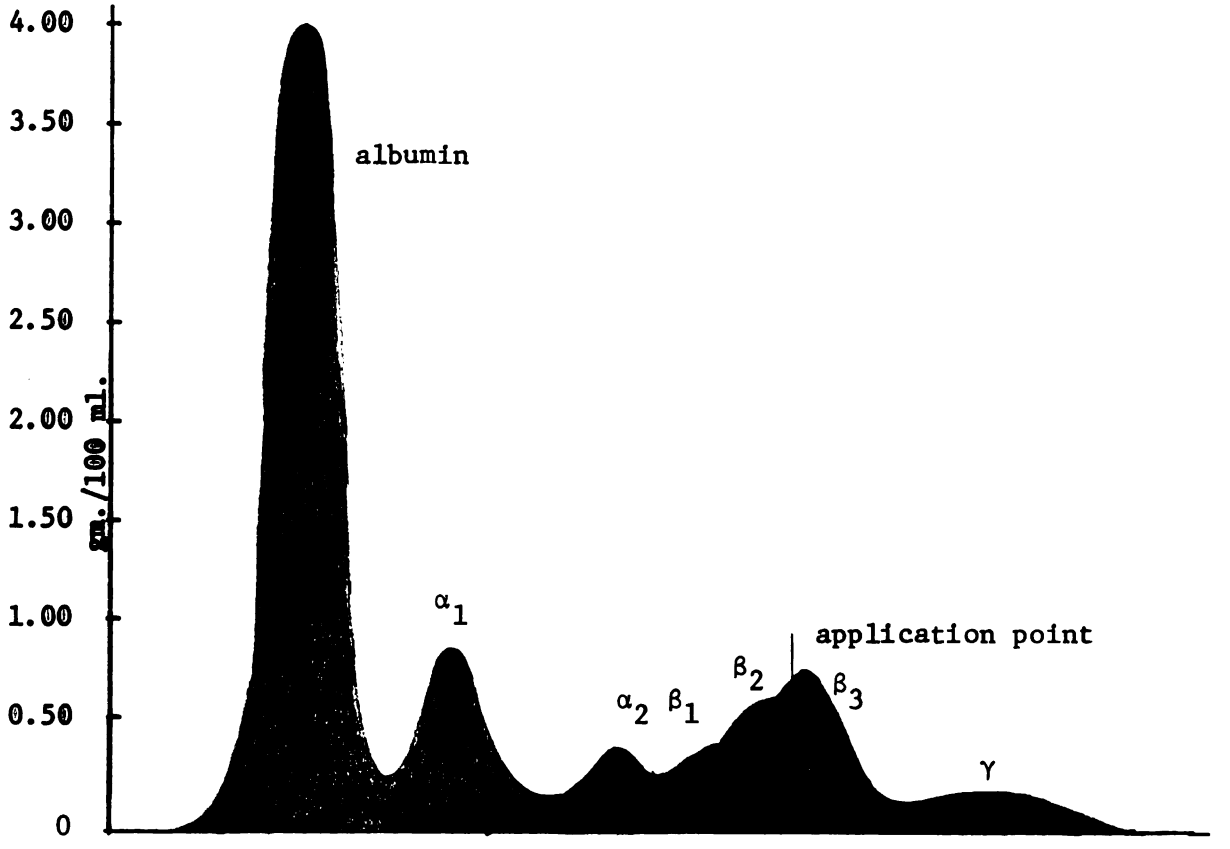

Figure 2. Tracing of electrophoretic analysis of plasma of Dog 12.

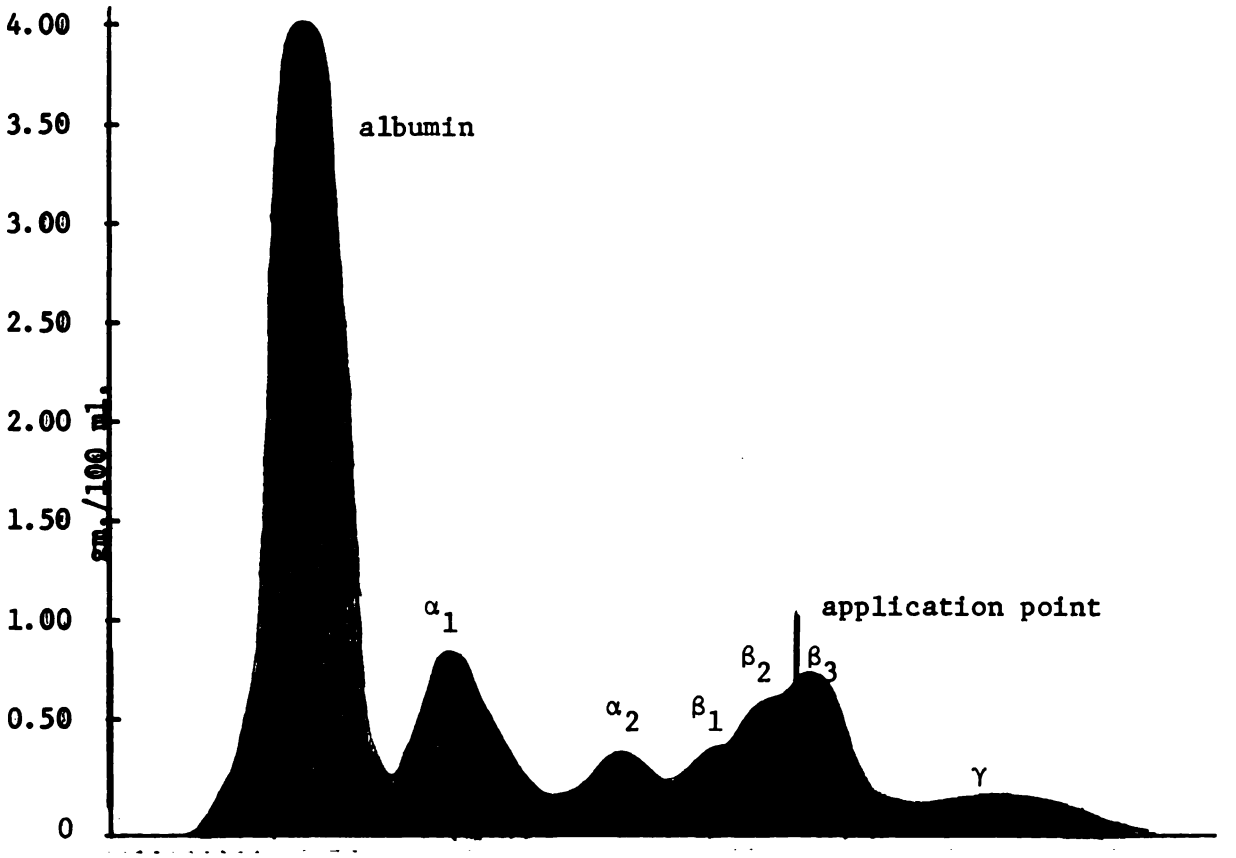

Figure 3. Tracing of electrophoretic analysis of plasma of Dog 12.

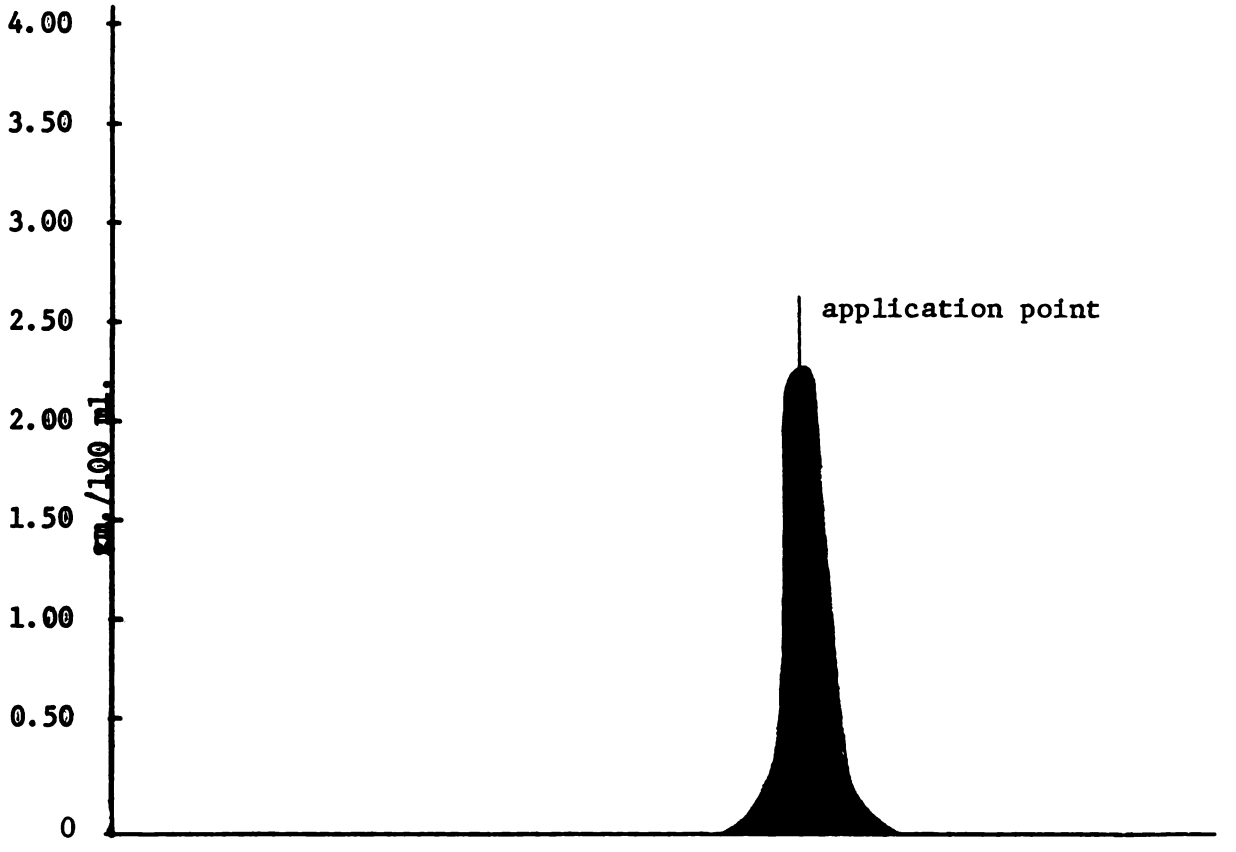

Figure 4. Tracing of electrophoresis of isolated, concentrated fibrinogen of pooled dog plasma.

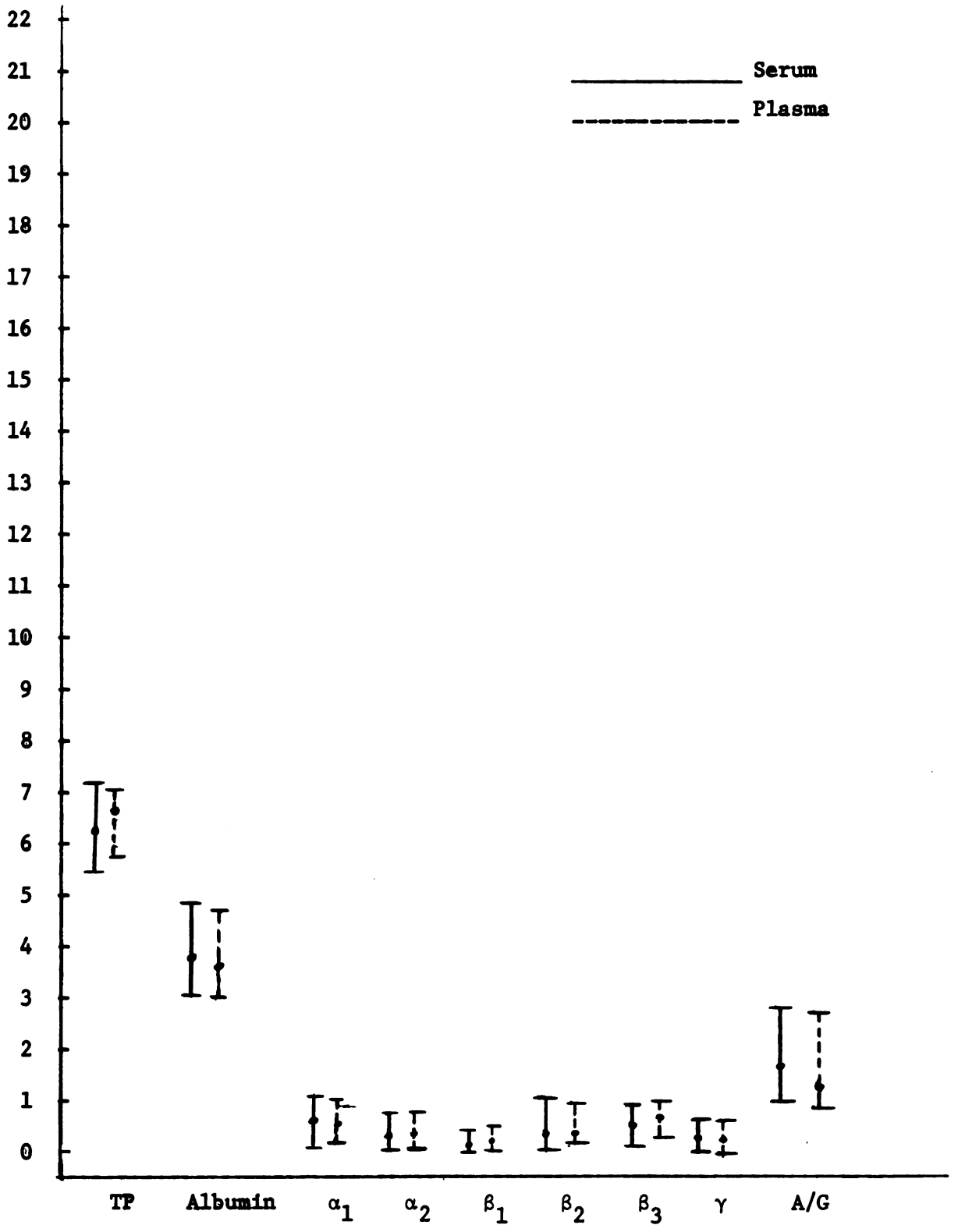

Figure 5. Comparison of electrophoretic analyses of serum and plasma. Means and ranges of protein concentrations for 24 dogs.

Table 1. Comparison of means, ranges and standard deviations of concentrations of plasma and serum proteins of normal specimens for 24 dogs

		Mean (gm./100 ml.)	Standard Deviation	Range (gm./100 ml.)
Total Protein	S	6.32*	0.429	5.50 - 7.22
	P	6.86†	0.387	5.85 - 7.10
Albumin	S	3.85	0.367	3.18 - 4.99
	P	3.71	0.400	3.14 - 4.88
Alpha-1 Globulin	S	0.63	0.182	0.27 - 1.17
	P	0.61	0.172	0.39 - 1.13
Alpha-2 Globulin	S	0.30*	0.113	0.11 - 0.71
	P	0.40†	0.114	0.19 - 0.75
Beta-1 Globulin	S	0.19*	0.066	0.08 - 0.44
	P	0.25†	0.091	0.11 - 0.52
Beta-2 Globulin	S	0.49	0.270	0.22 - 1.20
	P	0.46	0.129	0.27 - 0.80
Beta-3 Globulin	S	0.50*	0.134	0.28 - 0.81
	P	0.72†	0.203	0.40 - 1.14
Gamma Globulin	S	0.35	0.121	0.14 - 0.63
	P	0.35	0.134	0.07 - 0.63
A/G Ratio	S	1.67_2^1	0.404	1.13 - 2.88
	P	1.38^2	0.381	0.93 - 2.79

S = Serum
P = Plasma

* = Significantly different from † ($P<0.01$).

1 = Significantly different from 2 ($P<0.05$).

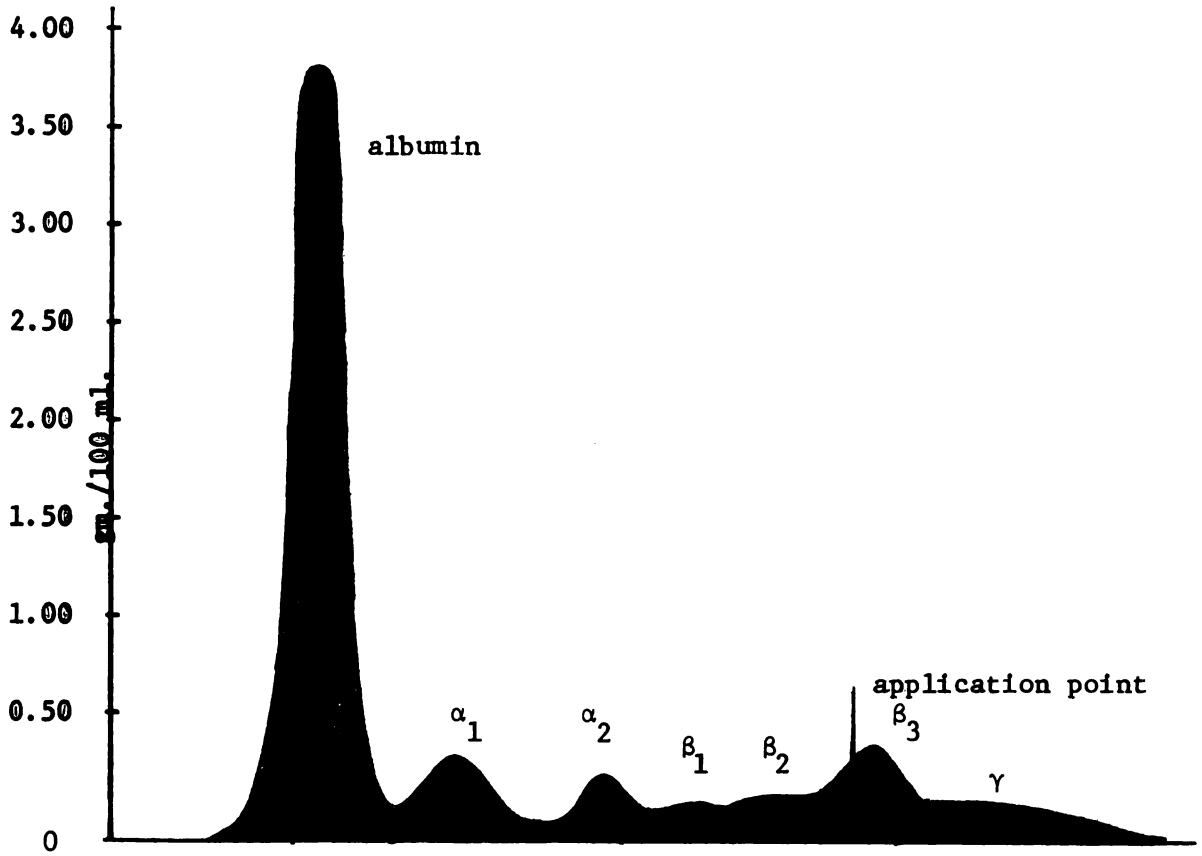

Figure 6. Tracing of electrophoretic analysis of serum of Dog 18.

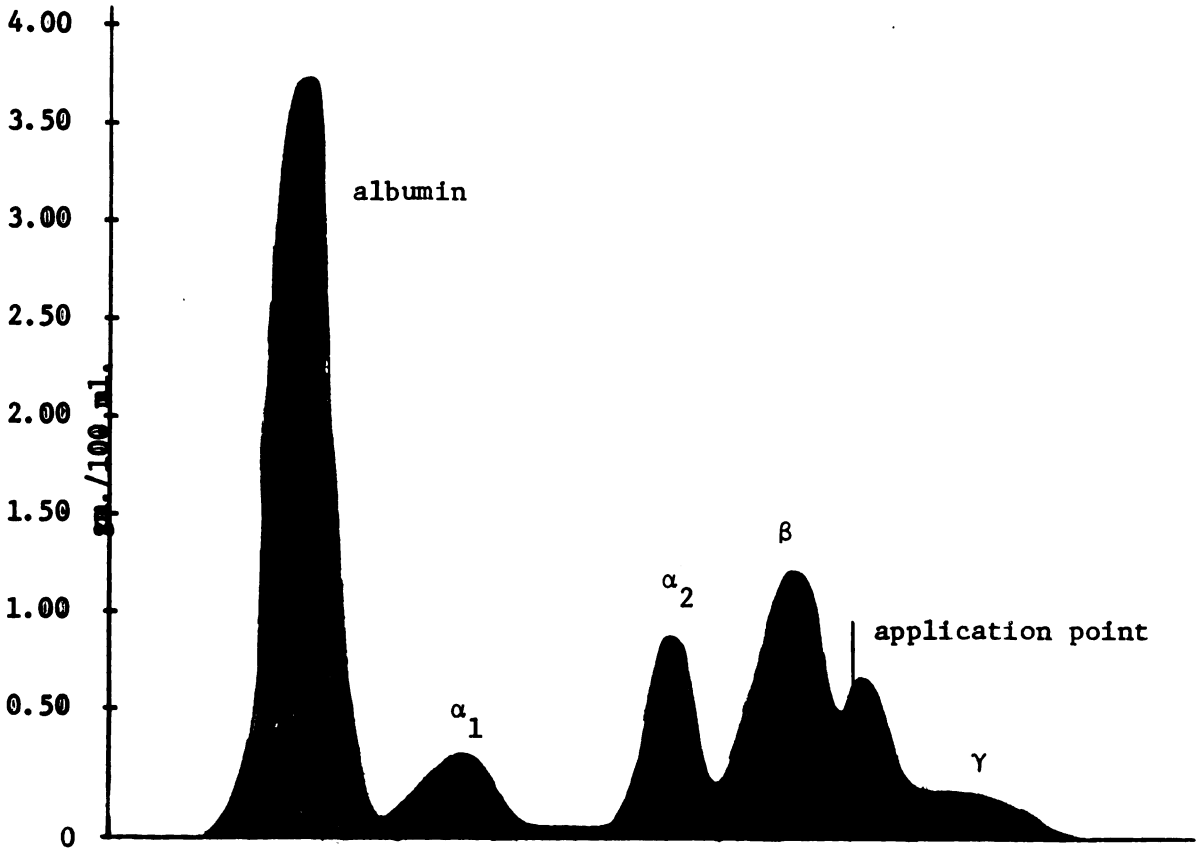

Figure 7. Tracing of electrophoretic analysis of serum from partially hemolyzed blood of Dog 18.

increased ($P<0.01$) and the β globulins tended to be slurred and only 2 β peaks were identifiable. The presence of approximately 50 mg. of free hemoglobin/100 ml. of serum or plasma was sufficient to produce slurring of the β globulins (Figures 8 and 9). Furthermore, there was an elevation of the α_2 globulin fraction ($P<0.01$) as a result of the formation of the haptoglobin-hemoglobin complex (Figure 7). Finally, in some dogs, there was an apparent retardation in the migration of the α_2 globulin fraction resulting in the formation of a distinct valley between it and α_1.

In Figure 11 it can be seen that hemoglobin migrates with the β globulins. By contrast, haptoglobin and the haptoglobin-hemoglobin complex both migrate as α_2 globulins (Figures 13 and 15).

Haptoglobin determinations were carried out on each of the 24 samples and were found to be from 0-235 mg./100 ml. with a mean of 133 mg./100 ml. and a standard deviation of $\pm$ 34.03.

The means and ranges for normal serum and for serum of partially hemolyzed specimens are graphically illustrated in Figure 16 and are shown in Table 2. In Table 2 it can be seen that albumin concentration was somewhat higher in hemolyzed specimens than that of unhemolyzed specimens. However, as determined by the Student's t test, there were no statistically significant differences between them.

Experiment 3
Comparison of the Electrophoretic Patterns of Normal Plasma and of Plasma from Partially Hemolyzed Samples

Representative electrophoretic patterns comparing normal plasma and plasma of hemolyzed samples are shown in Figures 17 and 18. As was the case with serum, hemolysis resulted in slurring of the β globulins. There was a tendency of the whole electrophoretic pattern to deviate somewhat

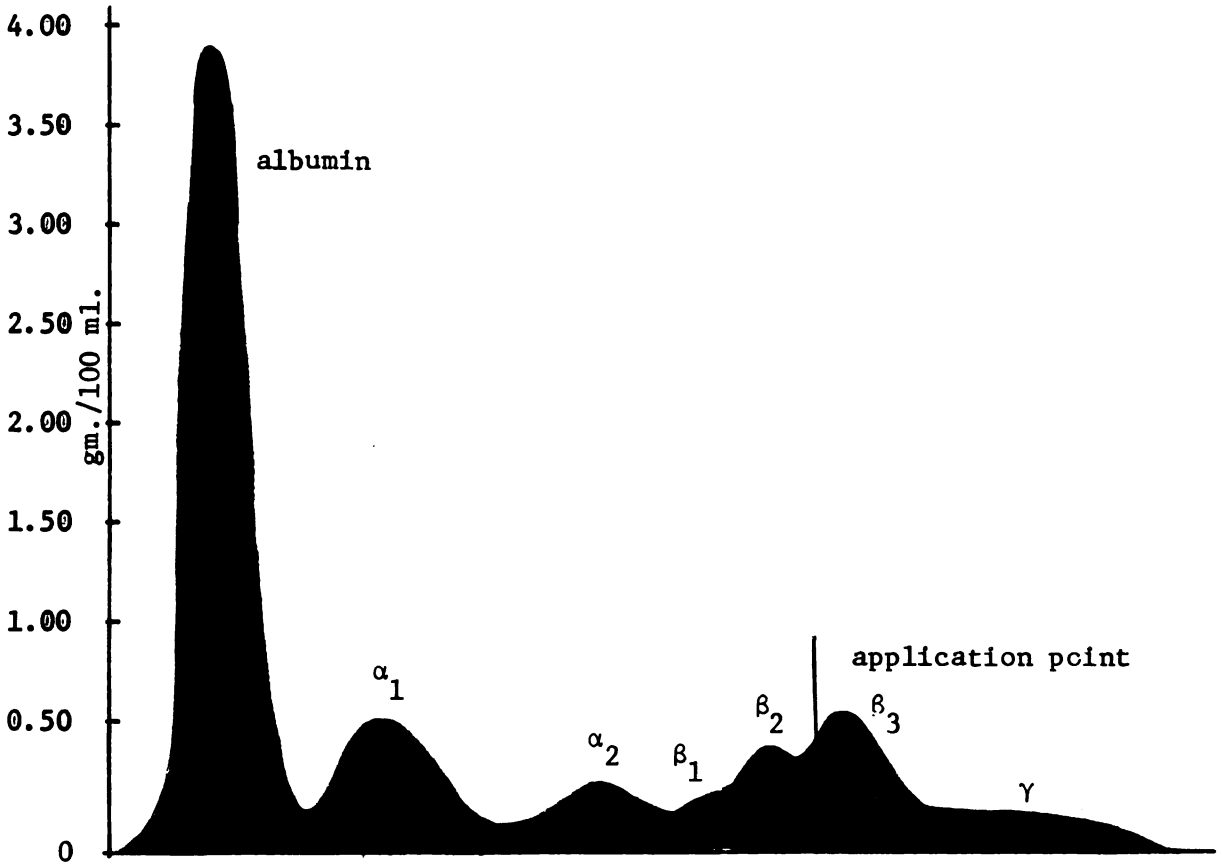

Figure 8. Tracing of electrophoretic analysis of plasma of Dog 5 with free hemoglobin concentration of 44 mg./100 ml.

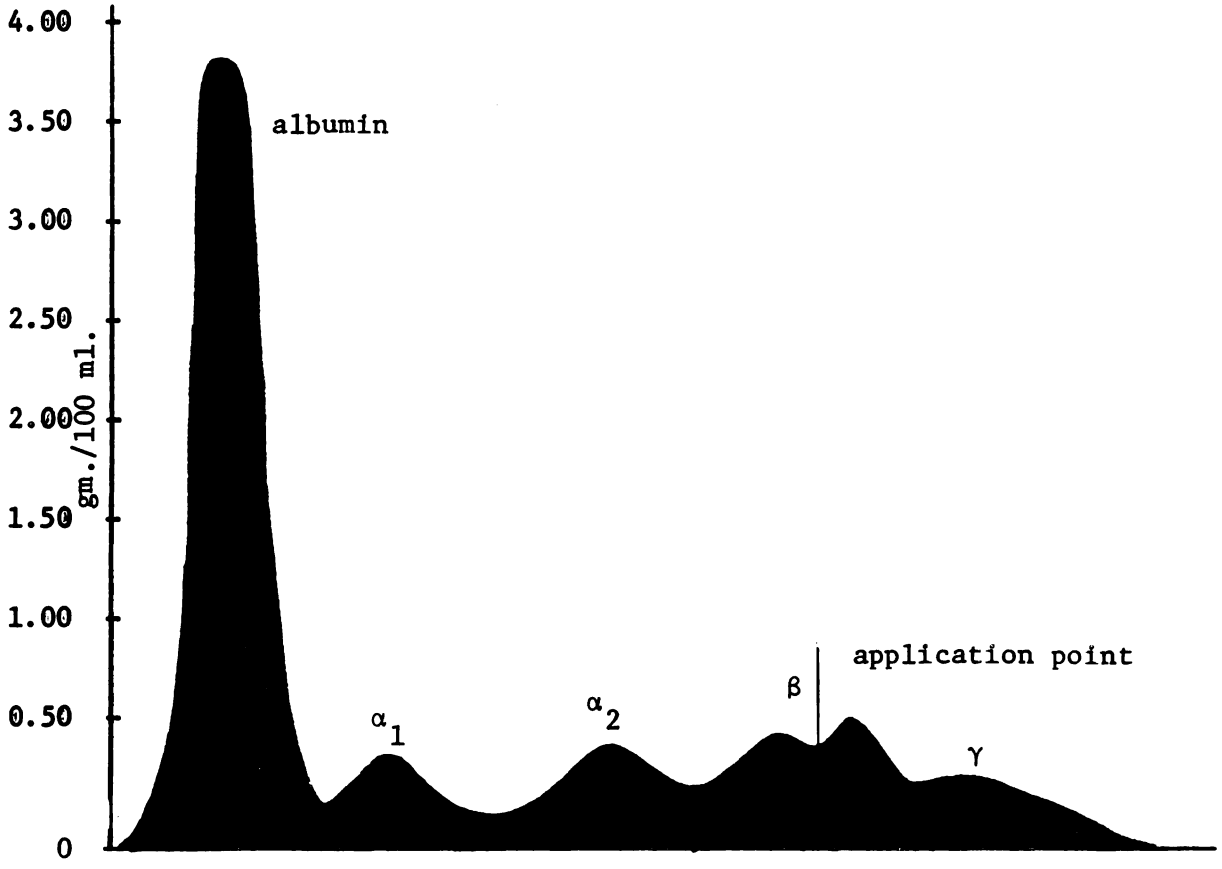

Figure 9. Tracing of electrophoretic analysis of serum of Dog 15 with free hemoglobin concentration of 60 mg./100 ml.

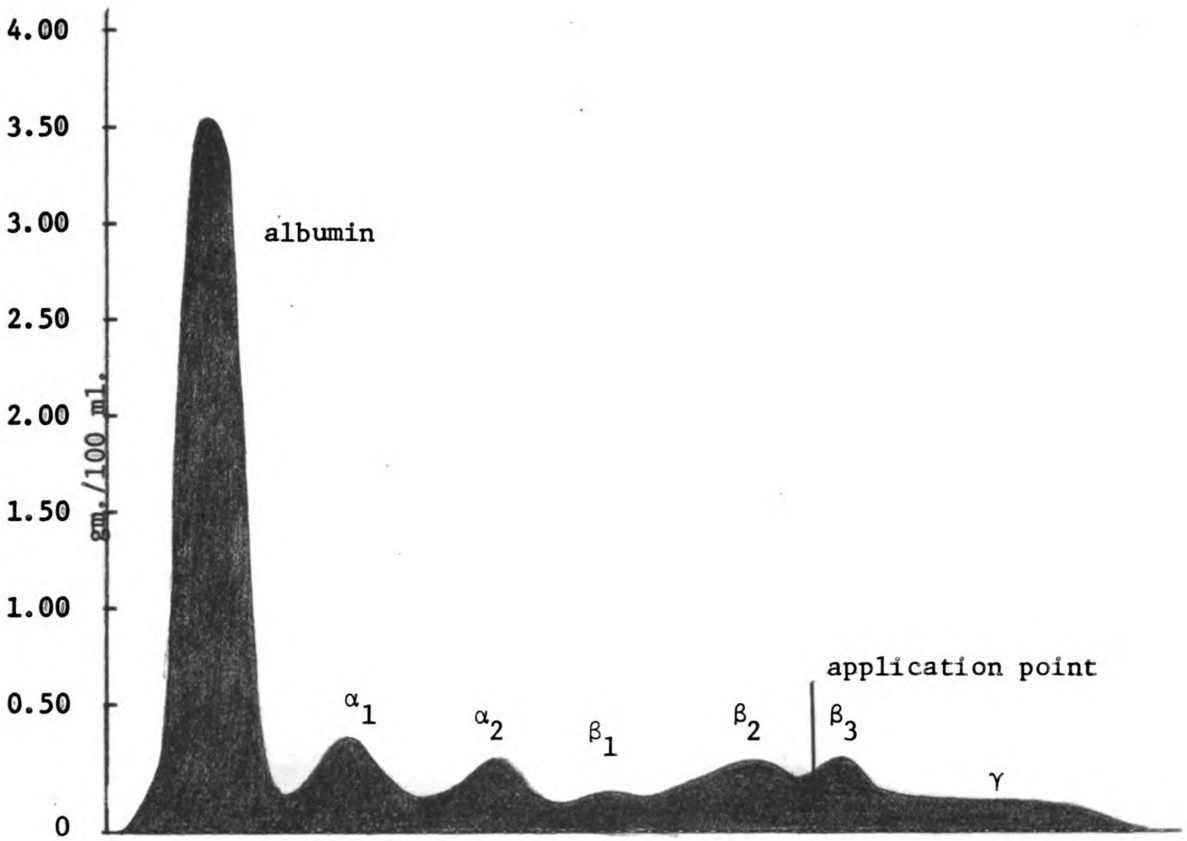

Figure 10. Tracing of electrophoretic analysis of serum of Dog 15.

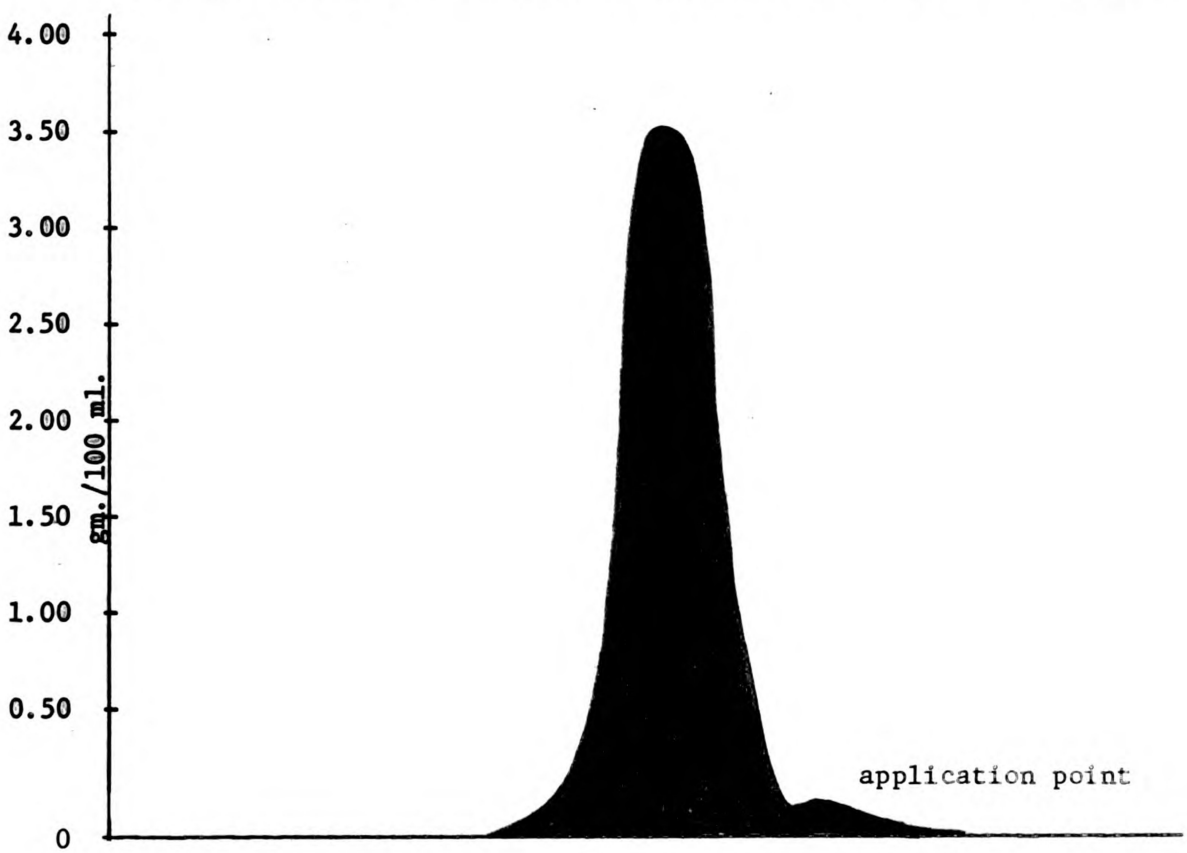

Figure 11. Tracing of electrophoresis of isolated, concentrated hemoglobin of Dog 15.

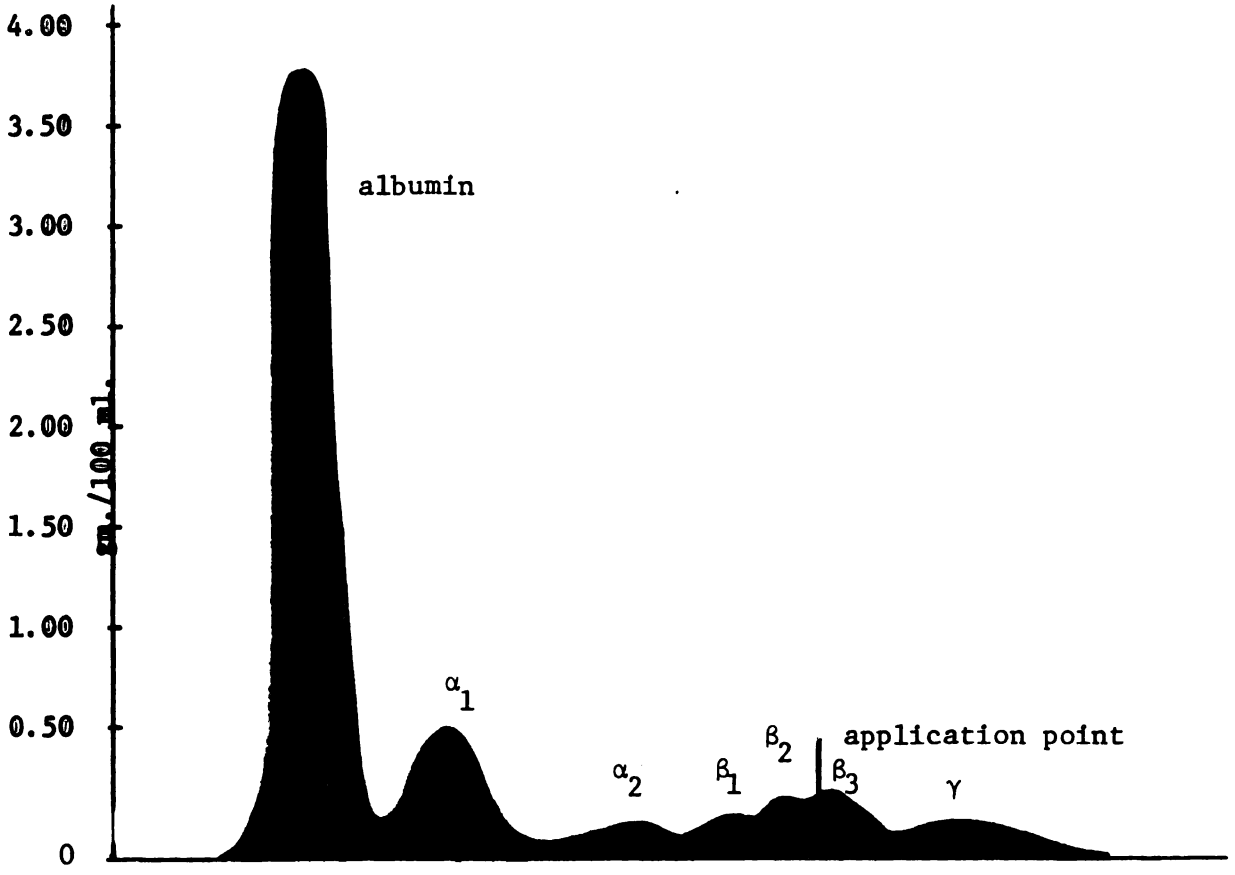

Figure 12. Tracing of electrophoretic analysis of serum of Dog 12.

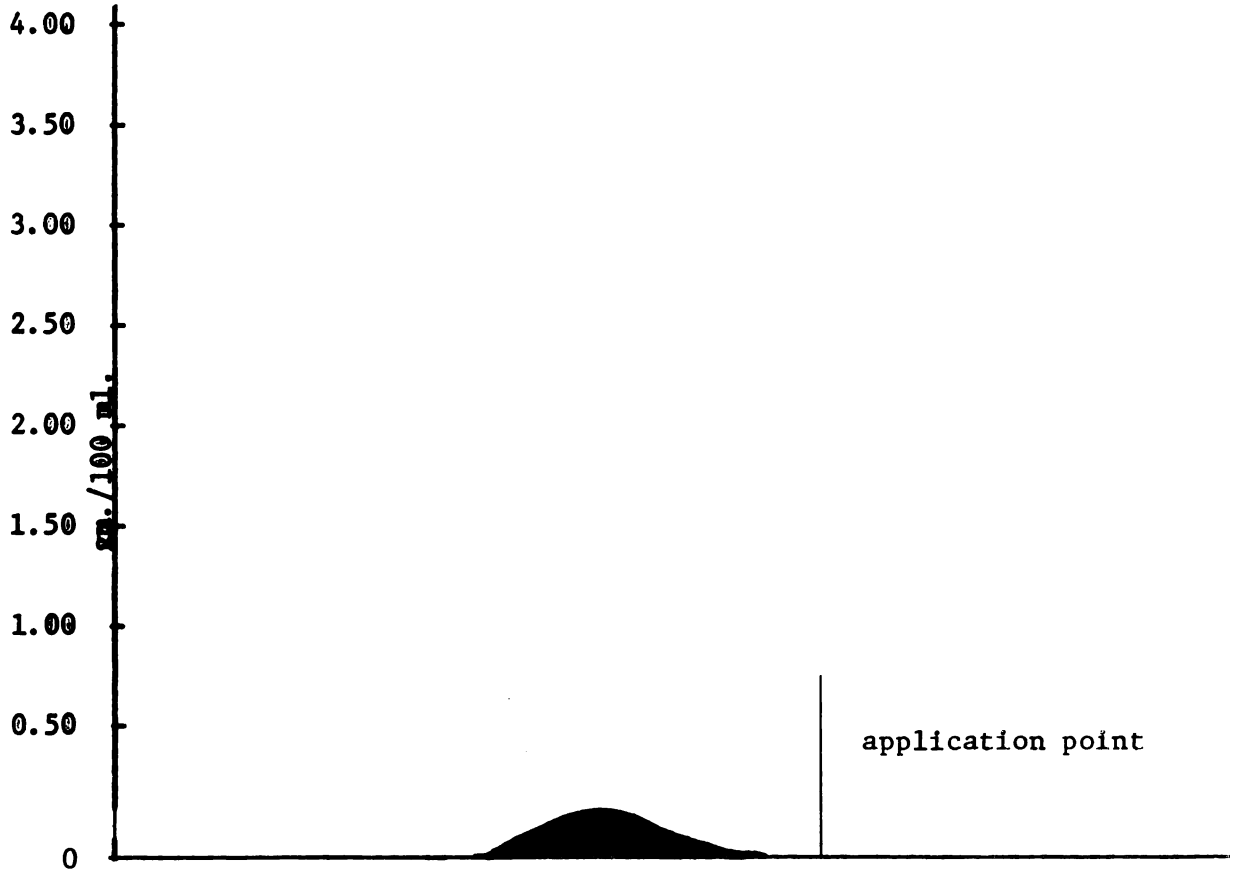

Figure 13. Tracing of electrophoresis of isolated, concentrated haptoglobin of pooled dog serum.

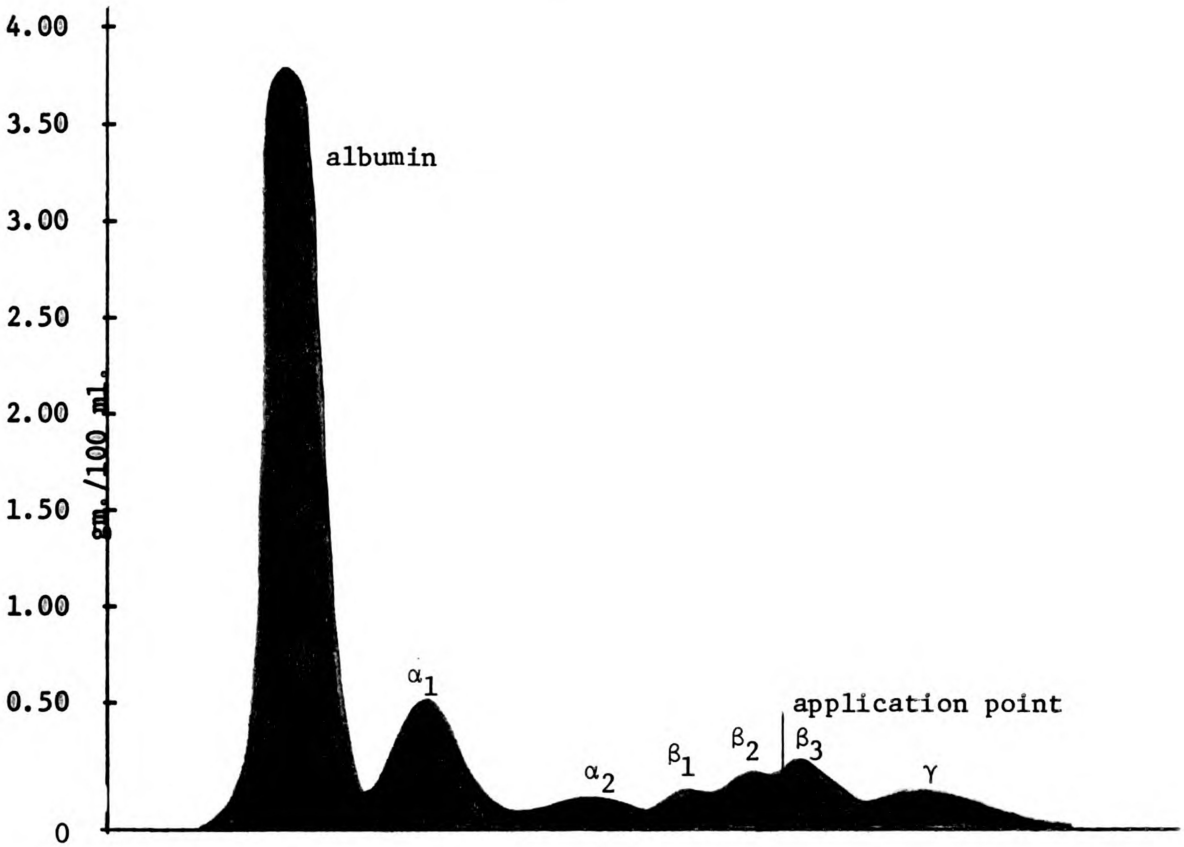

Figure 14. Tracing of electrophoretic analysis of serum of Dog 12.

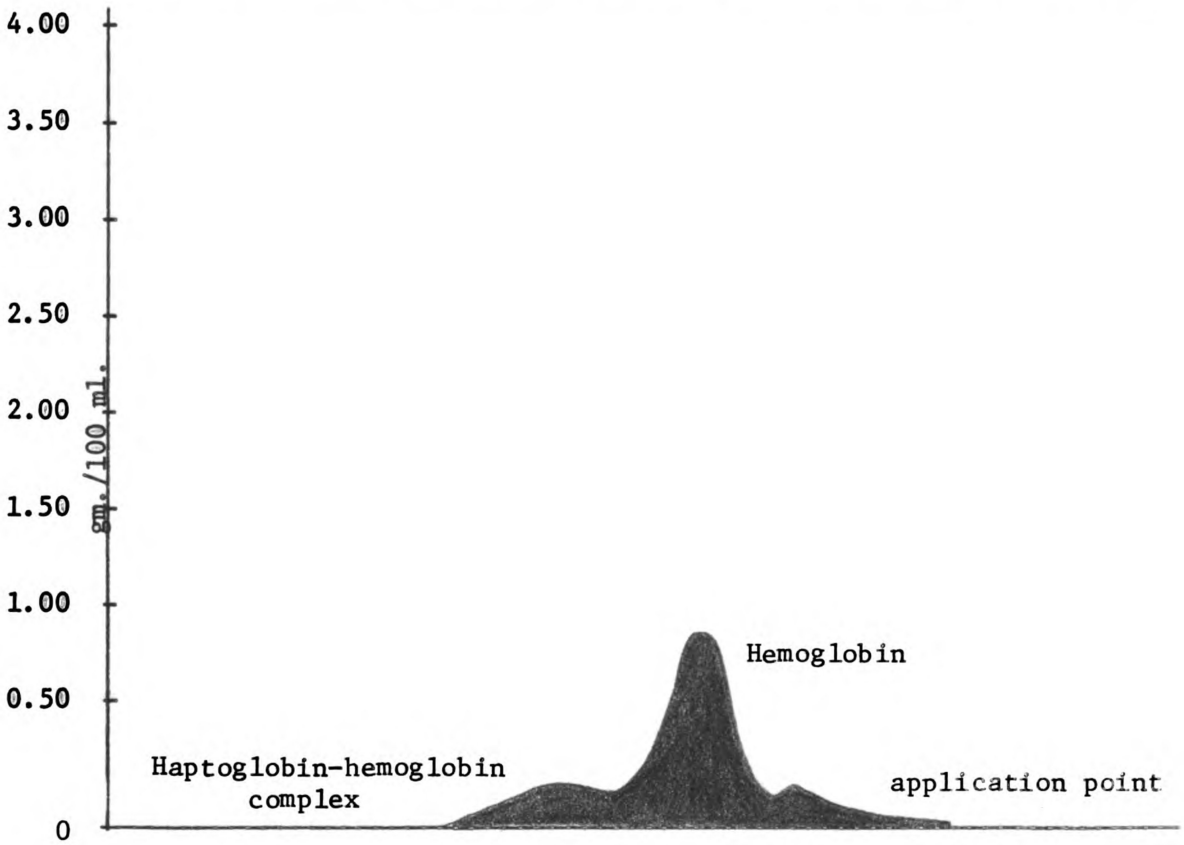

Figure 15. Tracing of electrophoresis of haptoglobin-hemoglobin complex and free hemoglobin.

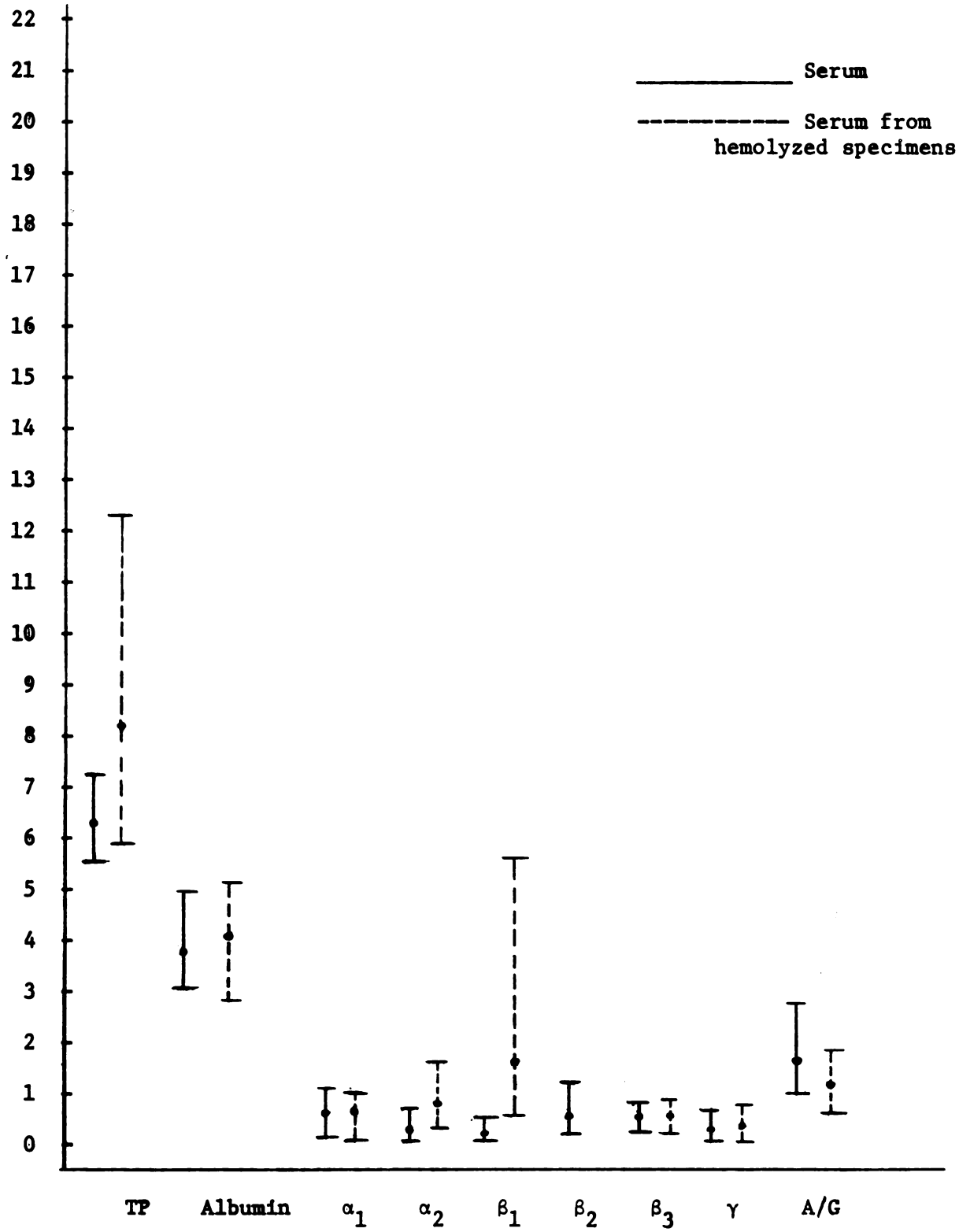

Figure 16. Comparison of electrophoretic analyses of serum and serum of hemolyzed specimens. Means and ranges of protein concentrations for 24 dogs.

Table 2. Comparison of means, ranges and standard deviations of concentrations of serum proteins of normal specimens and partially hemolyzed specimens for 24 dogs

		Mean (gm./100 ml.)	Standard Deviation	Range (gm./100 ml.)
Total Protein	S	6.32^{1}_{2}	0.429	5.50 - 7.22
	HS	8.08	1.289	5.98 - 12.35
Albumin	S	3.85	0.367	3.18 - 4.99
	HS	4.06	0.579	2.97 - 5.28
Alpha-1 Globulin	S	0.63	0.182	0.27 - 1.17
	HS	0.69	0.205	0.19 - 1.12
Alpha-2 Globulin	S	0.30^{1}_{2}	0.113	0.11 - 0.71
	HS	0.75	0.354	0.36 - 1.79
Beta-1 Globulin	S	0.19	0.066	0.08 - 0.44
	HS	1.57	1.162	0.53 - 5.67
Beta-2 Globulin	S	0.49	0.270	0.22 - 1.20
	HS	*	*	*
Beta-3 Globulin	S	0.50	0.134	0.28 - 0.81
	HS	0.58	0.197	0.29 - 0.95
Gamma Globulin	S	0.35	0.121	0.14 - 0.63
	HS	0.43	0.161	0.15 - 0.84
A/G Ratio	S	1.67^{1}_{2}	0.404	1.13 - 2.88
	HS	1.10	0.361	0.56 - 1.91

S = Normal serum
HS = Serum from partially hemolyzed specimens
* = β_1 and β_2 fractions were not separately resolved.
1 = Significantly different from 2 ($P<0.01$).

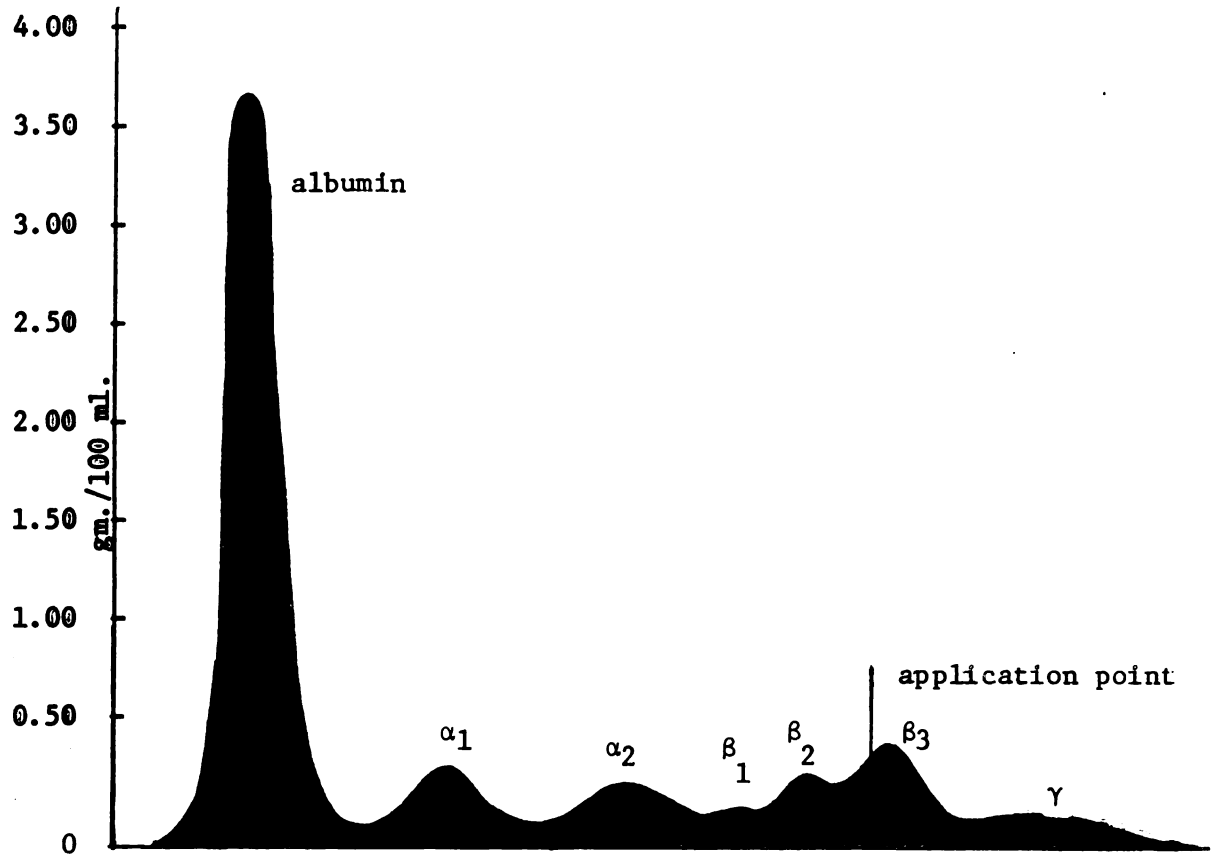

Figure 17. Tracing of electrophoretic analysis of plasma of Dog 11.

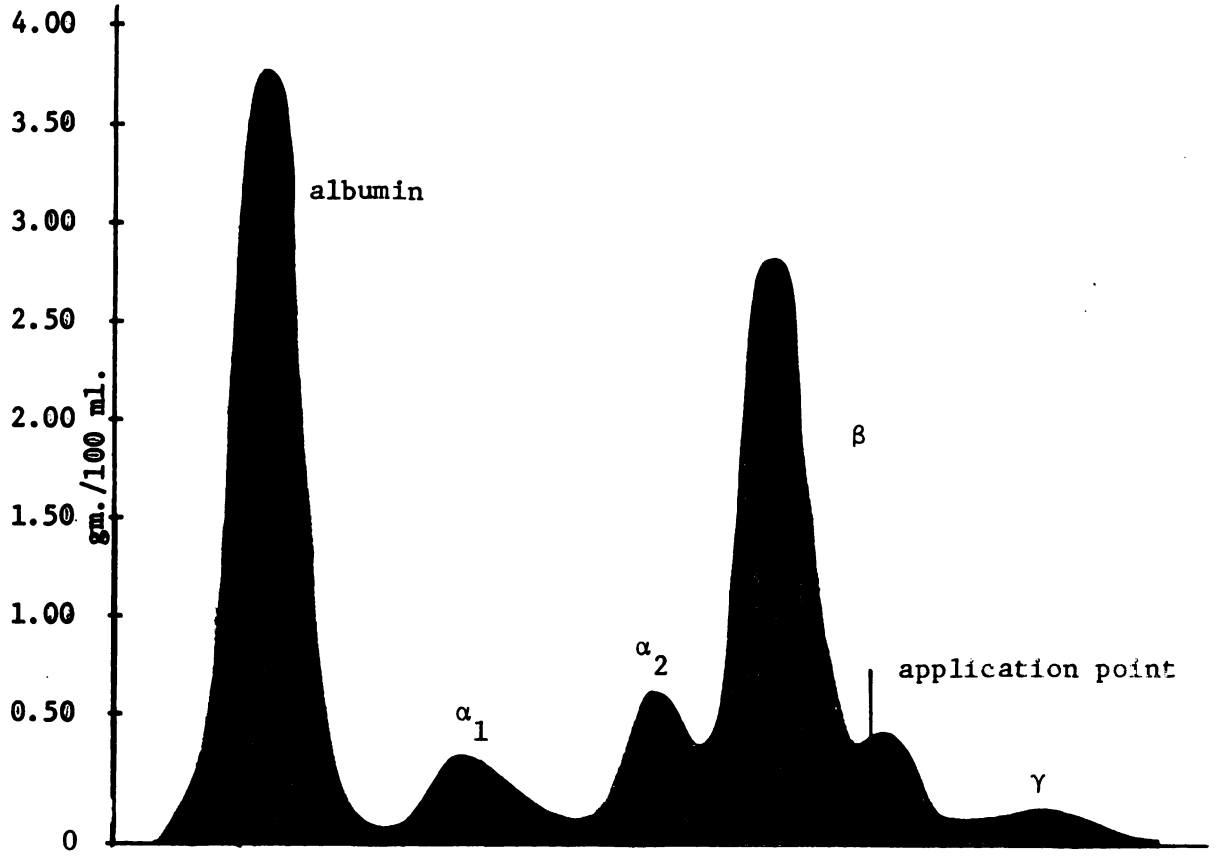

Figure 18. Tracing of electrophoretic analysis of plasma from partially hemolyzed blood of Dog 11.

from the normal pattern. This deviation from normal was principally due to a slowing of the rate of migration of the α_2 globulin fraction resulting in the formation of a valley between it and α_1. This effect was previously observed in the electrophoretic patterns of serum of hemolyzed specimens. Moreover, the α_2 peak was increased as a result of the binding of hemoglobin by the haptoglobin in this fraction.

The means and ranges are summarized in Figure 19 and shown in Table 3. In Table 3 it can be seen that there were also statistically significant differences in the total protein ($P<0.01$), albumin ($P<0.05$) and α_2 ($P<0.01$) fraction, all of these fractions being in higher concentrations in hemolyzed samples than in samples free from hemolysis.

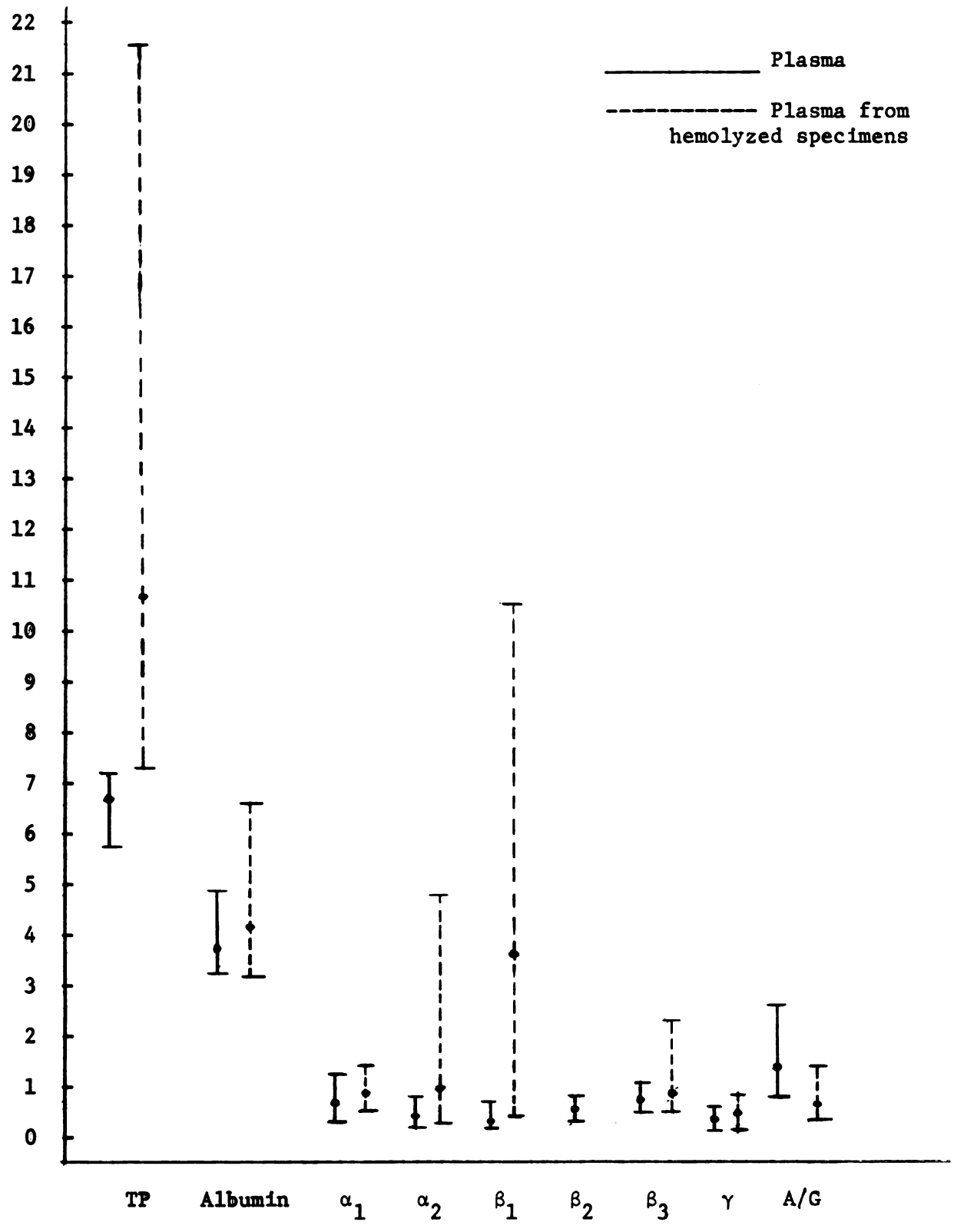

Figure 19. Comparison of electrophoretic analyses of plasma and plasma from hemolyzed specimens. Means and ranges of protein concentrations for 24 dogs.

Table 3. Comparison of means, ranges and standard deviations of concentrations of plasma proteins of normal specimens and partially hemolyzed specimens for 24 dogs

		Mean (gm./100 ml.)	Standard Deviation	Range (gm./100 ml.)
Total Protein	P	6.86^{1}_{2}	0.387	5.85 - 7.10
	HP	10.79^{2}	11.619	7.22 - 21.50
Albumin	P	3.71^{3}_{4}	0.400	3.14 - 4.88
	HP	4.09^{4}	0.762	3.03 - 6.58
Alpha-1 Globulin	P	0.61	0.172	0.39 - 1.13
	HP	0.71	0.200	0.44 - 1.19
Alpha-2 Globulin	P	0.40^{1}_{2}	0.114	0.19 - 0.75
	HP	0.98^{2}	0.856	0.33 - 4.80
Beta-1 Globulin	P	0.25	0.091	0.11 - 0.52
	HP	3.70	2.921	0.43 - 10.56
Beta-2 Globulin	P	0.46	0.129	0.27 - 0.80
	HP	*	*	*
Beta-3 Globulin	P	0.72	0.203	0.40 - 1.14
	HP	0.87	0.465	0.43 - 2.26
Gamma Globulin	P	0.35	0.134	0.07 - 0.63
	HP	0.45	0.214	0.10 - 0.93
A/G Ratio	P	1.38^{1}_{2}	0.381	0.93 - 2.79
	HP	0.72^{2}	0.297	0.29 - 1.36

P = Normal plasma
HP = Plasma of partially hemolyzed specimens
* = β_1 and β_2 fractions were not separately resolved.
1 = Significantly different from 2 ($P<0.01$).
3 = Significantly different from 4 ($P<0.05$).

DISCUSSION

The use of electrophoresis has gained world-wide importance in protein chemistry in recent years. It is an accurate method not only for the determination of the albumin-globulin ratio but also for differentiation, quantitation, and characterization of proteins. Depending on the medium and material used, the number of isolated protein fractions may be from 5 to as many as 30 or more.

At the pH of normal serum (7.35) or higher, all serum proteins exist as anions and hence migrate toward the anode. However, the various fractions migrate at different velocities in the electric field depending on several factors. Albumin, the smallest, moves with the greatest velocity and, upon staining, represents the band of greatest intensity. This is followed by alpha, beta, and gamma globulins with decreasing velocities.

Haptoglobin, an α_2 glycoprotein, binds with 2 molecules of hemoglobin (globin moiety) to form a stable complex of 310,000 molecular weight (Laurell and Nyman, 1957; Nyman, 1960; Giblett, 1961). The complex also moves with the α_2 globulins, as has been reported by previous investigators in human beings and animals, including dogs (Louderback and Shanbrom, 1968; Allen and Archer, 1971; Shim *et al.*, 1971).

In normal human beings, haptoglobin concentration is about 128 $\pm$ 25 mg./100 ml., which quantity can bind 100 to 150 mg. of hemoglobin/100 ml. of plasma (Bauer *et al.*, 1968; Mengel *et al.*, 1972). In dogs,

there has been little work concerning haptoglobins. Dobryszycka (1970), using the peroxidase method of Jayle, reported values of 138.6 mg./100 ml. in 2 dogs and 189.8 mg./100 ml., also in 2 dogs. This agrees quite well with the mean value of 133 mg./100 ml. in the present experiment, although in one animal no haptoglobin could be detected by the method used. This observation merits further investigation. It is known that ahaptoglobinemia affects certain members of the Negro race. It is possible that a similar condition exists in some dogs, or that the concentration of haptoglobin is so low as to be undetected by the method used.

In man, the haptoglobin-hemoglobin complex is removed from the circulation by the reticuloendothelial system at the rate of about 13 to 15 mg. of hemoglobin/100 ml. of plasma/hour, and not through the renal glomeruli into the urine (Laurell and Nyman, 1957; Murray *et al.*, 1961). Several studies (Aber and Rowe, 1960; Nyman, 1960; Muller-Eberhard and English, 1967; Mengel *et al.*, 1972) reveal that upon saturation of the binding capacity of haptoglobin, the excess hemoglobin is degraded into heme and globin. Heme combines with either a heme-binding beta globulin to form hemopexin or with albumin to form methemalbumin. If the binding capacities of these proteins are exceeded, hemoglobinuria occurs.

In conditions characterized by intravascular hemolysis, haptoglobins are eliminated from the blood stream. Hemoglobin, in the absence of haptoglobin, is filtered by the renal glomeruli.

In cases of intravascular hemolysis, the absence or depletion of haptoglobin can be demonstrated in an electrophoretogram by an increase in the beta fraction and a simultaneous reduction in the alpha-2 fraction. These 2 changes reflect, respectively, the presence of free hemoglobin and the removal of the haptoglobin-hemoglobin complex by the

reticuloendothelial tissues. In this experiment, there was an elevation of the α_2 globulin fraction due to the *in vitro* formation of the haptoglobin-hemoglobin complex. The retardation of the migration of the α_2 fraction of serum and plasma of partially hemolyzed specimens was presumed to be due to the much greater size of the haptoglobin-hemoglobin complex (molecular weight 310,000) as compared with uncomplexed haptoglobin (molecular weight 81,000). The slurring of the β globulin fraction suggests that the hemoglobin binding capacity of haptoglobin was exceeded, the free hemoglobin then migrating as a β globulin.

In this work, free hemoglobin in excess of 50 mg./100 ml. was sufficient to cause discernible alterations in electrophoretic patterns. Total protein concentration was elevated as a result of hemolysis. Furthermore, there was a significant increase in the albumin of plasma of hemolyzed samples ($P<0.01$) which may possibly be due to the binding of hemoglobin when haptoglobin is saturated. However, this was not observed to occur in the serum of hemolyzed specimens. These findings await further investigation.

Fibrinogen, which is classified as a globulin on the basis of its solubility characteristics, constitutes 3 to 6% of the total plasma proteins. It migrates between the β and γ globulins in most types of apparatuses (Abramson, 1964; Damm, 1965). In agar gel, it does not migrate at all due to its precipitation by the agar. This finding is in good correlation with the present investigation. Wieme (1965) cites Bockmuller and Oerter as being able to show that fibrinogen migrated in a 0.75% highly purified agar gel with phosphate/citrate buffer at pH 8.0. Henry (1965) noted a slight tendency of fibrinogen to migrate toward the anode in a Durrum type cell using paper electrophoresis.

However, he did not elaborate. For quantitation of fibrinogen, colorimetric determinations are easier, better and less cumbersome than electrophoresis.

In man, the normal concentration of fibrinogen is 200 to 400 mg./100 ml. (Tietz, 1970; Dobryszycka, 1970). Previous workers have reported ranges of 300 to 600 mg./100 ml. in normal dogs (Smith, 1964; Schalm, 1972). In this experiment, the range was 297 to 695 mg./100 ml. with a mean of 438 mg./100 ml. These values are somewhat higher than those cited. However, in the present work, fibrinogen was determined colorimetrically by the biuret method, whereas the earlier reports were based on the determination of total clottable protein and change of total protein concentration after heat precipitation at 56 C. In addition to the increased concentration of the β_3 fraction in plasma as compared to serum, there were also statistically significant ($P<0.01$) increases in the concentrations of the α_2 and β_1 fractions. The reasons for these changes were not clear and merit further investigation.

Gamma globulin, the slowest moving of the plasma proteins, seems to migrate in agar gel toward the cathode. This is due to the phenomenon of electro-osmosis, whereby water becomes positively charged upon contact with the medium. Water is free to move to the cathode carrying the buffer salts and the components of the mixture with it. This stream is in the opposite direction to that of protein migration, and γ globulin, being very slow and weakly charged, is carried back beyond the origin and appears to have travelled in the reverse direction to that expected.

It is often difficult to compare the results of electrophoresis of one laboratory with those of another because of differences in the media, buffers and apparatus employed. Hence, it is recommended that each laboratory establish its own normal electrophoretic patterns as standards for comparison.

SUMMARY AND CONCLUSIONS

Collection of satisfactory blood specimens from animals is often difficult. At times it is necessary to use plasma where serum would be preferred, and hemolysis is a continuing problem in clinical laboratories. The presence of hemoglobin or fibrinogen results in certain characteristic observations in patterns derived from the electrophoretic analysis of plasma and serum proteins.

1. The fibrinogen of plasma migrated as a β_3 globulin and therefore produced an elevation of this peak as compared with the corresponding peak of serum.

2. Free hemoglobin migrated as a β globulin, and the presence of approximately 50 mg. of free hemoglobin/100 ml. of serum or plasma was sufficient to produce slurring of the β globulin peaks and an increase in the area beneath the tracing of the β region.

3. There were some differences in the effects of *in vitro* hemolysis on the electrophoretic patterns of serum and plasma. In both cases there was an increase in α_2 globulins due to the binding of hemoglobin by the haptoglobin of this fraction. Furthermore, an additional change observed was an apparent retardation of the rate of migration of all globulins from α_2 through γ in some dogs. This caused the peaks representing those fractions to be shifted toward the cathode and resulted in the formation of a wide valley between the α_1 and α_2 peaks.

Due to variations in methods and techniques employed by different laboratories, it is advisable for each laboratory to have known normal tracings as reference standards.

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APPENDICES

APPENDIX A

REAGENTS

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REAGENTS

Modified Cawley and Eberhardt Method for Protein Electrophoresis

Ionagar[R] Reagent, 1%. Ionagar, 1.0 gm., and 100 mg. of sodium azide are added to 50 ml. of B_2 Buffer,[1] previously diluted 1:2. Boil until the solution is clear. Dispense 20 ml. in screw-cap tubes and refrigerate. Melt agar just before use.

Veronal Buffer Reagent. Dissolve the contents of one package of B_2 Buffer in 1 liter of deionized water.

Thiazine Red, 0.2%. Thiazine Red R,[2] 1.0 gm., is dissolved in 100 ml. of 10% acetic acid.

Fielding Method for Plasma Hemoglobin Determination

Calibration Curve Solution. Prepare several concentrations of hemoglobin solutions with deionized water ranging from 5 mg./100 ml. to 100 mg./100 ml. using commercial hemoglobin control[3] of 8.7 gm./100 ml.

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[1]Barbital-Sodium Barbital Mixture, pH 8.6, ionic strength 0.075, Harleco, 60th & Woodland Avenue, Philadelphia, Pennsylvania 19143.

[2]Harleco, Philadelphia, Pennsylvania 19143.

[3]Hycel Hemoglobin Control, Hycel, Inc., Houston, Texas.

Owen *et al.* Method for Plasma Haptoglobin Determination

Guaiacol Reagent. Guaiacol (A.R. 1.0 gm./ml.), 3.72 ml., is added to 700 ml. of deionized water and 100 ml. of 1 M acetic acid. The pH of the mixture is adjusted to 4.0 with 1 N NaOH, using a glass electrode pH meter. The volume is finally made up to 1 liter with deionized water.

Hydrogen Peroxide, 0.05 M. Prepare immediately before use by diluting a stock solution with deionized water to 0.05 M.

APPENDIX B

CALIBRATION OF STANDARD CURVE

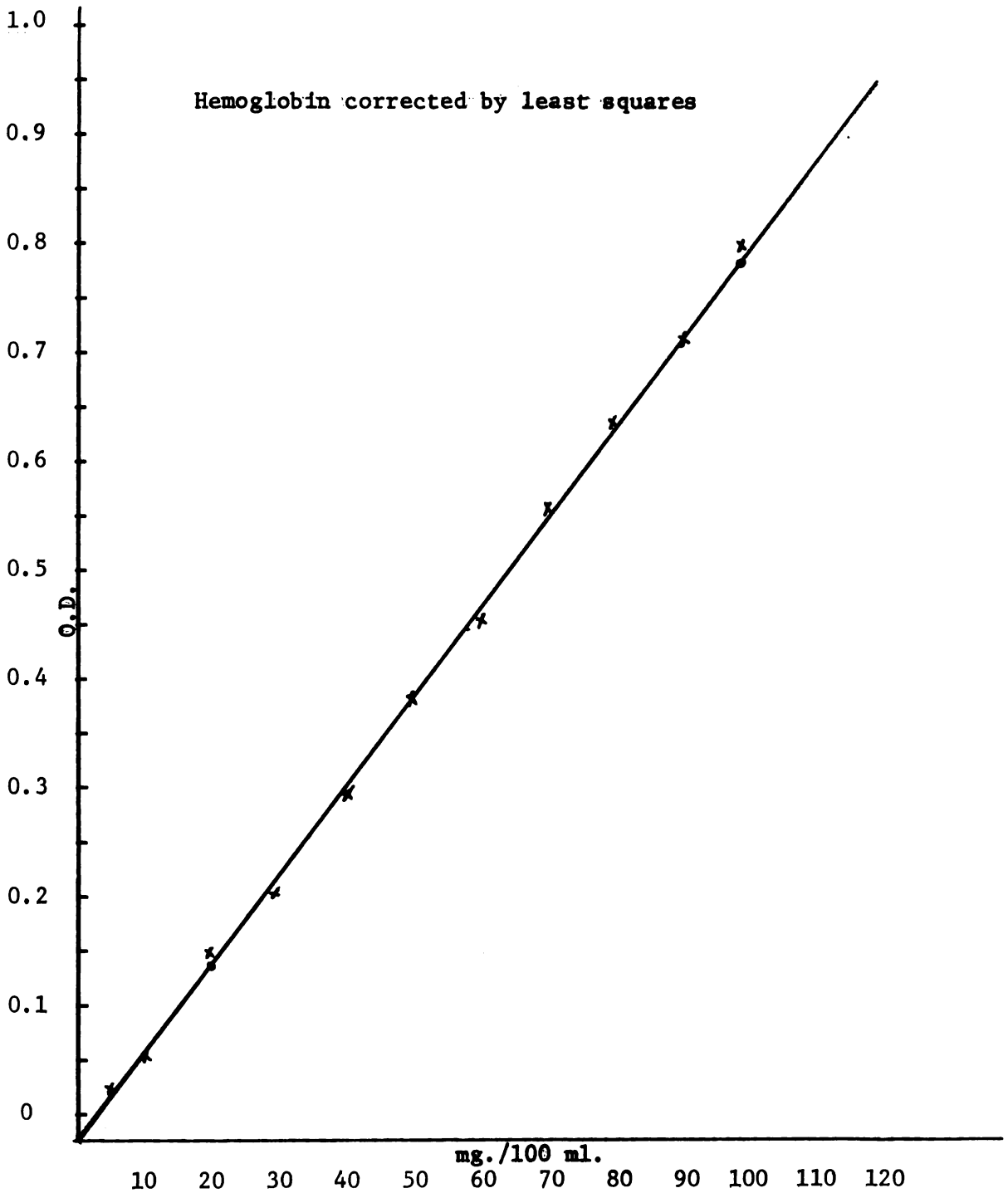

Figure B-1. Plasma hemoglobin standard curve.

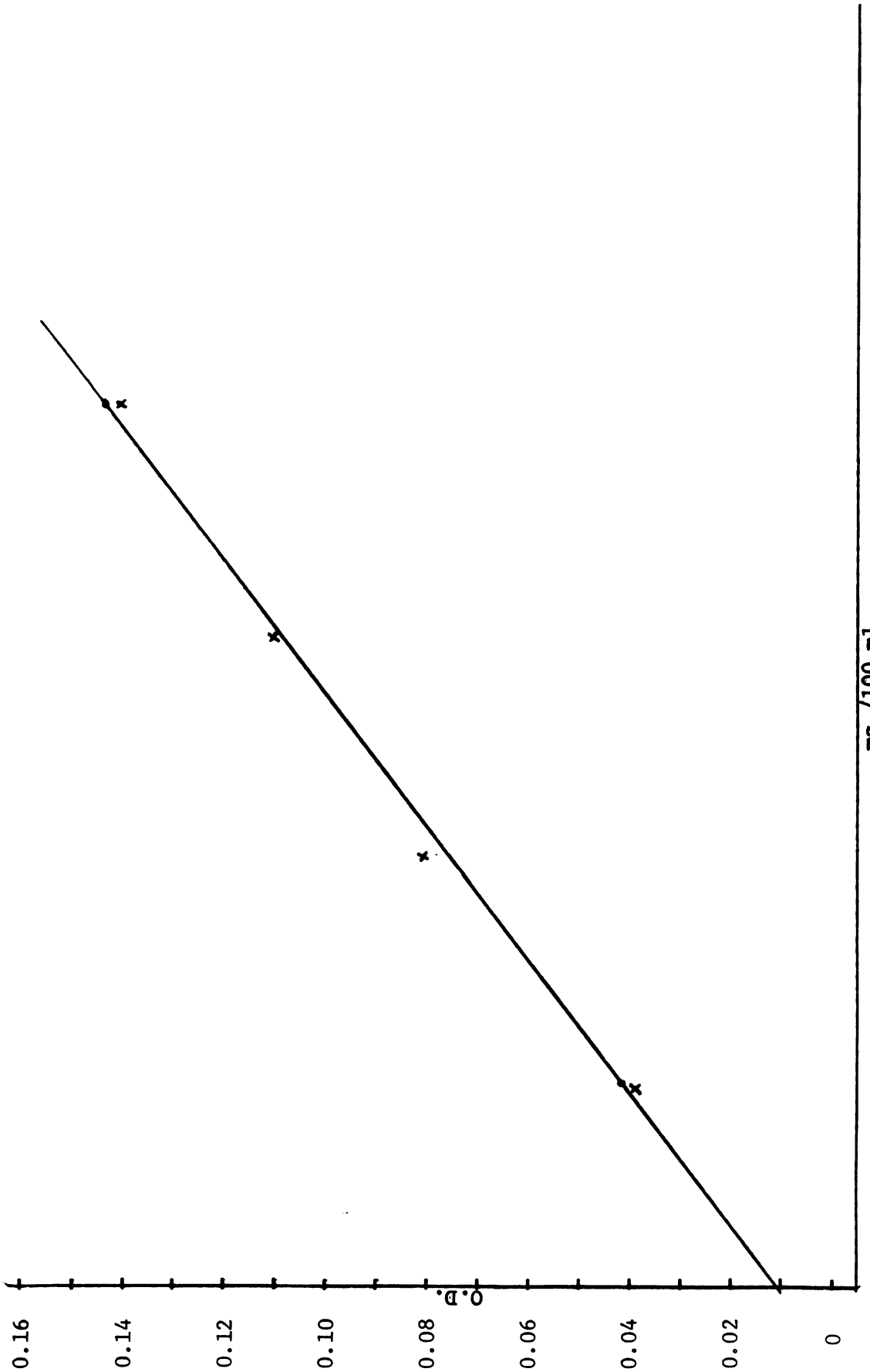

Figure B-2. Plasma fibrinogen standard curve.

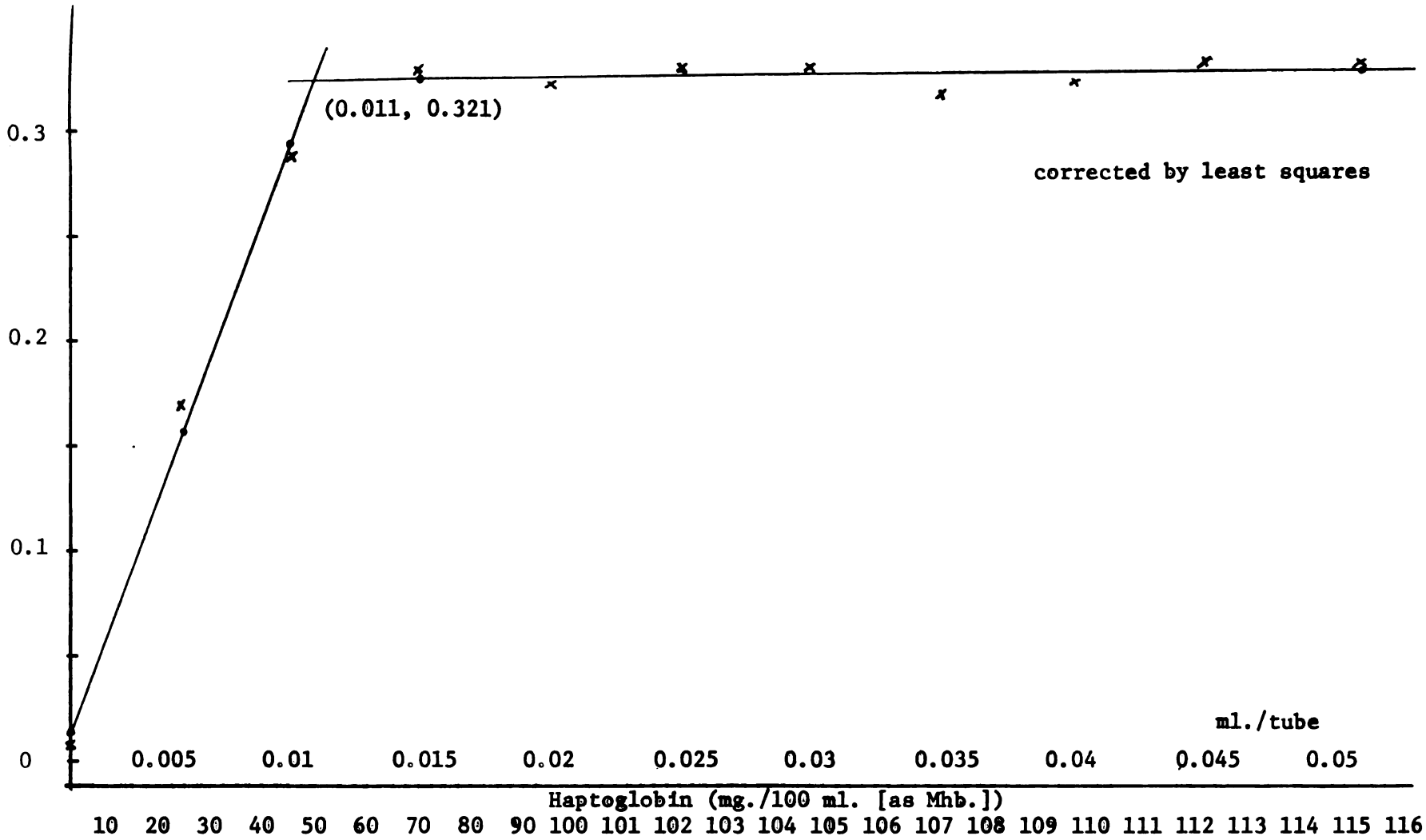

Figure B-3. Calibration curve for estimation of haptoglobin concentration. The abscissa shows at the point of change of gradient that the normal pooled plasma contains 50 mg. haptoglobin per 100 ml.

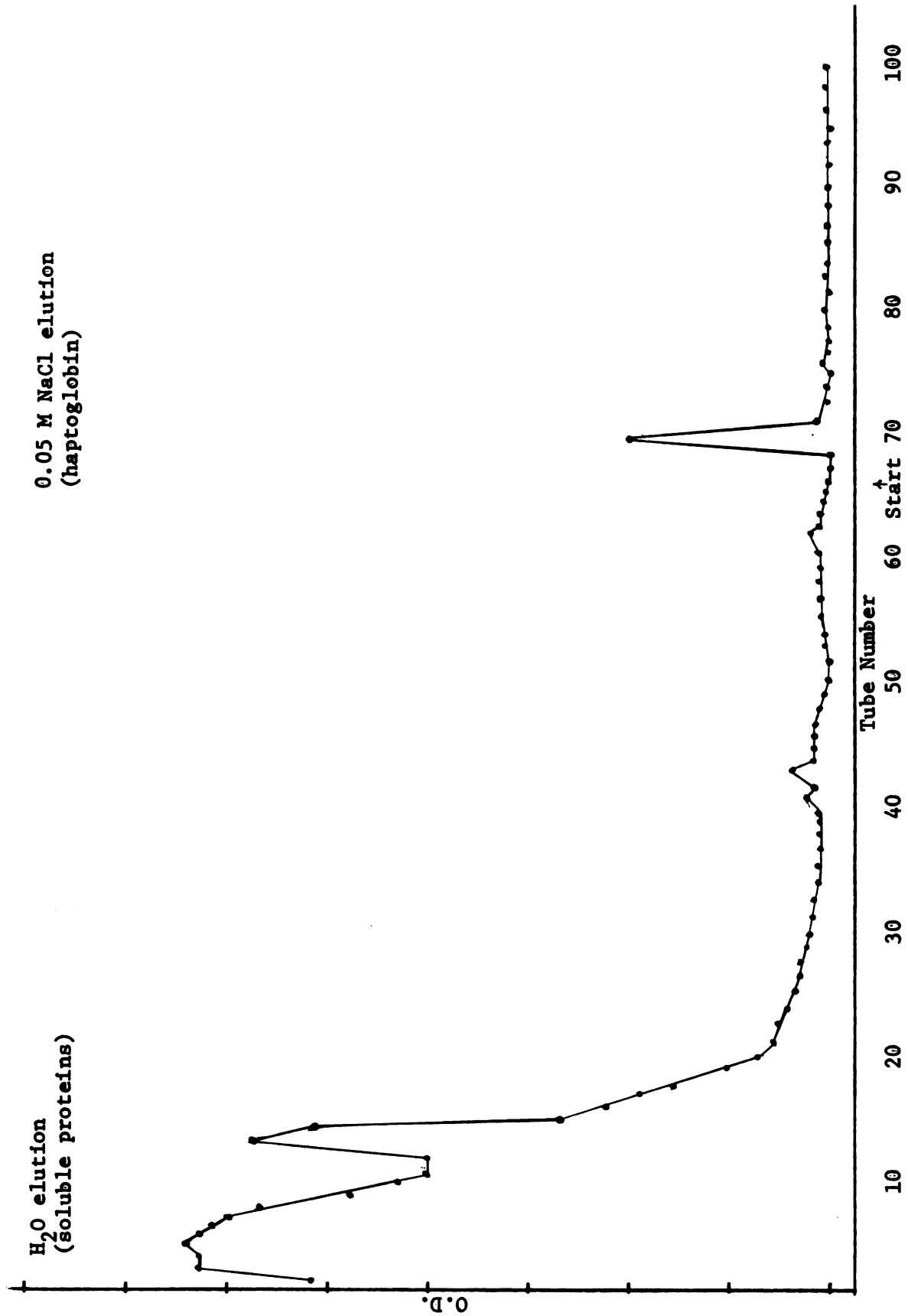

Figure B-4. Haptoglobin isolation elution curve.

APPENDIX C

CALCULATION FORMULA

APPENDIX C

CALCULATION FORMULA

Determination of Clottability of Fibrinogen Isolated

$$C = 100 \frac{d_1 - d_2 + d_3}{d_1}$$

where C = % clottability

d_1 = optical density of 0.1 ml. of fibrinogen preparation diluted with 4.0 ml. of 0.005 M citrate solution; read against citrate blank

d_2 = optical density of dilution as above with 0.2 ml. of thrombin solution

d_3 = citrate blank with 0.2 ml. of thrombin solution.

VITA

The author was born in Palauig, Zambales, Philippines, on April 10, 1944. She graduated from Centro Escolar University, Manila, Philippines, with her B.S. in Medical Technology in 1964.

After passing the Louisville Civil Service Board Examination in Kentucky, she joined the staff of the clinical chemistry laboratory of Louisville General Hospital in Louisville, Kentucky, in February 1967. In September 1969 she enrolled in a program of Graduate School in Clinical Laboratory Science in the Department of Pathology, Michigan State University, with the aid of a full-time job at the clinical laboratory, Veterinary Clinic, Department of Pathology.