ELECTROPHORESIS OF PROTEINS ON FILTER PAPER

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The recent success in the separation of amino acids and peptides by filter paper chromatography has led numerous investigators to attempt to separate proteins in an analogous manner. These efforts have thus far not met with definite success. However the possibility of employing filter paper as a framework to support the liquid medium used for the separation of proteins through electrophoresis has proven useful. The observations of Durrum (1), Cremer and Tiselius (2), Wieland (3, 4), and Turba and Enenkel (5) have indicated that the serum proteins would separate on filter paper into components similar to those found in free electrophoresis. Furthermore by cutting the paper into segments and eluting the dye used to stain the separated serum proteins a curve could be obtained which resembled the usual electrophoretic pattern (2).

Filter paper electrophoresis in addition to being extremely simple possesses advantages in some respects over the classical methods in free solution. First, absolute separation of components takes place rather than just separation into concentration gradients. Second, smaller amounts of material at lower concentrations can be investigated. Third, isolation of all the components which have been separated is possible. Fourth, the paper support makes it possible to develop techniques of two dimensional electrophoresis and devices for continuous flow preparative work (6, 7). Whether it is possible to achieve the accuracy obtained with the sensitive optical methods employed in free electrophoresis for localizing and quantitating components, is doubtful. In the present report a description is given of the separation and isolation of a large number of different proteins employing a method of paper electrophoresis between glass plates in which disturbing factors such as evaporation, heating, and buffer concentration gradients were reduced to a minimum. An attempt was made to employ the method for determining the mobilities of certain proteins.

Material and Methods

In a previous report from this laboratory (2) a method was described for filter paper electrophoresis in which the paper strip, immersed in buffer and containing the specimen to be analyzed, was clamped between glass plates and immersed under a solution of chlorobenzene and connected to electrode vessels. This apparatus was

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improved and simplified by Schneider (8) and by Wallenius (9). It possessed the advantage that the chlorobenzene prevented evaporation from any portion of the filter paper and acted as a partial cooling system. Since the buffer in the electrode vessels formed a continuous system through the chlorobenzene, the levels of the liquid at the two electrodes were always equal preventing secondary liquid flow. This method worked very well but due to the work under chlorobenzene the apparatus lacked versatility. Satisfactory results were obtained with a simpler apparatus by sealing the edges of the glass plates with silicone grease, thus preventing evaporation at the edges.

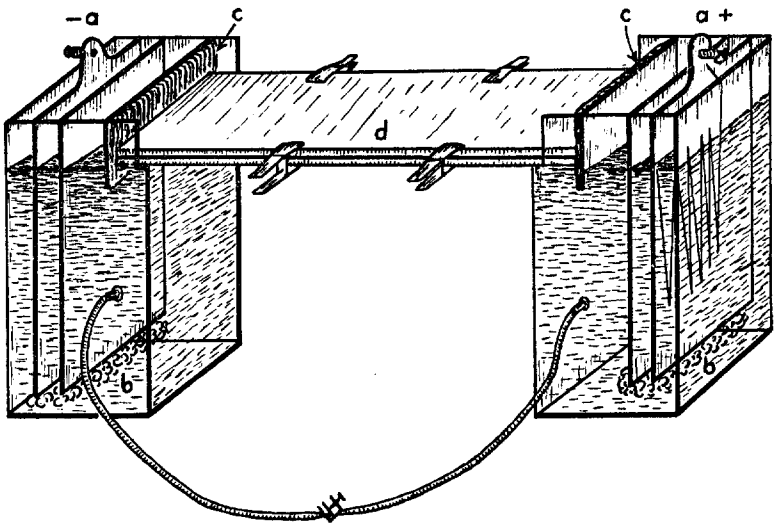


FIG. 1. Illustration of the apparatus used for filter paper electrophoresis. (*a*) Coiled platinum electrodes; (*b*) glass wool at the juncture between electrode chambers; (*c*) highly porous paper carrying liquid from electrode vessels to the filter paper; (*d*) glass plates surrounding the filter paper.

The two ends of the filter paper simply dipped into buffer at the electrode vessels on which the glass plates rested. Fig. 1 illustrates the usual apparatus employed.

The electrode vessels were built of perspex of sufficient size so that there was no pH change at the filter paper ends after 24 hours at a current of 5 ma., when barbital buffer at pH 8.8 and $\mu = 0.1$ was employed.

The volume in cubic centimeters that the hydrogen ion travels is expressed by the formula:

$$V = \frac{u_H \cdot it}{\kappa}$$

in which κ, the conductivity of the barbital buffer, equals $3.08 \cdot 10^{-3}$ and u the mobility of the hydrogen ion is $223 \cdot 10^{-5}$.

Substituting these values in the equation with the current i and the time t:

$$V = \frac{223 \times 10^{-5} \cdot 0.005 \cdot 8.64 \times 10^{4}}{3.08 \times 10^{-3}}$$

$$= 313\text{cc.}$$

For such an experiment it is important to have a volume of buffer in the electrode vessels somewhat greater than this figure to allow for diffusion and convection. Actually 800 cc. were used in the electrode vessels employing coiled platinum wire electrodes in a separate compartment. By placing glass wool at the bottom of the vessels where the compartments formed a junction further insurance against pH changes at the filter paper ends was obtained.

A rubber tube connected the electrode vessels to balance the liquid levels. This was closed during all experiments except those measuring electroosmotic flow. The vessels were freely movable on a heavy sheet of glass to accommodate various lengths of filter paper between glass plates. A cooling system was arranged by placing the entire apparatus in a cold room and resting the glass plates on a large metal block. This conducted the heat away from the glass plates and made it possible to carry out the electrophoretic separation at 3°C., the temperature of the cold room.

Alternating current which had been passed through a rectifier furnished a stable source of current giving any desired voltage between 50 and 1000 volts. This was regulated by means of a resistance applied as a potentiometer. For most experiments 80 to 400 volts were used giving a current ranging from 4 to 20 ma.

Procedure for Preparing the Filter Paper.—A number of types of filter paper were employed. In general, a relatively hard, thick, even type was found most useful (Munktell 20, 150 g.; Ford blotting paper, extra heavy). Some separation could be obtained on almost any type except the thinner varieties. The Munktell 20 paper was used most frequently and was found more suitable between glass plates than the Whatman 2 and S & S 598 g. or 413 g. used by other workers. One advantage was the relatively small electroosmotic flow.

The paper was cut into sheets which were slightly narrower than the glass plates (approximately 0.5 cm. at each side) and approximately 4 cm. longer. A line was drawn at the point at which protein specimens were to be applied and the paper dipped in the buffer used in the electrode vessels. The paper was then placed on another sheet of filter paper for approximately 1 minute to permit the removal of most of the liquid. This barely moist paper was then placed on one glass plate and the protein applied. Usually 0.01 to 0.04 cc. of solution was used for a single sheet of paper. Depending on the breadth of the paper, multiple spots could be applied. The second glass plate was then placed over the paper and, with the plates held tightly between the fingers, silicone grease was forced between the plates to the margin of the paper on both sides. Metal clamps were then applied to hold the plates together. The glass plates were polished prior to use with a cloth that contained a considerable amount of silicone grease. This left a fine coating on the plates which aided considerably in preserving sharp protein spots. In order to achieve even pressure on the plates, metal plates were placed on the upper glass plate but this was not necessary for all experiments. The glass plates were then placed on the lips of the electrode vessels with the ends of the paper dipping into the buffer. Some liquid from the buffer went into the paper and equilibrium was established in approximately 10 minutes. If the protein

spot was placed half way between the electrodes, it remained at the origin. However, if the protein was closer to the cathode, as was usually the case, the protein moved toward the anode as the liquid entered the paper. For many purposes this did not matter, but for experiments on electroosmosis and mobility measurements it was important to keep the protein right at the origin prior to the application of current. With some experience this could readily be done by always using a colored protein solution at one spot and keeping it at the origin by dipping one end of the plate in buffer more than the other until equilibrium was established.

Serum with just enough bromphenol blue added to give it a distinct blue color was found extremely useful in various parts of the procedure in which it was of value to observe the initial spot. The added bromphenol blue became bound to the albumin of the serum and this made it possible to follow the migration of albumin during the course of the experiment. Other serum components did not bind the dye and the mobility of the albumin was not altered significantly. The dye-albumin mixture was used as a reference standard in most experiments.

Serum was added in the undialyzed form for most experiments. However for accurate mobility measurements and for separation in acid solution it was important to bring the protein solution to the pH of the buffer.

A current ranging from 2 to 20 ma. was then applied depending on the type of experiment and the use of a cooling system. For the average size filter paper significant heating was not obtained when 7 ma. or less was used at room temperature without a cooling system. With metal plates in contact with the glass plates at 3°C. in the cold room as much as 20 ma. could be used without significant heating. For a given size paper and using the same buffer medium, the field strength remained relatively constant at the same amount of current. For example for one type of plate that was often used, the resistance of the filter paper ranged from 19,000 to 20,500 ohms in five different experiments. It was difficult to say whether better results were obtained at low currents over long periods of time than at high currents. For standardized experiments, particularly those for quantitative determination of serum components, plates 25 cm. long and 9 cm. wide were usually employed. These permitted the use of 3 or 4 specimens of serum which were allowed to separate overnight (14 hours) at a current of 4 ma. Shorter plates with shorter strips of paper had many advantages particularly for preliminary studies with unknown protein mixtures.

At the finish of the separation the ends of the paper were torn off and the clamps carefully removed from the glass plates. In a vertical position with one end of the plates resting on the table, the plates were pried apart at the top, and, with a rapid movement, separated. It was very important not to let the plates slide over each other and disturb the protein spots. With some experience, this could readily be done. The paper was then dipped into the staining solution. For some experiments particularly those employing dextran the paper was dried at 110°C. prior to staining.

For preparative purposes numerous strips of long paper, piled on top of each other, were used. Four sheets were usually employed but as many as ten were used. After three sides of a square had been cut through the layers of paper and a flap lifted and the paper moistened, the protein was applied to the flap. The flap pointed in the direction of migration. This permitted a straight edged starting point. The outline of such a flap can be seen in the strips illustrated in Fig. 4. Long glass plates 50 × 6 cm. were found most convenient. The multilayered paper was placed between the glass

plates, silicone grease applied at the edges, and current applied, usually 30 ma. At the end of the experiment the upper sheet was stained in the usual manner and the spots located, while the lower sheets, held together by moisture, were cut up as desired. For these experiments lined filter paper with numbered segments was used to correlate the stained and unstained strips.

The degree of elution of the protein from the paper segments varied somewhat for different proteins. One hundred per cent elution for albumin and γ-globulin could be obtained by soaking the paper for 24 hours in 0.1 N NaOH and this procedure was used for determining protein concentration. Isotonic saline removed approximately 75 per cent of the protein from the paper in 24 hours as long as the paper was kept moist while being partitioned.

Procedure for Two Dimensional Electrophoresis.—A broad sheet of filter paper with two lines crossing the long axis of the paper separated by a distance equal to the breadth of the paper was placed between broad glass plates, following application of the protein in the upper cathode corner of the paper. After carrying out one dimensional electrophoresis in the usual manner, the paper was removed from the plates and cut along the previously drawn lines which lay beyond the spread out protein on both sides. The middle portion of the paper was again placed on one glass plate after being turned 90°. The cut ends of the strip were placed along the former sides of the paper to provide contact with the buffer and the upper plate applied. The paper, without having been dried, was then used for separation in the second direction.

Protein Staining and Estimation.—The protein-staining method used was a modification of that employed by Durrum (1) using the dye bromphenol blue. An ethyl alcohol solution containing 1 per cent bromphenol blue and saturated with $HgCl_2$ was usually used, but an aqueous solution containing 1 per cent $HgCl_2$, 0.05 per cent bromphenol blue, and 2 per cent acetic acid also worked well for the staining. The critical point for maximal staining of the protein on the paper was the use of an acid water solution for washing the stain from the paper. A 0.5 per cent acetic acid solution was usually employed and the paper rinsed at least four times over a period of 20 minutes. The paper was then dried at room temperature. The yellow acid color could be converted to the deeper blue color by passing the paper through ammonia vapor. The binding of bromphenol blue and other similar dyes by proteins is greatly increased in acid solution and, unless acid water is used for washing, a large amount of dye is lost. Washing the paper with methanol and ethanol (2) did not give maximal staining but the appearance of the paper was neater. The latter method was used for many of the paper strips illustrated in the present paper.

For the quantitative estimation of the protein on the paper, stained in the manner described above and washed in acid solution, segments of 0.5 or 1 cm. in length of partially dried paper were cut and placed in a solution of 0.01 N NaOH for 30 minutes. The dye was thus eluted quantitatively and the tubes read in a Beckman spectrophotometer at 575 mμ. The yellow dye was converted to the blue by the alkaline eluting solution. Fig. 2 *a* illustrates the curves relating optical density and protein concentration of the paper. These were obtained by placing various amounts of protein on paper and then eluting the dye as described above. The albumin showed somewhat greater binding of dye per unit of protein as calculated from nitrogen determinations.

The protein on unstained paper was also determined directly in segments as above

by a modification of the Heidelberger-MacPherson procedure for determining tyrosine groups (10). Numerous other methods were tried including the ninhydrin procedure (11) but these were not of practical use. The strips of paper were placed in 2 cc. of 0.1 N NaOH for 60 minutes after which 1.5 cc. of a copper sulfate solution (20 parts 4 per cent Na_2CO_3, 1 part 2 per cent sodium tartrate, 1 part 1 per cent $CuSO_4$) was added. After standing for 30 minutes, 0.5 cc. of Folin-Ciocalteu reagent diluted with 2 parts distilled water was added. The color in the tubes was read after 30 minutes in a Beckman spectrophotometer at 750 mμ. The optical density was transferred to protein concentration from the curve shown in Fig. 2 *b*. Despite considerable work

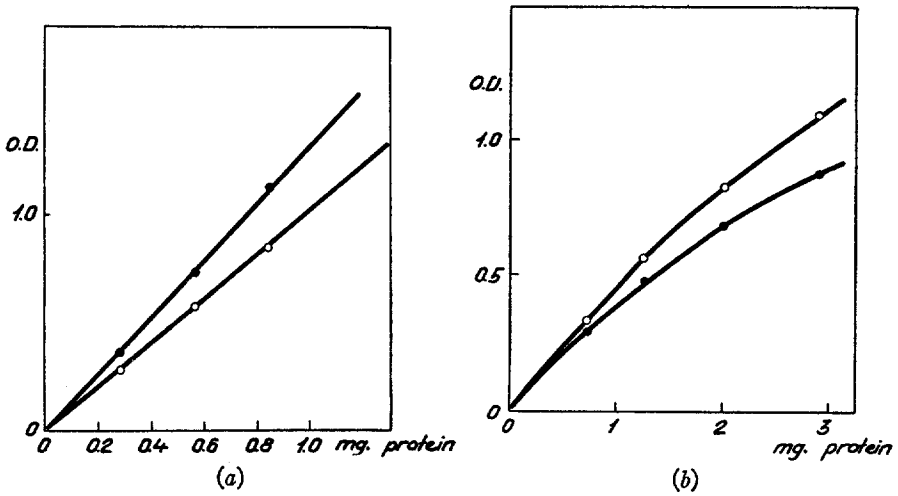


FIG. 2. Comparison of the intensity of color produced by albumin (●) and γ-globulin (○) per unit of protein (in terms of nitrogen) with (*a*) bromphenol blue elution method; (*b*) modified Folin method.

with the method a straight line relationship was not obtained. γ-Globulin gave a more intense color than serum albumin.

Methods for Electroosmosis and Mobility Studies.—The polysaccharide dextran, which was used as a reference substance, was stained on the paper by means of bromphenol blue in ethanol in the same manner as protein. However it was necessary to wash the paper with organic solvents to preserve the dextran stain. Methanol was usually employed. The dextran could also be localized as a white spot when the dried paper was first placed in the deeply colored ethyl alcohol solution of bromphenol blue. The dried dextran spot failed to take up the dye as readily as the rest of the paper. This procedure was also used for localizing other polysaccharides on the paper.

The dextran solution was placed on the line of origin in the same manner as the protein but always in a separate spot; 0.005 cc. of a 3 per cent solution was used. A dextran spot was placed above and below the serum spot and at the end of the experiment a line drawn through the center of each dextran spot. If the dextran spot lost its globular shape during the course of the experiment or if the two spots were not equal distances from the origin, the results were discarded.

In the experiments on the determination of electroosmotic flow for different papers a small rubber tube between the electrode vessels was kept open during the experiment to prevent accumulation of water at the cathode with resulting pressure changes. This tube carried a small fraction of the total current and a small correction for this shunt was made in calculations of field strength in the experiments on mobilities.

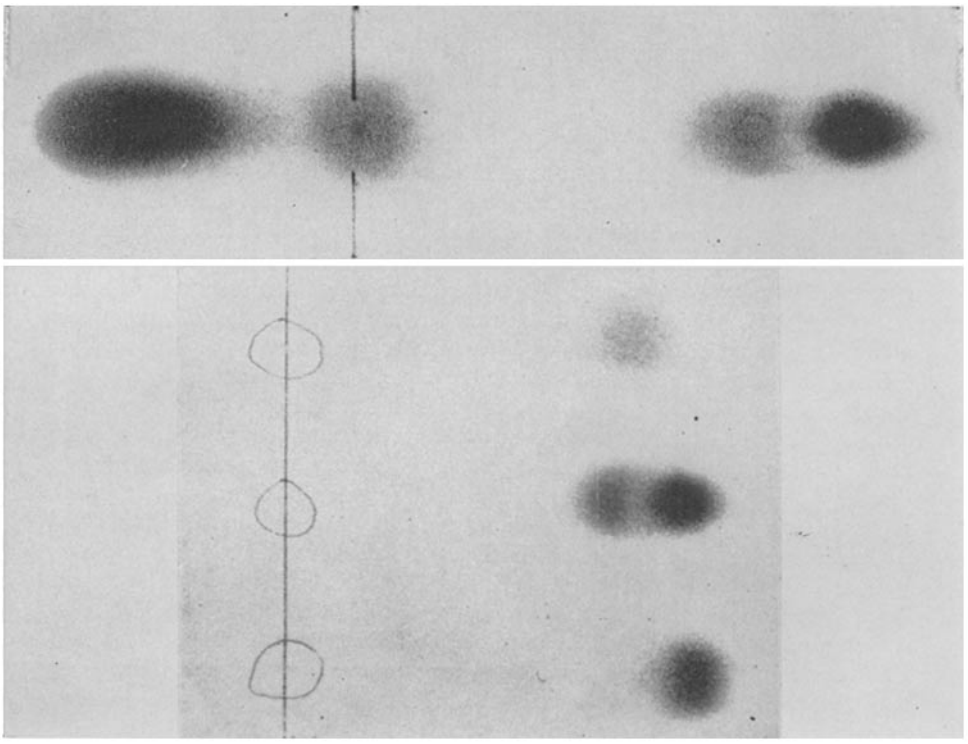
For accurate measurements of the resistance of the paper strip, specially coated platinum electrodes were dipped in the buffer at the end of the paper strips and the resistance determined with alternating current by means of a Philips universal measuring bridge, Type GM 4144, with an L.F. oscillator.

The cross-sectional area of the buffer in the paper, q_a, was determined by weighing the glass plates and filter paper before and after buffer had entered the paper, calculating the volume of buffer from the specific gravity, and dividing the volume by the length of the filter paper. In these experiments the filter paper was the same length as the glass plates. The ends of the plate and filter paper were forced against a mass of special, highly porous, filter paper dipping in buffer that was attached to vertical perspex plates at the electrode vessels (Fig. 1). This served as a good conducting medium between the ends of the glass plates and the solution in the electrode vessels. It was only 0.5 to 1 cm. in length depending on the thickness of the glass plates and was so saturated with buffer that it offered no significant resistance. The path of ionic migration in the paper (l') was determined for various electrolytes by measuring the resistance, the available cross-sectional area, and the conductivity in free solution. Similar results were obtained when multiple layers of paper were used between the glass plates as for a single sheet. Measurements of mobility were made in barbital buffer at pH 8.8 $\mu = 0.1$ unless otherwise specified. Low currents along with the cooling system preserved a temperature of 3°C.

Separation of Mixtures of Purified Proteins

The study of a large number of highly purified proteins, either alone or in artificial mixtures, showed that proteins that were soluble in alkaline buffers migrated as discrete spots on filter paper and could be separated. Fig. 3 *a* shows the separation of a mixture of crystalline lysozyme, myeloma protein, crystalline β-lactoglobulin, and crystalline human albumin on a strip of filter paper stained with bromphenol blue. The line across the paper indicates the starting point. The area of the initial spot was equal to the size of the gap between the lines and slightly less than the area of the myeloma protein which is seen at the origin. This protein had a mobility of -1.2×10^{-5} in free solution but due to the flow to the cathode caused by electroosmosis it remained close to the origin. If the electroosmosis was neutralized by arranging a counterflow of liquid this protein moved to the right and did not adhere to the origin. The lysozyme which has an isoelectric point near pH 11 moved to the left (cathode) since the pH of the buffer was below its isoelectric point. Crystalline trypsin not illustrated in this figure also moved toward the cathode at pH 8.8. β-Lactoglobulin and human albumin moved to the anode with a comparatively rapid mobility.

In various experiments the shape of the spots on filter paper showed consid-



(c)

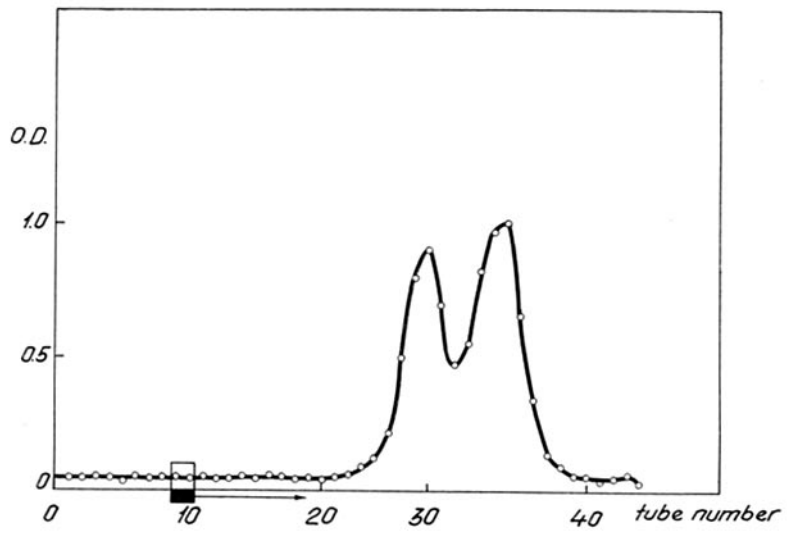


FIG. 3 (*a*). Separation of an artificial mixture of crystalline lysozyme, purified myeloma protein, crystalline β-lactoglobulin, and crystalline human albumin by paper electrophoresis. The line indicates the origin of the initial spot. (*b*) Crystalline β-lactoglobulin (upper) and crystalline human albumin (lower) and the mixture of the two separated on paper. (*c*) Curve obtained when a paper containing the mixture of β-lactoglobulin and albumin was segmented and the fractions analyzed for protein concentration by the Folin method. The block indicates the site of application of the protein solution.

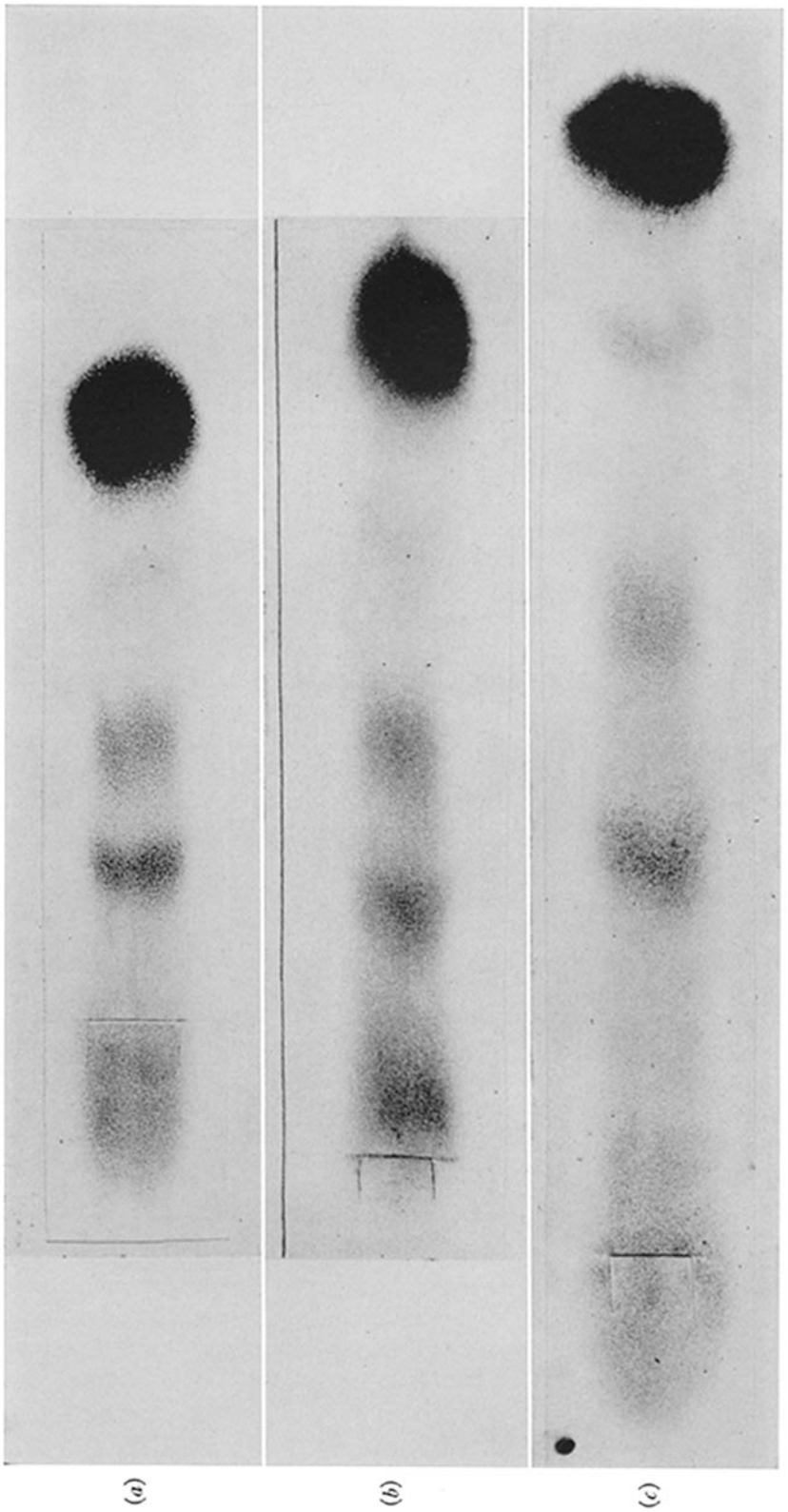
erable variation depending on the concentration of the individual protein and on the protein itself. In general proteins which gave very sharp peaks in free electrophoresis, such as the myeloma proteins, gave the roundest spots. Fig. 3 *b* illustrates the separation of a mixture of β-lactoglobulin and human albumin and the individual proteins alone on the same sheet of filter paper. When another paper strip containing this mixture was cut into segments and the protein content determined by the modified Folin procedure previously described, a curve showing two peaks was obtained (Fig. 3 *c*).

None of the protein remained at the origin as demonstrated by the flat line at this point. The paper itself gave a slight color in the reaction and accounted for the minor elevation above the base line. In this experiment no tail of protein that could be detected was left behind on the paper. Further experiments with higher concentrations of these proteins demonstrated that β-lactoglobulin showed no adsorption on the paper where as little as 0.3 per cent of the peak concentration could be detected. Human albumin, however, showed a little adsorption ranging from 0.5 to 1.5 per cent being slightly greater at low temperatures. None of the other proteins studied showed as much adsorption as albumin in alkaline buffers, with the exception of the colored protein phycoerythrin. At acid pH values the adsorption was greater for most proteins. Despite this limitation, acid buffers were found useful in certain instances. For example, a study of various preparations of insulin at pH 3.0 showed good localization of this protein with very little adsorption to the paper.

Other purified proteins that were studied which could be localized and separated by filter paper electrophoresis were crystalline ovalbumin, vitellin and legumin from peas, a phosphatase preparation, and a lactase preparation. Some efforts were made to stain enzymes specifically as they separated on paper. Partial success was achieved particularly with the lactase in which the colorless substrate (nitrophenyl galactoside) was split into a yellow compound which could be seen directly on the paper. One difficulty in such experiments was that in order to have sufficient protein so that peaks could be seen with bromphenol blue on the paper a great excess of enzyme was usually necessary and the peak concentration was difficult to localize by means of the substrate without dilution of the enzyme. Wallenfels and Pechmann (12) have recently reported the localization of various enzymes separated by filter paper electrophoresis.

Separation of Normal and Pathological Sera

The proteins in serum were particularly well suited for filter paper electrophoresis experiments because of their ready solubility in high concentrations in alkaline buffers. A high concentration of protein could be placed on the paper in a relatively small spot, an important factor in obtaining good resolution of components. Fig. 4 *a* illustrates the pattern of normal human serum


(a)
(b)
(c)

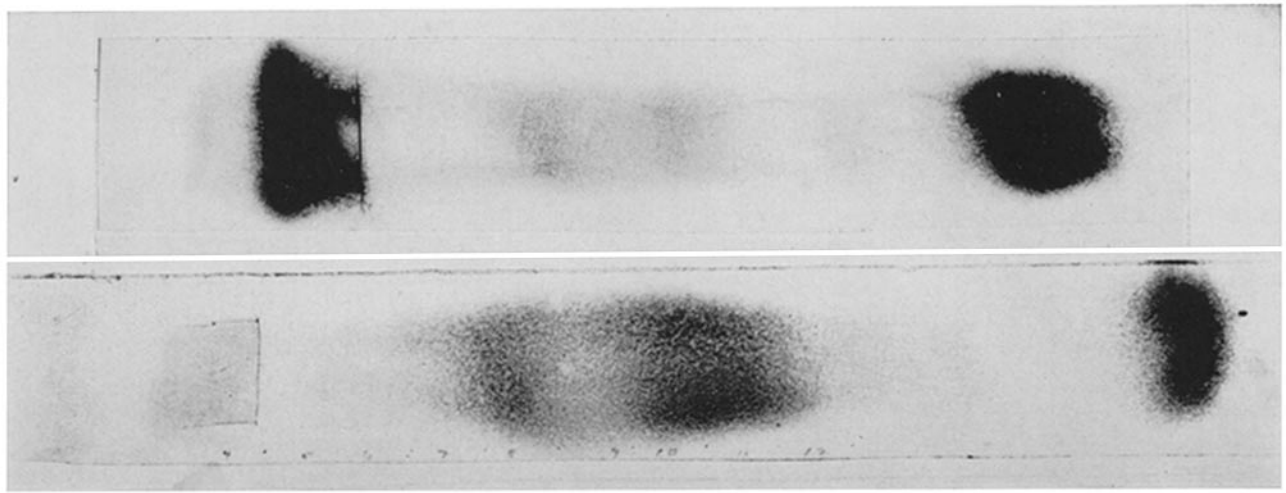


FIG. 4 (*a*, *b*, *and c*). Single paper strips of normal serum separated under different conditions. The serum was applied to a three-sided flap cut in the paper (4 *a*, *b* × ⅔; 4 *c* × ½). (*d*, *e*), Single paper strips of two pathological sera; (*d*), multiple myeloma; (*e*), nephrosis.

separated between glass plates 25 cm. long and 4 cm. wide. In this experiment the protein was applied to the flap which was cut in the paper. The protein solution just covered the area of the flap. Five main components are readily visible: reading from right to left, albumin as a very deeply stained round spot, α_1, as a very faint spot, α_2, as a fairly broad band, β, as a sharper band, and γ, as a very diffuse spot. Fig. 4 *b* represents a different normal serum in which the electroosmosis was partly counteracted by raising the level of liquid in the cathode compartment 2 cm. above that in the anode. This pattern also shows the five main spots seen in the upper pattern and well known from experiments with free electrophoresis. Other patterns of normal serum have occasionally shown evidence of a splitting of the β spot. The α_1 is not always visible as in these patterns. Fig. 4 *c* represents serum that was run on an unusually long strip (glass plates 50 by 6 cm.) over a period of 48 hours at 18 ma. It shows the broad distribution of γ-globulin on both sides of the origin and suggests separate bands in the γ-fraction. This was seen in several other strips particularly those in which the serum was separated slowly over long distances.

For many purposes it was found advantageous to use broader glass plates and broader paper. A number of specimens of serum could be run simultaneously and compared directly under the same conditions. Fig. 5 *a* illustrates the patterns of five different sera run between glass plates, 14 by 9 cm. The top pattern is from the serum of a patient with nephrosis. It shows an increased α_2 component and a very low γ-globulin. The serum was milky and as a result there was considerable adsorption at the origin. The next two patterns are from sera from two different patients with biliary cirrhosis. Various changes are apparent with increases in the β and γ components being most marked. The fourth pattern represents normal serum, showing the usual five components. The bottom pattern is from the serum of a young girl with cirrhosis of the liver with extreme elevation of the γ-globulin fraction.

Filter paper electrophoresis was particularly suited for the study and characterization of various types of myeloma sera. Fig. 5 *b* illustrates the results obtained with four different myeloma sera; a normal serum is shown in the middle for comparison. The unusual myeloma protein is readily apparent and its high concentration compared to that of albumin can be ascertained. The different mobilities of the myeloma proteins in the different sera can be seen. It was usually advisable to dilute sera containing high concentrations of abnormal components, although this was not done with the sera illustrated in Fig. 5 *b*. Too high a protein concentration sometimes produced irregularities in the field strength and therefore altered the mobilities of the components. Fig. 5 *c* illustrates a γ myeloma and a β myeloma pattern, as compared to the normal, in which the myeloma sera were diluted two times.

Quantitative Analysis of Serum Components

It was readily possible to partition into defined segments the various components of serum. Considerable accuracy could be achieved in cutting up the

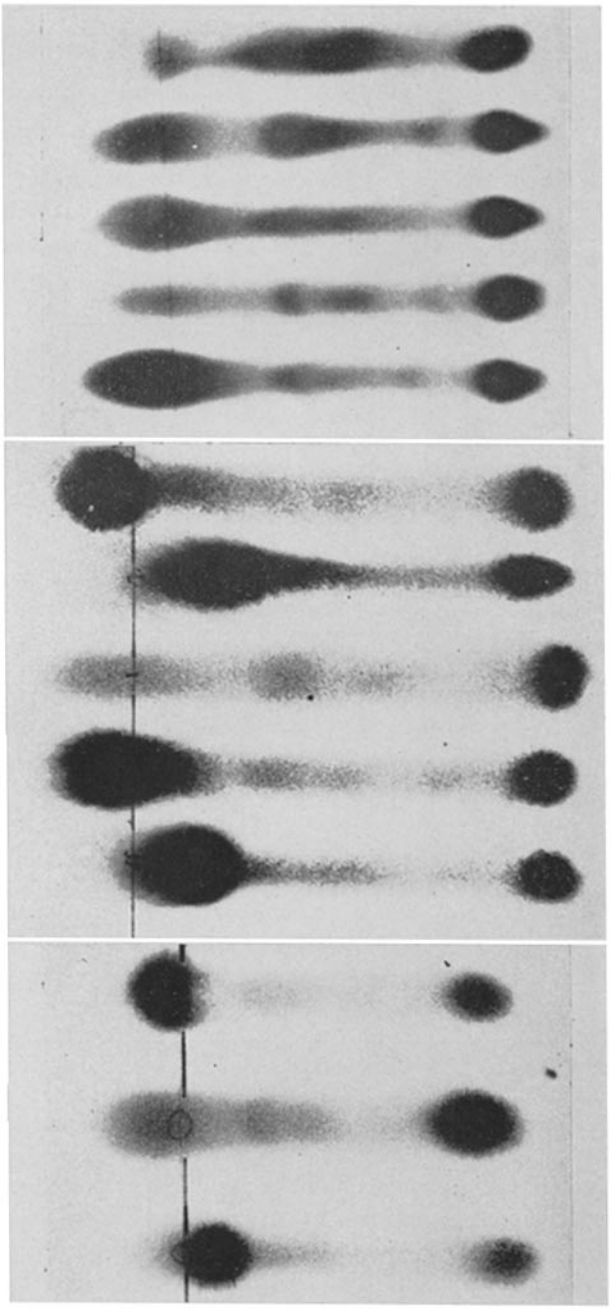


FIG. 5 (*a*). ($\times \frac{2}{3}$) Five sera separated together on the same sheet of paper. The upper serum is from a patient with nephrosis, the fourth from the top is normal serum, the remainder from different patients with cirrhosis of the liver. (*b*) Sera from four different patients with multiple myeloma compared with normal serum on the same sheet of paper. The high concentration of the abnormal myeloma component is readily apparent. (*c*) Two myeloma sera, diluted with an equal volume of saline, compared with a normal serum. The difference in the mobility of the abnormal myeloma proteins is apparent.

paper strips if the paper was allowed to dry partially. Fig. 6 illustrates the results obtained with normal serum by the two methods of protein determination discussed previously. The two patterns were obtained in separate experiments on the same serum. However, by using two or three sheets of filter paper, it was possible to determine the protein by both methods for the pattern obtained in a single experiment. Neither method furnishes a curve which

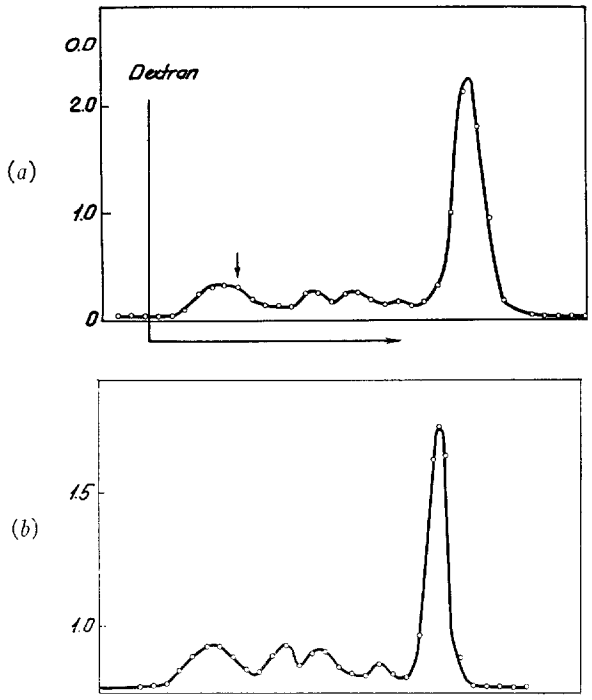


FIG. 6. Comparison of the curves obtained by segmentation of two papers containing the same normal serum with protein determined (*a*) by the dye elution method, (*b*) by the modified Folin method. The dextran line representing the true starting point is illustrated in the upper pattern. The arrow refers to the point of protein application.

agrees exactly with the nitrogen content of the different proteins. When comparisons were made between purified albumin and purified γ-globulin (Fig. 2) it was found that γ-globulin gave a more intense color per unit of nitrogen than did albumin by the modified Folin method but by the dye elution method the reverse was true and the albumin gave a more intense color. Table I shows the percentage composition of the areas of the components in the two curves illustrated in Fig. 6 as compared with the results for the same serum in free electrophoresis. Closer agreement could be obtained by correcting the albumin and γ-globulin values according to Fig. 2.

By means of the Folin method, the albumin and γ-globulin concentration could be accurately calculated as miligrams of protein from the curves in Fig. 2. When the volume of serum that was applied to the paper was accurately known, the concentration of these components in the serum could thus be determined directly and expressed as grams per 100 cc. This made it unnecessary to compute the relative areas under the curve. The chief difficulty with this procedure was that it became imperative that none of the applied protein be lost or left on the paper. By using glass plates made water-repellent by dipping in a silicone solution and drying, by using several sheets of paper between the plates, and by letting the paper segments soak in 0.1 N NaOH for 24 hours prior to developing the color reaction, it was possible to obtain 93 per

TABLE I

Comparison between Percentage Composition of Various Components of Normal Serum as Calculated from Areas under Curves Obtained by Paper Electrophoresis and Free Electrophoresis

Method	Composition				
	Albumin	α_1	α_2	β	γ
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Paper electrophoresis					
Modified Folin method..	52.9	6.3	12.2	10.1	18.5
Dye elution method.....	65.9	3.9	8.0	9.0	13.1
Free electrophoresis					
Ascending.....	53.33	6.21	10.87	13.79	15.62
Descending.....	52.06	5.28	11.26	14.66	16.74

cent recovery. The dye elution method of protein estimation was more difficult to use for direct determination of protein concentration in this way because of variations in the amount of dye bound in different experiments. However, it had many advantages, being simpler and more sensitive, and calculation of the relative areas of the components furnished reliable results, despite differences in dye-binding capacity for the different serum proteins.

Figs. 7 *a* and *c* illustrate the curves obtained for two pathological sera in which the protein was determined by the modified Folin method. Curve 7 *a* is from the serum of a patient with cirrhosis. For comparative purposes the electrophoretic pattern obtained with the same barbital buffer in free solution is also shown. The marked elevation in the γ-globulin is readily apparent. In addition each of the three intermediate components between the γ-globulin and albumin, although present in low concentration, is evident. Curve 7 *c* is from a patient with lipoid nephrosis. An ordinary electrophoretic pattern could not be obtained on this serum because of extreme milkiness. The total lipid level was 3200 mg. per cent, as determined by the Van Slyke lipid carbon

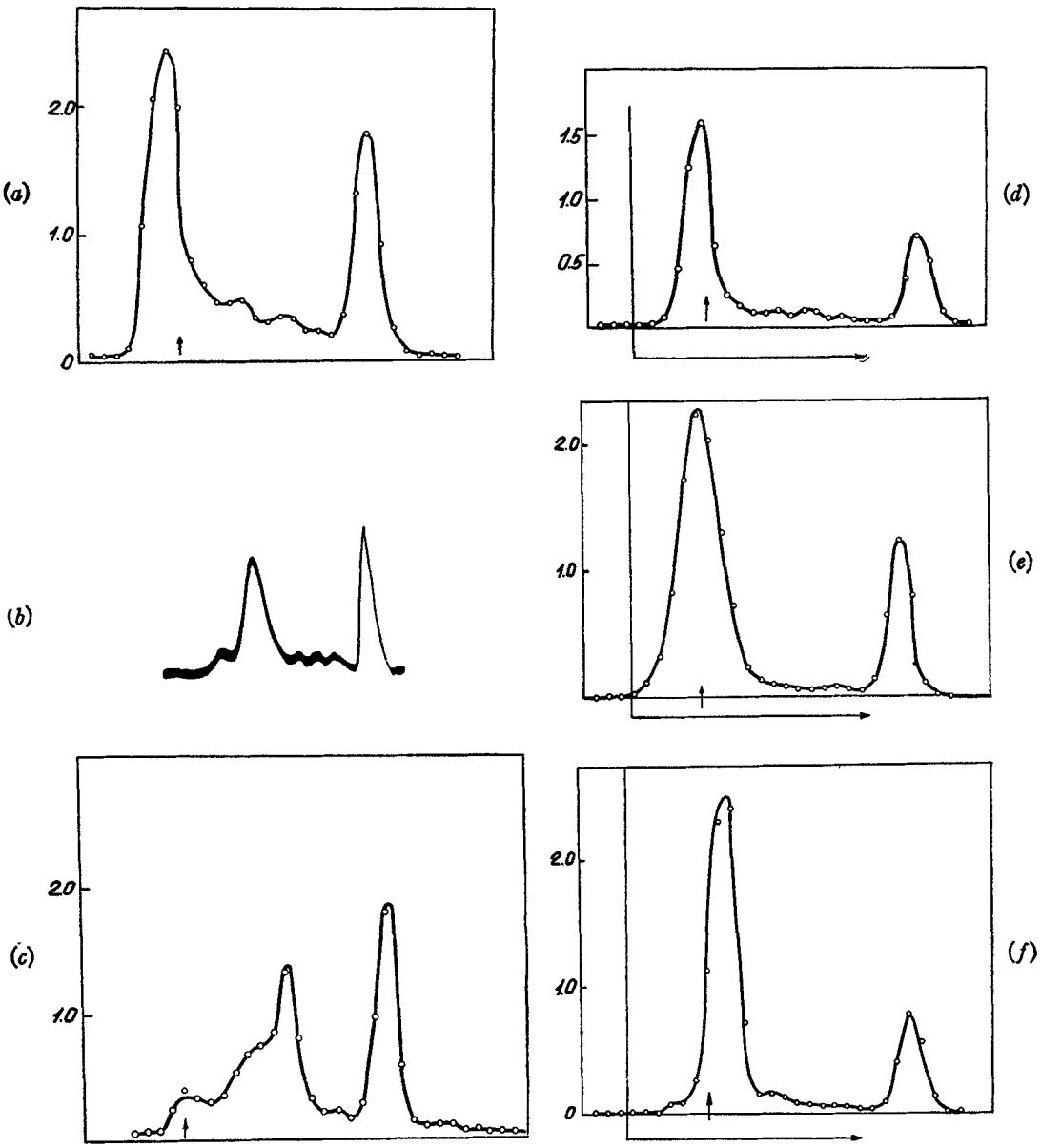


FIG. 7 (*a*, *c*). Curves obtained by the modified Folin method on paper segments for two pathological sera (*a*) cirrhosis, (*c*) nephrosis. The descending electrophoretic pattern in free solution (*b*) is illustrated for the cirrhosis serum. (*d*, *e*, *f*) Curves obtained by the dye elution method for three different myeloma sera separated on the same sheet of paper. The dextran line of true origin is illustrated.

method, which was predominantly neutral fat. The extreme elevation in the α_2 and β fraction is apparent. In this experiment some protein remained at the origin giving an abnormally high point at this site.

Figs. 7 *d*, *e*, *f* show the curves obtained with the dye elution technique for three different myeloma sera. All three patterns were run on the same sheet of filter paper. In this experiment, the separation was carried out overnight and the next morning one technician plotted the three curves after staining the paper, eluting the segments, and reading the color in the spectrophotometer. The entire procedure was carried out in approximately 3 hours. The lower curve (7 *f*) illustrates a myeloma protein with somewhat more rapid mobility than the others, where the normal γ-globulin is just visible to the left of the myeloma component. However in free solution the mobility of this component was somewhat greater relative to the other components.

Two Dimensional Electrophoresis

By using a very broad sheet of filter paper between glass plates, it was possible to obtain separation of proteins in a second direction following the first separation. Fig. 8 illustrates the pattern obtained for normal serum employing the same barbital buffer at pH 8.6 in both directions. Here the components have become grouped in a diagonal line. Although no new components are evident by this procedure, it has a distinct value. Each component has migrated "around the corner" and therefore any adsorbed protein or trailing is left in the path of the protein and does not interfere with the other components. In Fig. 8 no trailing is visible. However, a faint blue color was visible in the path of the albumin under certain special conditions of staining. This was too faint to be visible in a photograph. In other experiments some trailing of the β-globulin was obtained "around the corner."

The greatest value of this technique was in demonstrating that the dark areas between the distinct spots represent a multitude of minor components with different mobilities. From the one dimensional pattern it might be inferred that they represent tails or spreading out of the major components in the course of the experiment. However, the fact that these areas became arranged in a diagonal band after "turning the corner" indicates the same defined mobility in both directions. If for example the γ-globulin represented a component with a single mobility that in one direction spread out into a fairly broad band due to boundary disturbances and random distribution of the individual protein particles, then it would migrate as a horizontal band in the second direction with particles at both ends of the band going the same distance. This is what happened for albumin in Fig. 8 in which no diagonal arrangement of the particles took place. However, the γ-globulin became arranged in a distinct diagonal showing different mobilities in the second direction for the two ends of the one dimensional spot, proving in a definite manner the heterogeneity of this component. This was confirmed in separate

experiments on a purified preparation of γ-globulin (Squibb II-1, 2). Although this material gave a single peak in free electrophoresis and one broad spot in one dimensional paper electrophoresis, it gave a distinct diagonal band when two dimensional experiments were carried out.

Relatively little success was achieved in further separating serum proteins by employing a different pH in each direction. This was chiefly due to the

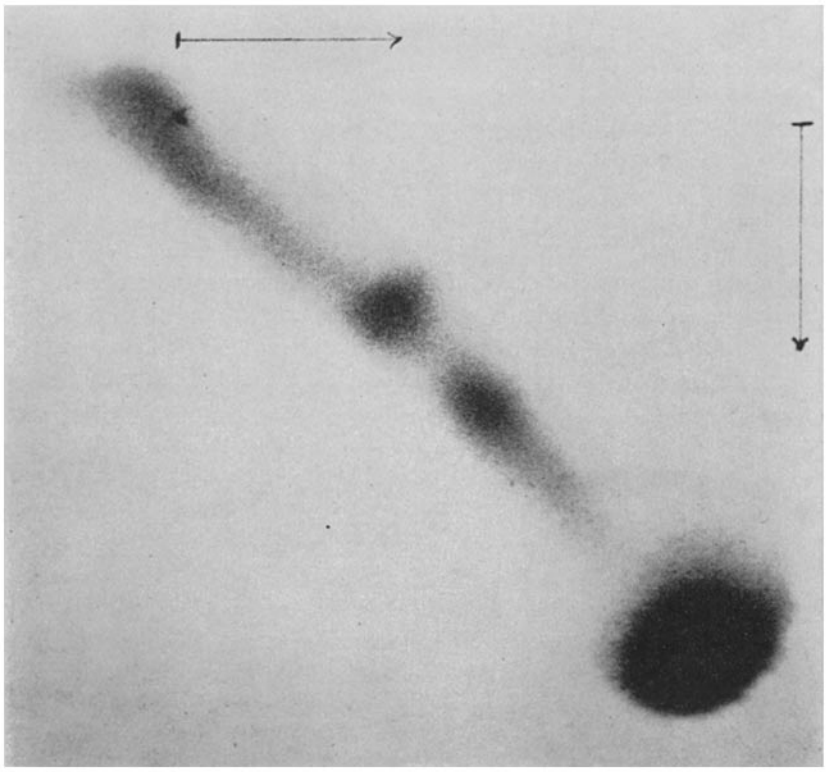


FIG. 8. Two dimensional pattern obtained for normal serum using the same barbital buffer at pH 8.6 in each direction. The diagonal arrangement of the proteins demonstrates the multiplicity of the components with different mobilities.

spreading out of various components, particularly albumin and the increased adsorption to the paper in acid solution.

Results of Preparative Experiments with Serum Components

By employing numerous sheets of filter paper between long glass plates it was possible to separate as much as 5 cc. of serum in a single experiment. Fig. 4 *c* illustrates the top sheet of a group of four that were used to isolate the normal components of serum. In this experiment 1 cc. of serum was placed

on the paper flap and good resolution of the components was obtained. This procedure, followed by elution of the protein from segments, was used with considerable success for the isolation of the different myeloma proteins from the other serum components. These preparations were used for immunological studies and were shown to be free, at least, of all traces of albumin by testing with albumin antiserum. The results of these experiments will be published separately.

Studies on Electroosmosis and Mobility Determinations

In all the experiments on filter paper electrophoresis described in this report a flow of buffer was observed in the direction of the cathode when current was applied to the paper. This caused a shift of the γ-globulin to the cathode side of the origin on many of the papers. The streaming of liquid resulted from the phenomenon of electroosmosis. The walls of the many pores in the filter paper acquire a negative charge relative to the buffer medium just like the walls of glass tubes in classical experiments on electroosmosis. Since the filter paper walls are fixed in a stationary position and cannot move toward the anode as a result of their negative charge, the buffer solution alone moves toward the cathode since its charge is positive relative to the filter paper.

In order to study this phenomenon more thoroughly and to quantitate the degree of electroosmosis in various filter papers, a search was made for some substance without significant charge which would not have a mobility of its own in an electric field but would move with the electroosmotic liquid flow and thus give an index of the degree of this flow. Such a material would also serve as a reference for measurements of mobility. Various substances were tested, starch, amylose, sucrose, and numerous other substances particularly polysaccharides. They were not used extensively because of difficulty in staining, extensive diffusion, or inherent mobility of their own. The most suitable substance proved to be dextran which showed the least mobility of any substance studied and could be stained readily with bromphenol blue along with proteins on the paper. In free electrophoresis this material showed only a very slight mobility toward the anode in barbital buffer at pH 8.8 ($u = -0.16 \times 10^{-5}$). At the bottom of Fig. 5 *a* the dextran spot is lightly stained with bromphenol blue and has moved further toward the cathode than any protein in serum. Fig. 9 illustrates the movement of the dextran toward the cathode on two different types of paper along with serum protein patterns. The lower paper was considerably thicker and coarser than the upper one and considerably greater movement of the dextran was obtained as well as greater back migration of the proteins. In the experiments illustrated a high concentration of dextran was used in a relatively large spot for purposes of illustration. However for maximal back migration small spots of constant size and containing a lower concentration of dextran were used. The latter method was employed for localizing the dextran in Figs. 6 *a* and 7 *d*, *e*, *f* and for the re-

sults in Table II. On the basis of a large number of experiments it appeared that the dextran movement gave a close approximation of the extent of the electroosmotic flow.

Since the two phenomena electroosmosis and electrophoresis are very closely related, it should be expected that a relationship could be found between the distance of electroosmotic flow as indicated by dextran and the distance of

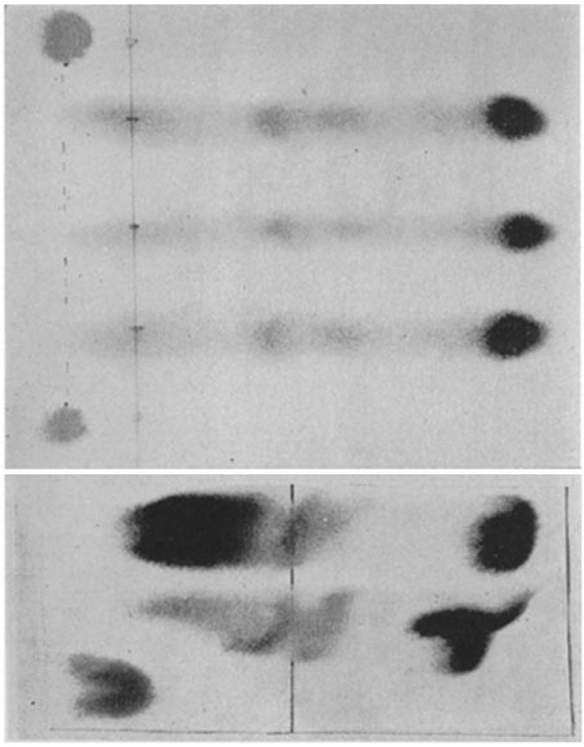


FIG. 9. Serum patterns on two different types of paper illustrating differences in electroosmotic flow (*a*) Munktell 20 paper with slight back migration of the dextran spot, (*b*) a thick soft paper, not suitable for general use, but showing marked back migration of the dextran spot.

migration of a specific protein such as albumin on the paper. For example, if d_{alb} is the distance that the albumin travels on the paper from the origin and d_{dex} is the distance that the dextran travels in the opposite direction, the total distance that the albumin travels is $d_{\text{alb}} + d_{\text{dex}}$. The mobility, $-u_{\text{alb}}$, is expressed by the formula:

$$-u_{\text{alb}} = \frac{d_{\text{alb}} + d_{\text{dex}}}{F\ t} \tag{1}$$

in which F is the field strength and t, the time. In the same way, the mobility

of the electroosmotic flow may be expressed by the formula:

$$u_{el} = \frac{d_{dex}}{F\,t} \tag{2}$$

Combining the two formulas:

$$\frac{d_{dex}}{d_{alb} + d_{dex}} = \frac{u_{el}}{-u_{alb}} \tag{3}$$

The relationship $\frac{u_{el}}{-u_{alb}}$ is constant regardless of distance, time, or field strength and therefore $\frac{d_{dex}}{d_{alb} + d_{dex}}$ should also be constant. Table II shows the result of experimental determinations of this ratio. The ratio remained constant in different experiments despite variations in the current applied and in the time over which the separation was carried out. The relationship $\frac{d_{dex}}{d_{alb}}$ was therefore also a constant, and once this was determined for any brand of paper it was always possible to calculate the distance of electroosmotic flow and then the total distance that any ion migrated. For the Munktell 20, 150 g. paper, which was usually used, $\frac{d_{dex}}{d_{alb}}$ was determined as 0.35 at 3°C. and pH 8.8 and this factor could subsequently be used without further need of measurements with dextran. For paper No. 3, a thick soft type, $\frac{d_{dex}}{d_{alb}}$ was 1.6, with the dextran showing greater movement to the cathode than albumin to the anode.

TABLE II

Observed Values for Distance of Electroosmotic Flow and Albumin Migration and Calculated Mobility of Electroosmotic Flow

Type of paper	Distance from origin		$\frac{d_{dex}}{d_{alb}}$	$\frac{d_{dex}}{d_{dex} + d_{alb}}$	$u_{el} \times 10^5$
	d_{dex}	d_{alb}			
1. Munktell 20	0.7	1.9	0.36	0.27	1.83
	2.5	7.4	0.34	0.25	1.59
	1.3	3.8	0.35	0.26	1.69
	1.5	4.3	0.35	0.26	1.61
2. Ford, blotting paper	2.7	3.2	0.84	0.47	2.20
	3.5	5.0	0.70	0.41	1.98
	3.5	4.2	0.83	0.47	2.33
3. Thick, soft paper	4.2	2.5	1.7	0.63	
	9.9	6.4	1.5	0.61	

The results presented in Table II were all obtained with barbital buffer at pH 8.8. At lower pH levels the dextran also showed a positive mobility which gradually decreased until it approached zero at approximately pH 3. Fig. 12

shows dextran mobilities at various pH levels used in the determination of the isoelectric point of human albumin on Munktell 20 paper.

Employing $d_{dex} + d_{alb}$ as the distance of migration of albumin, an attempt was made to calculate the mobility of this protein on filter paper. Fig. 10 shows the distance of migration of albumin, made visible with bromphenol blue, plotted as a function of time. A straight line was always obtained when the current was kept constant.

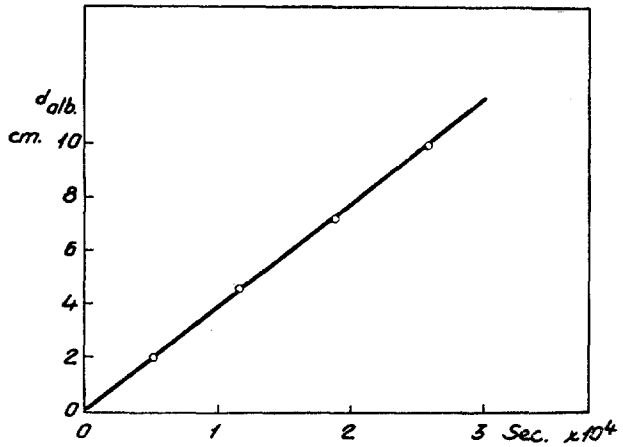


FIG. 10. The distance of migration of purified human albumin plotted as a function of time.

The determination of mobilities on paper involved several new considerations because the formulas used in free solution:

$$u = \frac{dl}{iV} \tag{4}$$

and

$$u = \frac{dq\kappa}{ti} \tag{5}$$

in which V is the voltage, l the length of the channel, q the cross-sectional area, κ the conductivity, and i the current, are not applicable to liquid in a highly porous supporting medium. It could readily be shown that the expressions for field strength, $\frac{V}{l}$ and $\frac{i}{q_a\kappa}$ were not equal. This was due to the fact that l did not represent the true distance of voltage drop through the paper. This can best be seen from a consideration of Fig. 11 in which a tortuous channel is envisioned in the paper (l'). This, of course, represents a great simplification of the intricate channeling of liquid that actually exists in the paper.

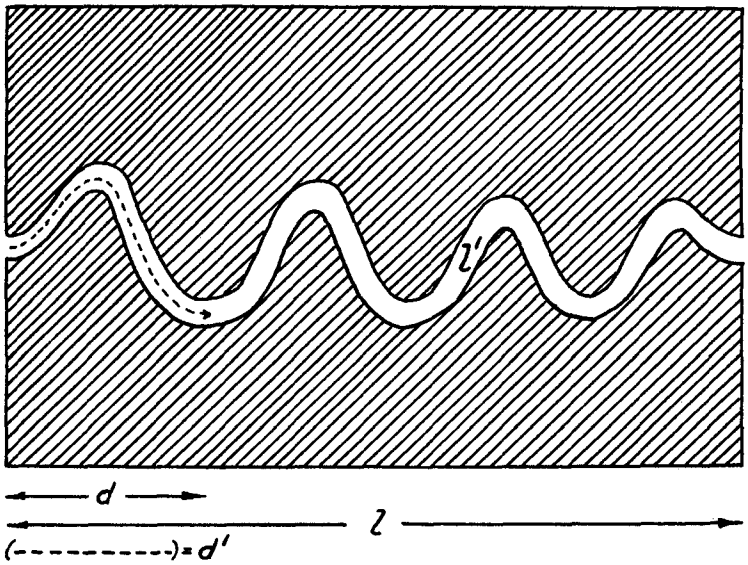


FIG. 11. Schematic drawing illustrating a theoretical path of ionic migration through filter paper.

In accordance with the diagram the protein particle takes the tortuous path d' which follows l' and is a fraction thereof and not the observed distance d. Since

$$l' = l\left(\frac{l'}{l}\right) \tag{6}$$

therefore

$$d' = d\left(\frac{l'}{l}\right) \tag{7}$$

In free solution the protein particle migrates through a distance:

$$d = ut\frac{V}{l}$$

But in the paper the distance is

$$d' = ut\frac{V}{l'}$$

Substituting (6) and (7)

$$d\left(\frac{l'}{l}\right) = \frac{utV}{l\left(\frac{l'}{l}\right)}$$

$$d = ut\frac{V}{l}\left(\frac{l}{l'}\right)^2 \tag{8}$$

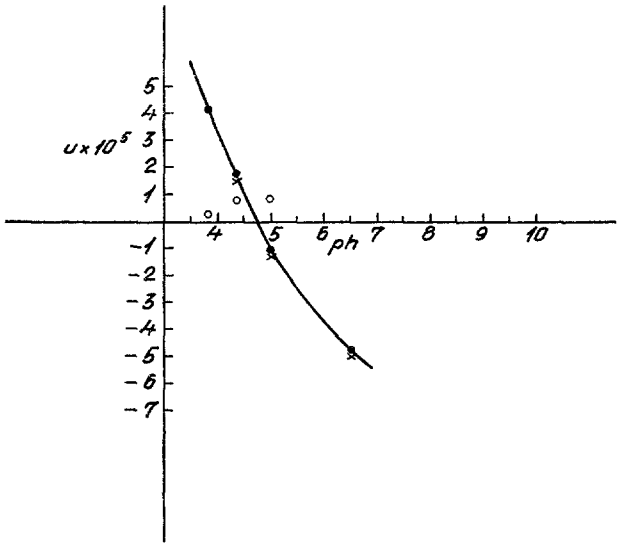


FIG. 12. Mobility values plotted against pH for human serum albumin on filter paper (●) and in free solution (×). The individual points (○) represent dextran mobilities.

Also, since in free solution the protein particle migrates through a distance:

$$d = \frac{uti}{q\kappa}$$

In paper this becomes:

$$d' = \frac{uti}{q_a\kappa}$$

Substituting (7)

$$d\left(\frac{l'}{l}\right) = \frac{uti}{q_a\kappa}$$

$$d = \frac{uti}{q_a\kappa}\left(\frac{l}{l'}\right) \tag{9}$$

Equations (8) and (9) differ from those for free solution in that they contain the factor $\frac{l}{l'}$ which may be termed the correction factor for calculation of mobility on paper. It was possible to determine this factor for various types of filter paper using the equation

$$l' = Rq_a\kappa$$

in which R is the resistance of the paper strip.

TABLE III

Mobility Values for Human Albumin in Barbital Buffer pH 8.8, μ = 0.1 for Various Types of Paper

Values for the correction factor $\left(\frac{l}{l'}\right)$ used in obtaining the true mobility u_e are shown.

Medium	u_a	$\left(\frac{l}{l'}\right)$	u_e
Munktell 20 paper	4.01 ± 0.09	0.77 ± 0.03	6.78
Ford, blotting paper	3.21 ± 0.12	0.70 ± 0.04	6.53
S. & S. 413 paper	2.34 ± 0.21	0.58 ± 0.06	6.85

TABLE IV

Absolute Mobilities of Serum Components Obtained with Munktell 20 Paper

Experiment	Mobilities × 10^5				
	Albumin	α_1	α_2	β	γ
1	6.18	4.86	3.96	3.15	1.45
2	6.53	5.04	4.09	3.06	1.24
3	6.62	5.32	3.77	2.84	1.12
4	6.60	5.38	4.04	3.05	1.25
Average	6.48	5.15	3.97	3.02	1.27

TABLE V

Relative Mobilities of α_1, α_2, and β Components of Serum Calculated by Assigning Values Obtained in Free Solution to Albumin and γ-Globulin

Electrophoresis method		Mobilities × 10^5				
		Albumin	α_1	α_2	β	γ
Free solution		6.43	5.47	4.51	3.06	1.11
Paper	Experiment 1		5.04	3.68	2.80	
	Experiment 2		5.12	3.85	2.96	
	Experiment 3		4.90	3.57	2.68	
	Experiment 4		5.20	3.90	2.94	
	Average		5.05	3.74	2.85	
	Difference from that in free solution		0.42	0.77	0.21	

The factor $\frac{l}{l'}$ represents a characteristic of each type of filter paper and is independent of the liquid medium. Experiments with KCl, barbital buffers, phosphate buffers, and acetate buffers gave similar results. Table III shows the values for $\frac{l}{l'}$ and the mobility values for human albumin, u_a, uncorrected, employing formula (4) and u_c, corrected, employing formula (8). Whereas the uncorrected mobilities vary for different types of filter paper, the corrected mobilities were approximately the same. In addition the uncorrected mobilities were very low while the corrected mobilities showed good agreement with those in free solution. This is also apparent from Fig. 12 in which the corrected mobilities on paper in acetate and phosphate buffers ($\mu = 0.1$) for human albumin are compared with those in free solution for the determination of the isoelectric point, employing the same buffer solutions in each case.

The mobilities listed in Table III were for albumin in the isolated state. Slightly higher mobilities were always obtained in this way than for albumin in whole serum. Table IV illustrates the corrected mobility values obtained for the various serum components.

The mobilities of the middle components of serum were also calculated relative to γ-globulin and albumin assigning average values obtained from descending patterns in free solution to the latter two components and thus comparing the relative mobility of the middle components directly with those in free solution (Table V), thus avoiding electroosmosis considerations and absolute mobility calculations. The measurements were made from four quantitative curves obtained for different normal sera resembling Fig. 6 *a*. For each component the value obtained gave a slightly lower mobility for the component on paper than in free solution. This was most marked for the α_2 component and least for the β. As a result, these two components migrated closer together than in free solution.

DISCUSSION

Although the basic apparatus and technique for filter paper electrophoresis were relatively simple, there were many minor pitfalls in the procedure, making it necessary to acquire considerable experience before satisfactory results could be obtained. The use of a colored protein solution as a guide aided considerably in overcoming the difficulties, particularly in the application of the initial spot and the clamping and separation of the glass plates. Each type of protein required special conditions because of variations in concentration, solubility in various buffers, mobility, adsorption, and numerous other factors. A small detail such as the presence of a small amount of precipitate in a protein solution could readily spoil an experiment, due to the continual dissolving of the pro-

tein in buffer on the paper thus producing a band extending from the origin. However, with particular attention to the technical details and experience with the procedure, many applications could be found.

The greatest advantage of the paper method over that in free solution lay in the fact that actual separation of the protein components was accomplished. Each point on the curves obtained from paper segments that were illustrated above, represented a test tube containing that fraction of protein in an isolated form. This made it possible to carry out other analyses on these fractions in addition to just the protein concentration. Curves were obtained for the lipid concentration in the various segments which could be plotted along with the protein curve. In the case of cholesterol, preliminary results have demonstrated the presence of at least three peaks in normal human serum, the major peak corresponding to the β-globulin.

Another application of the technique was to immunological studies of the various fractions of normal and pathological serum. The method was particularly suited for such a study because of the small amounts of protein necessary for immunological analysis. Concentrations of antigen determined by quantitative immunological methods were plotted along with the ordinary total protein concentration curve of serum, and thus the component reacting with any given antiserum could be identified.

In the experiments in which 1 cc. of serum was applied to the flap on four sheets of filter paper and separation carried out over a long distance, it was possible to observe the separation of the natural pigments of serum. Three definite colored spots were readily visible when the paper between the plates was held against the light in the moist state, a definite yellow spot at the albumin peak, another yellow spot at the α_2 region, and a reddish brown band at the site of the β-globulin. Light transmitted through multiple layers of paper containing a relatively high concentration of the serum fractions made the colors stand out.

The use of broad sheets of paper between glass plates made it possible to run a number of specimens at once and thus compare them with each other under identical conditions. In several experiments as many as ten different sera were separated at one time. These sera could be stained together and then each one cut out, segmented, and the dye eluted from the segments, furnishing a curve of the components of each serum. This method was extremely simple and surprisingly accurate. The chief defect lay in the variation of the dye-binding capacity of different proteins. Direct estimation of protein in the paper segments by a modified Folin procedure gave a curve which was closer to that obtained by optical methods in free solution but lacked the sensitivity, simplicity, and precision of the dye elution method.

A third method used for obtaining a curve of protein concentration from

the paper was by direct measurement of the dye on the paper by means of a special photometer. For this purpose the photograph negative of the filter paper strip was usually employed and the curve of the light transmitted obtained on a motor-driven recording device as the negative was slowly passed on a motor-driven sliding plate before a lamp. Curves similar to those obtained by segmenting the stained paper could be recorded. The method was feasible but in view of the complexity of the apparatus required and the ease of cutting up the strips and eluting the dye, it was not generally used. A similar procedure, employing the stained paper strip directly, has recently been described by Grassman and Hannig (13, 14).

The determination of the extent of electroosmotic flow was particularly important in accurately defining the isoelectric point of a protein. When electroosmotic flow was neglected, mobility curves at different pH levels showed irregular shapes with an S type configuration. The isoelectric point was shifted giving a falsely high value. This shift was relatively small for proteins with low isoelectric points but was considerable in the higher range. The curve reported by Schwarz (15) for horse serum albumin can be chiefly explained on this basis.

The dangers inherent in interpretations of non-electrolyte transport and the importance of regarding non-electrolytes as reference substances rather than as inert materials have been well discussed by Longsworth (16). Dextran represented a convenient substance that behaved more like an electrically inert substance on filter paper than any other of a number of non-electrolytes tested. In the present study the assumption was made that it represented an inert substance because it was not certain that the slight mobility at pH 8.8 in free solution was applicable to the paper and because of a lack of precise information regarding pH effects. Since the shift in the isoelectric point on paper from that in free solution is an index of the electroosmotic flow at the isoelectric point, the close agreement on the isoelectric point for albumin found in the present study indicates that dextran gave a close approximation of the extent of electroosmotic flow.

The experimental observations on mobilities calculated with the aid of a formula, modified from that used for free electrophoresis, demonstrate the validity of assuming an irregular pathway for ionic migration in the paper. For human serum albumin approximate agreement was obtained with the mobility of this protein in free solution although the slightly different temperature conditions made the exact comparison difficult. Since the factor $\frac{l}{l'}$ used for the calculations in paper is squared, great accuracy in the determination of l' is important. Technical problems particularly in the measurement of the available cross-sectional area in the paper make this difficult. However, once this factor is determined for a given brand of paper, it can be used for mobility

calculations under widely varying conditions. In view of the difficulties with considerations of the $\frac{l}{l'}$ correction factor, the assumption of dextran as an inert material represented a relatively small error in mobility calculations.

The slight adsorption of serum albumin to the paper and the difficulties in obtaining 100 per cent elution from the paper suggest that the paper may have specific effects beyond simply acting as an inert supporting medium. In addition, the negative charge of the paper so evident from the electroosmotic flow experiments undoubtedly plays a role. However, the mobility results reported above suggest that these effects in the case of serum albumin are relatively small.

SUMMARY

A simplified procedure for filter paper electrophoresis is described in which disturbing factors such as evaporation, heating, buffer concentration gradients, and pH changes in the electrode vessels were reduced to a minimum. Artificial mixtures of highly purified proteins could be separated and the components isolated. The application of the method to a variety of studies on serum proteins is demonstrated. Protein concentration in paper segments was determined by two different methods of protein estimation. Curves were obtained showing the same five major peaks for normal serum as found by the classical methods of free electrophoresis. Comparisons were made of the areas of the various components under the curves obtained with the different methods.

Two dimensional electrophoresis was applied to serum and serum components. It proved of value in demonstrating the heterogeneity of fractions such as the γ-globulin of serum.

The polysaccharide dextran was used as an index of the extent of electroosmotic flow during the course of the various experiments. The ratio of the distance of electroosmotic flow and the distance of protein migration was shown to be constant for a given type of paper. For serum albumin on Munktell 20 paper this ratio was 0.35. A formula for mobilities applicable to liquid in a highly porous supporting medium is presented. Mobility values for human serum albumin at various pH levels on paper showed approximate agreement with those obtained in free solution giving a similar isoelectric point.

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