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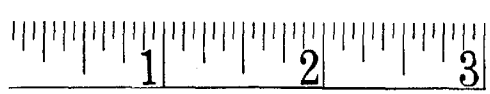

THE OPERATION AND APPLICATION OF THE TISELIUS ELECTROPHORESIS APPARATUS

By

Chester R. Hardt

A THESIS

Submitted to the Graduate School of Michigan State College of Agriculture and Applied Science in partial fulfilment of the requirements for the degree of

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The Operation and Application of the Tiselius Electrophoresis apparatus

INTRODUCTION

When an electrical current is passed through a solution or suspension of charged particles, the particles migrate to the anode or cathode. The phenomenon is known as electrophoretic migration. Abramson (1) has used the following illustrations to show that electrophoretic migration depends only upon the nature of the surface and is independent of the size, shape, and nature of the bulk of the particles. Figure 1 indicates the direction of electrophoretic migration of a wide variety of charged particles in a neutral salt solution. Figure 2 shows the effect of adding a small amount of neutral gelatin solution. It will be noted that the ferric oxide particle has reversed its direction of migration and the following conclusions are evident. "The electrophoresis of gelatin coated particles is primarily concerned with and determined by a property of gelatin surfaces. In addition there is every reason to believe that we are investigating something very closely connected to the properties of gelatin as it exists in solution" (1).

Two widely different methods have been developed to study electrophoretic migration, the microscopic and the moving boundary methods.

The advantages of the microscopic method have been considered by Abramson (2) and are as follows:

" (1) During the period of observation the environment of the particle does not change perceptibly.

" (2) The electrophoretic mobilities of particles may be compared by watching them move simultaneously. Detection of slight differences between particles that are visually indistinguishable may be accomplished by this method.

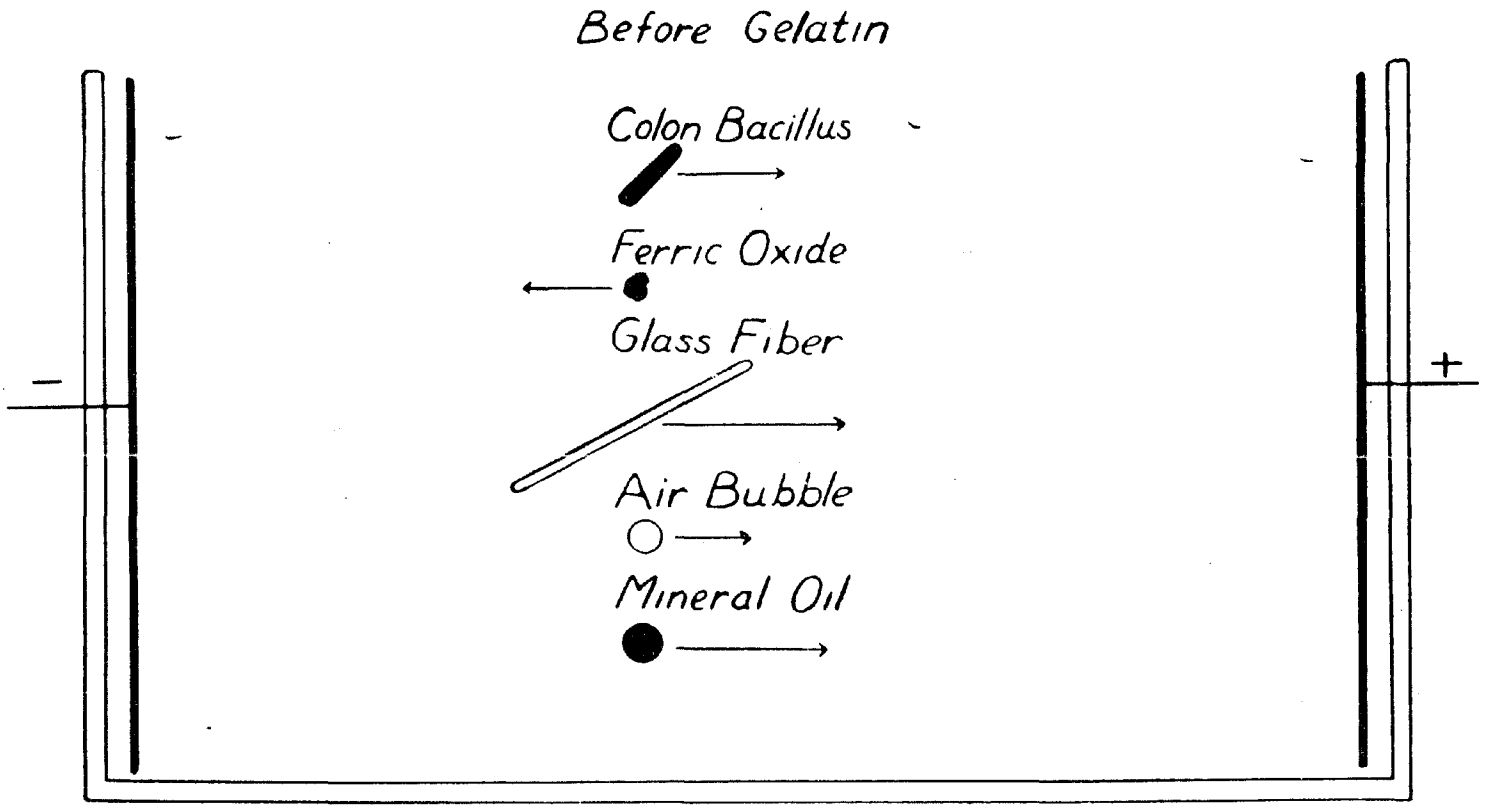


Figure 1. The arrows indicate the direction and approximate velocity of particles of different chemical constitution, size, shape, and orientation in an electric field. (Abramson, Moyer, and Gorin,1)

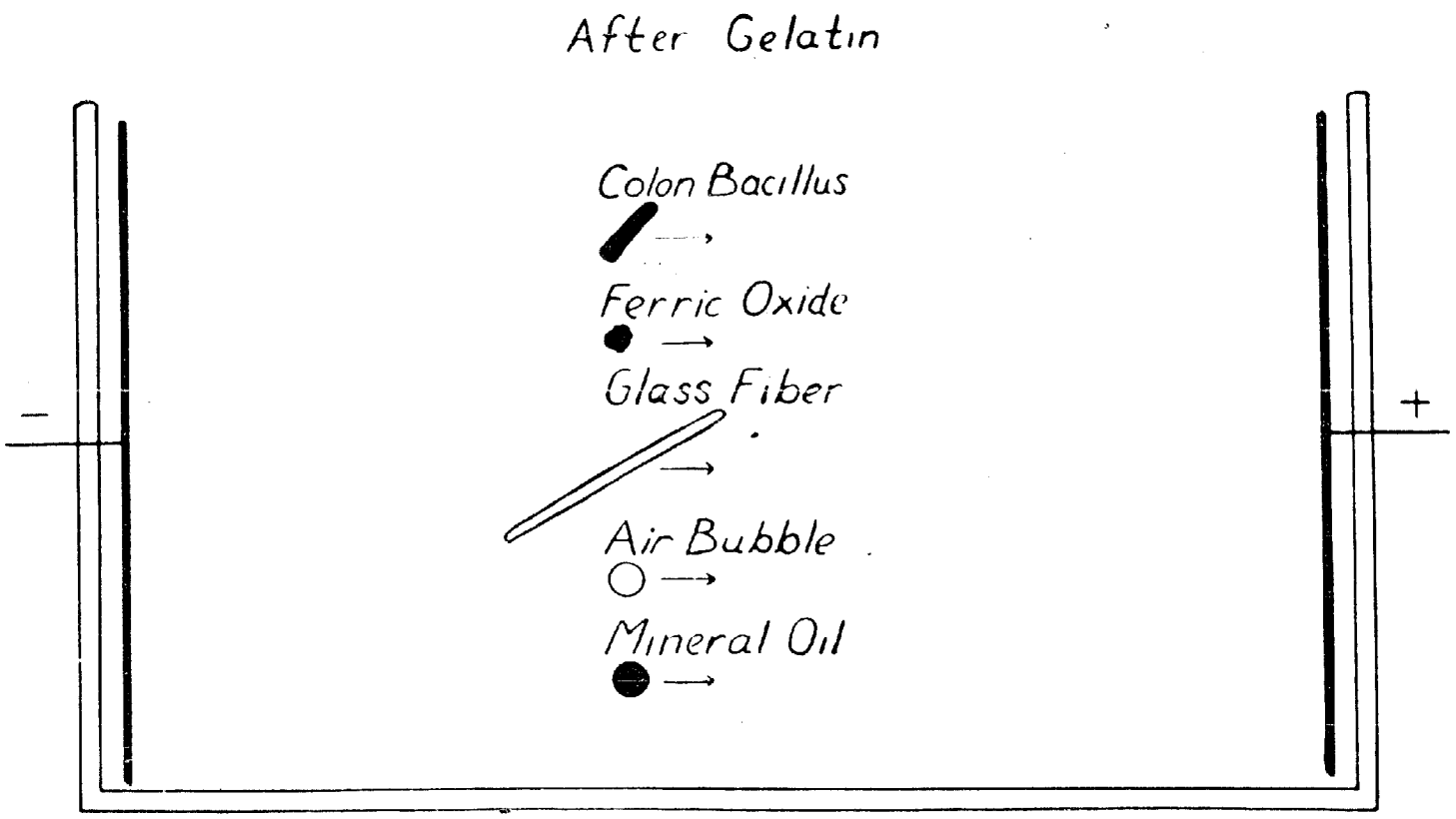


Figure 2. After addition of gelatin the direction and approximate velocity of particles of different chemical constitution, size, shape, and orientation in an electric field. (Abramson, Moyer, and Gorin,1)

" (3) High magnification may be employed, resulting in great sensitivity.

" (4) The size, shape, and orientation of the particles under observations are directly observed.

" (5) Measurements of electric mobility may be made not only in fairly concentrated salt solutions (up to about M/5) but also in very dilute solutions.

" (6) The method is the only way in which certain biological systems may be accurately investigated. Thus bacteria, fungi, blood cells, and protein particles are most easily examined in this way.

" (7) There is no difficulty in calculating from the curve, relating electric mobility to depth in the cell, the electroöosmotic mobility of the liquid relative to the wall of the cell itself.

" (8) By means of the vertical cell, the same particle may be observed over a comparatively long period. Changes in the surface of the particle during this time may be followed.

The microscopic method may be employed only with microscopically visible particles thus limiting its range of adaptation. It is not the object of this paper to discuss this method. For the interested reader an excellent review of the literature on this method has been presented by Abramson (1).

The moving boundary method as developed by Tiselius (3) is applicable to dissolved materials. According to Abramson (1) Longsworth has claimed the following advantages for this method.

" (1) It is applicable to a wide variety of high molecular weight substances in solution in both their native and denatured forms;

(2) it is applicable to mixtures of these substances, and when applied to such mixtures yields information as to, (a) the number of electrically separable components in the mixture, (b) the degree of electrical homogeneity of each component, (c) the concentration and (d) the mobility of each component;

(3) moreover, the moving boundary method may be used to separate in a pure state the components of a mixture;

(4) the mobilities of a given material as a function of pH may be studied over the entire aqueous pH scale;

(5) since the method is applicable over a wide range of concentrations of the substance in a given solvent it may be used to study the interaction between the solvent and the substance and between the particles of the substance itself."

It is evident from the advantages of the two methods that they supplant each other. The microscopic method is designed for the study of particles, whereas the moving boundary method is designed essentially for dissolved material.

Many excellent treatises have been presented on the moving boundary technique of studying electrophoretic migration. Many of these papers are of a theoretical nature and although much has been written on the practicle application it is widely scattered throughout

the literature. For this reason it seemed highly desirable to survey the literature and supplement this material with the practicle experience gained in this laboratory with the sole purpose in view of preparing a working manual on the moving boundary technique of electrophoretic analysis. Much of the following material has been drawn from the excellent works of Tiselius and co-workers abroad; Longsworth and the Rockefeller Institute group in this country and from Abramson and co-workers. To the seasoned worker in the electrophoresis field this treatise will seem rather elemental but it is hoped that newcomers in the field may find much fundamental and practica/ help on the subject as it is treated in this thesis.

HISTORICAL

The Russian physicist Reuss (4) appears to have been the first to observe the phenomenon of electrophoresis. In the year 1807 he constructed an apparatus consisting of two glass tubes driven into a clump of moist clay. In the bottom of each tube was placed a thin layer of sand and the tubes were then partially filled with water. Upon passing a current from a voltaic pile through the apparatus he noted that the water surrounding the positive pole became cloudy due to the electrophoretic migration of the clay particles, while the water surrounding the negative pole remained clear, but increased in volume, due to electroösmosis.

More complete electroösmosis experiments were reported by Porret (5) in 1816. He divided the inside of a glass jar into two compartments by means of a moistened bladder. One compartment (anode) was filled with water and the other (cathode) was only partially filled. Upon passing a current through the compartments he observed that the liquid moved from the anode to the cathode. After one half hour the levels in the two compartments were equal. He summarizes his results as follows: "I think that by the above experiment I have demonstrated the existance of a power not before noticed in the voltaic current, namely, that of conveying fluids through minute pores not otherwise pervious to them, and of overcoming the force of gravity."

A similar experiment using muscle strips as membranes was performed by Kuhne (6). He too reported an increase in the volume of water at the cathode accompanied by a decrease in volume at the anode.

Stern reports (7) that the first quantitative electrophoresis experiments were undertaken by Wiedemann in 1856.

Using the microscopic method Quincke (8) studied the migration of microscopic particles of many types. All particles investigated bore a negative charge in distilled water but many were positively charged in other liquids. He also conceived the idea of the electrical double layer and showed that the rate of migration of particles in an electric field was a linear function of the potential gradient.

Jürgensen (9) in 1860 reported on some results of experiments by Heidenhain. It was found that the passage of a current through cells of "Vallisneria" caused a migration of chlorophyll particles and other ill defined bodies towards the anode. This migration could be reversed. Jürgensen investigated this phenomena in vitro and found that the particles moved towards the anode.

Lodge (10) used the moving boundary technique to follow the electrophoretic migration of colored ions.

Picton and Linder (11) (1892-1897) were the first to study proteins by the electrophoretic method. They utilized a V shaped cell, the forerunner of the U tube to study hemoglobin.

In 1899 Hardy (12) carried out electrophoretic studies on denatured egg albumin particles. He observed the behaviour of the particles under three different conditions with respect to the acidity of the solution. "When the fluid has an alkaline reaction the effect of the passage of a constant current is the formation of an opaque white coagulum about the anode.

"When the fluid has an acid reaction the movements of the proteid particles and of the water are the reverse of those described.

"Fluid is neutral...There is now so little movement of the particles under the influence of a current that it is difficult to detect,

and what movement there is, is due to the fact that the material is not absolutely neutral."

Hardy in 1905 (13) used the "boundary" method for studying the migration of globulin suspensions.

Pauli (14) found that dissolved salt free serum albumin behaved in the same manner as the particles studied by Hardy.

Michaelis and associates (15) made use of the variation in the rate and direction of migration with changing hydrogen ion concentration to determine the isoelectric points of various proteins. They found that for each amphoteric substance there was a definite pH value, or zone of pH values, corresponding to the absence of migration.

The earlier work on the electrophoresis of proteins is reviewed by Pauli and Valko (16), Tiselius (3), and Prausnitz and Reitstotter (17).

Svedberg and Jette (18) were the first to report a direct observation of the electrophoretic migration of a soluble, colorless, protein boundary. In their novel method the green fluorescence emitted by the protein when irradiated with long wave ultraviolet light was utilized. The migration was followed by photographing the fluorescing material. No quantitative results were reported at this time. One of their origional observations is shown in Figure 3.

In a later paper Scott and Svedberg (19) described quantitative results by the fluorescence method on the mobility of egg albumin in acetate and phosphate buffers of different hydrogen ion concentrations. Figure 4 illustrates the results of one experiment.

Several disadvantages were inherent in the fluorescent method, namely, (1) a long period of exposure was necessary in photographing the

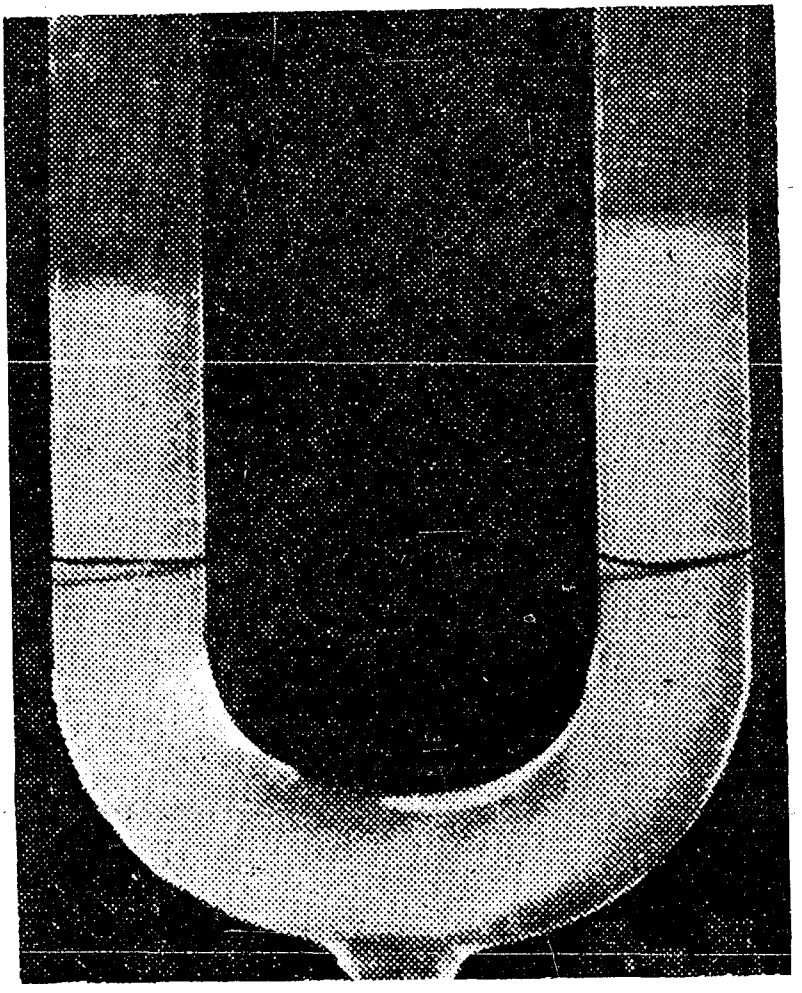


Figure 3. One of the earlier direct observations of the migration of colorless protein boundaries. Photograph made by irradiating the protein solution and photographing the resulting green fluorescence. (Svedberg and Jette, 18)

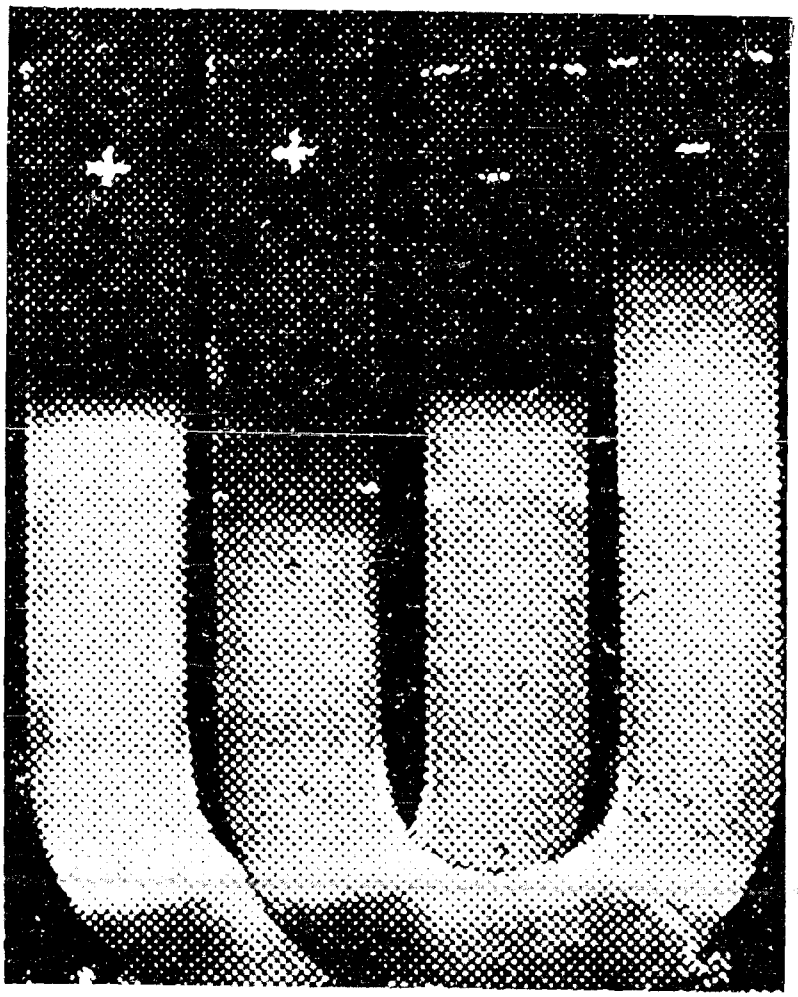


Figure 4. Photograph of the migrating boundaries of egg albumin solutions by the fluorescent method.
(Scott and Svedberg, 19)

boundary, (2) the U tube could not be immersed in a water bath, (3) contamination by other fluorescing substances was not uncommon. However this method was the basis of work which followed and revolutionized electrophoresis studies.

Svedberg and Tiselius (20) attempted to improve the method of optical observation of the moving boundary. They made use of the fact that protein solutions absorb rather strongly, radiation of wave lengths below 300 mμ. This method has been used previously by Svedberg and others to study the sedimentation of proteins in the ultracentrifuge. The general arrangement of the apparatus employed by them is shown in Figure 5. Figure 6 shows the type of electrophoresis cell used. The portion of the U tube in which the migration of the protein solution is recorded by ultra-violet photography consists of fused bubble-free quartz. Figure 7 shows the results of an experiment by this method.

Apparatus for preparative as well as analytical purposes was designed by Theorell (21). A number of successful applications with this apparatus have been made in the biochemical field. Of notable importance was the purification of the yellow oxidation enzyme of Warburg by Theorell. Stern (7) has reviewed these successful applications. An improved preparative apparatus of Theorell (22) is shown in Figure 8.

A new improved apparatus employing the "Schlieren" method of optical analysis was introduced by Tiselius in 1937 (23) (24). This new method of optical analysis using visible light was based on a principle used by Toepler (25) for detecting flaws in telescope lenses. The application of this method requires only that the refractive index of the solution differ from that of the solvent. The new cell and electrode assembly are shown in Figure 9.

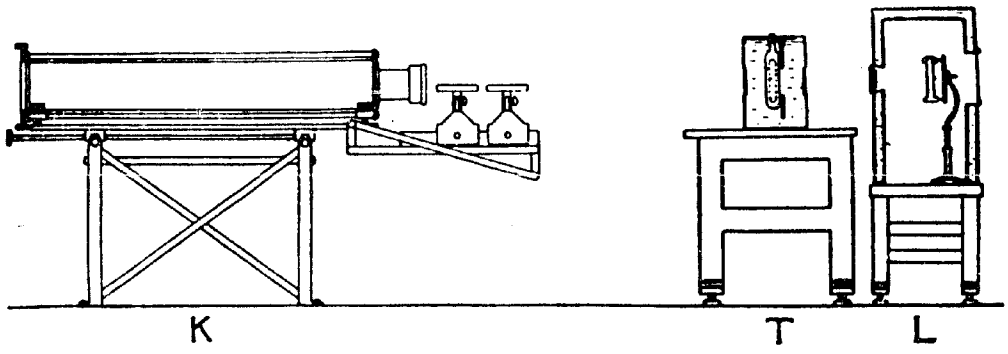


Figure 5. Tiselius electrophoresis apparatus for light absorption method. L, lamphouse, double walled and water cooled, containing as a light source a quartz mercury lamp run from storage batteries. In front of the housing is a screen of ground, fused quartz. T, water thermostat equipped with plane parallel windows of bubble-free fused quartz. The tank contains the electrophoresis cell(Figure 6). K, camera equipped with quartz lens and a combination of chlorine and bromine filters transmitting only radiations below 2800A°. (Svedberg and Tiselius,20)

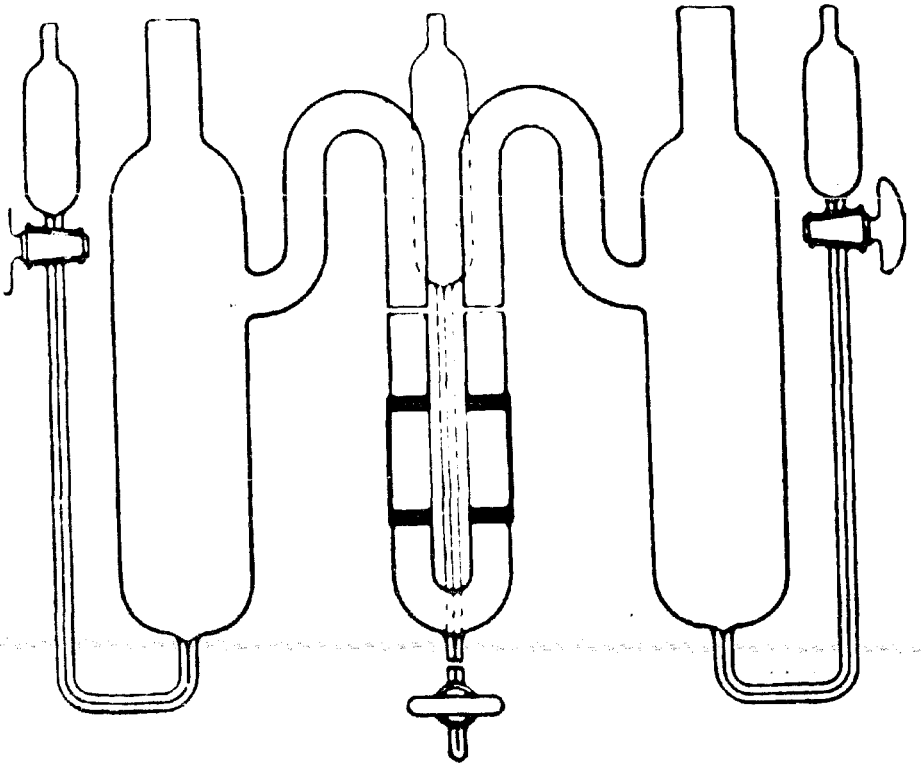


Figure 6. Electrophoresis cell according to Tiselius. The portion of the U tube in dark line is bubble free fused quartz. The two cylindrical limbs are ground with precision to yield a uniform cross section. The bottom of the U tube is connected through a capillary and stopcock with the storage vessel for the protein solution. The stopcocks at either side allow concentrated potassium chloride to be run around the electrodes in the electrode vessels. (Svedberg and Tiselius, 20)

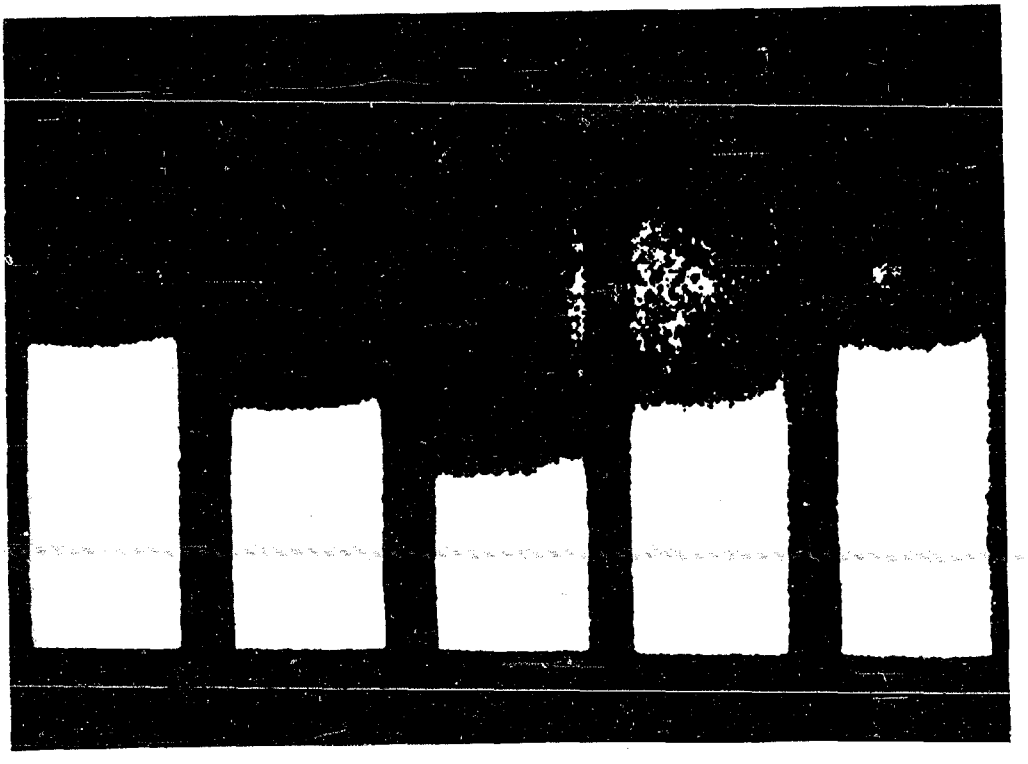


Figure 7. Electrophoresis of C-phycocyan, a chromoproteid from algae, by light absorption method. (Svedberg and Tiselius, 20)

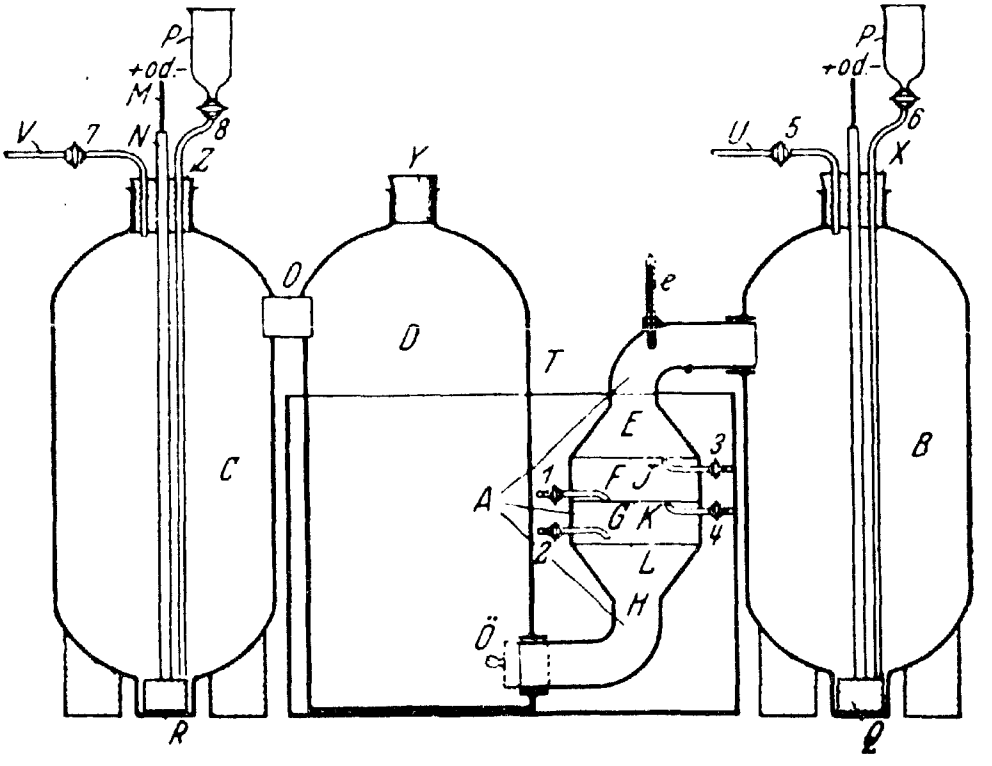


Figure 8. Preparative apparatus of Theorell's. A. Separation chamber; B and C electrode vessels (10 liter); D. Intermediary Chamber (10 liters); E and H communication pieces; F and G glass cylinders; J. K. and L. Membranes, two of which J and L are impermeable for the colloid to be purified, while K is permeable for it; 1, 2, 3, and 4, tops for admitting and withdrawing fluids; P storage vessels for saturated potassium chloride solution; T. water bath equipped with glass windows. (Theorell, 22)

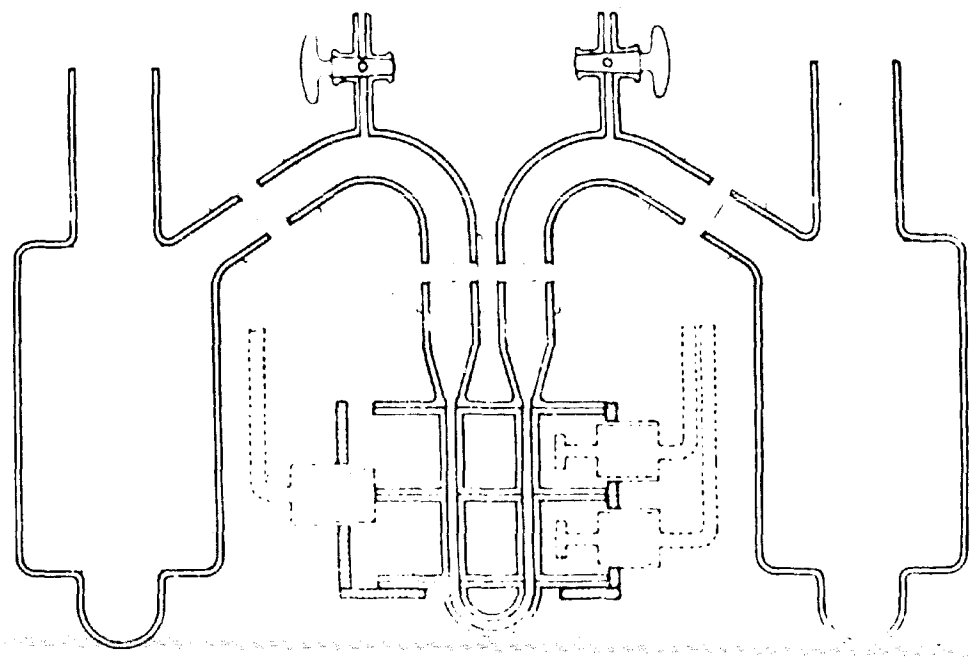


Figure 9. Cells and electrode vessels for electrophoretic analyses. E_1, E_2 represent the electrode tubes, T rubber tubings; I, II, III, IV, the four U tube sections of which II and III can be moved with the pneumatic arrangement P_1, P_2 and P_3. (Tiselius 23)

The glass U tube portion is divided into three separate compartments and is connected with rubber tubing to the electrode vessels. The glass sections are connected together with vaseline and are held in place under slight spring pressure. The sections are moved by a pneumatic arrangement (dotted lines) and can be used for separation studies. Silver-silver chloride electrodes were immersed in a buffer solution in the large electrode vessels and surrounded by a layer of saturated potassium chloride solution. A typical pattern obtained by using this technique is shown in Figure 10.

The advantages of this new apparatus are (26); (1) the reduction of the disturbing effects of heat convection in the electrophoresis tube by working at a temperature in the neighborhood of +4°C; (2) conventional optical observation of the migrating boundaries by the Toepler-Schlieren method or similiar methods depending upon the refractive index; (3) an improved method for forming the boundary and dividing the contents of the electrophoresis tube into portions for analysis; (4) a compensation arrangement for preventing the boundaries from migrating out of the tube in prolonged experiments, particularly valuable for separation studies.

Since this improved apparatus and its modifications are to be the subject of this paper no further details will be discussed at this time.

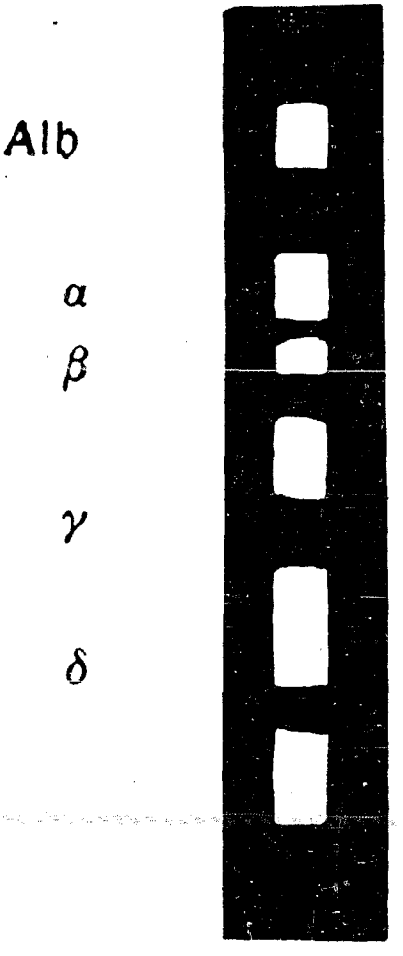


Figure 10. The migrating boundaries of normal horse serum, schlieren method. (Tiselius, 24)

APPARATUS

The purpose of this paper is to discuss apparatus as well as technique. In conjunction with the discussion of the apparatus as a whole some detail will be given as to the individual parts, the general arrangement and construction.

Support

The electrophoresis apparatus is supported by three concrete bases, A, B and A shown in Figure 11. The specifications of the B block are shown in Figure 12 and those of the A blocks in Figure 13. The bases extend into the soil below the floor and the sides are insulated from the floor by means of asphalt. Two steel channel irons (5" by 19 1/2') spaced 8" apart by means of steel plates, Figure 11 serve as the optical bench. They are anchored to the A blocks by means of threaded studs and rest in the channel of the B block. Figure 14 illustrates the manner in which the various optical elements to be described later are attached to the optical bench. This arrangement using standard structural steel shapes provides for flexibility of adjustment.

The electrophoresis apparatus on the optical bench consists of three main parts, (1) a light source, (2) thermostat, (3) and camera.

Light Source

The light source L, Figure 11 is a General Electric Mercury vapor lamp type H4. Placed in a horizontal position in the lamp housing behind the slit it serves as an adequate source of illumination. The curvature of the glass bulb eliminates a condensing lens (27). The details of the lamp housing and slit are shown in Figure 15. The position of the whole fixture may be adjusted by means of the slots in the angle iron.

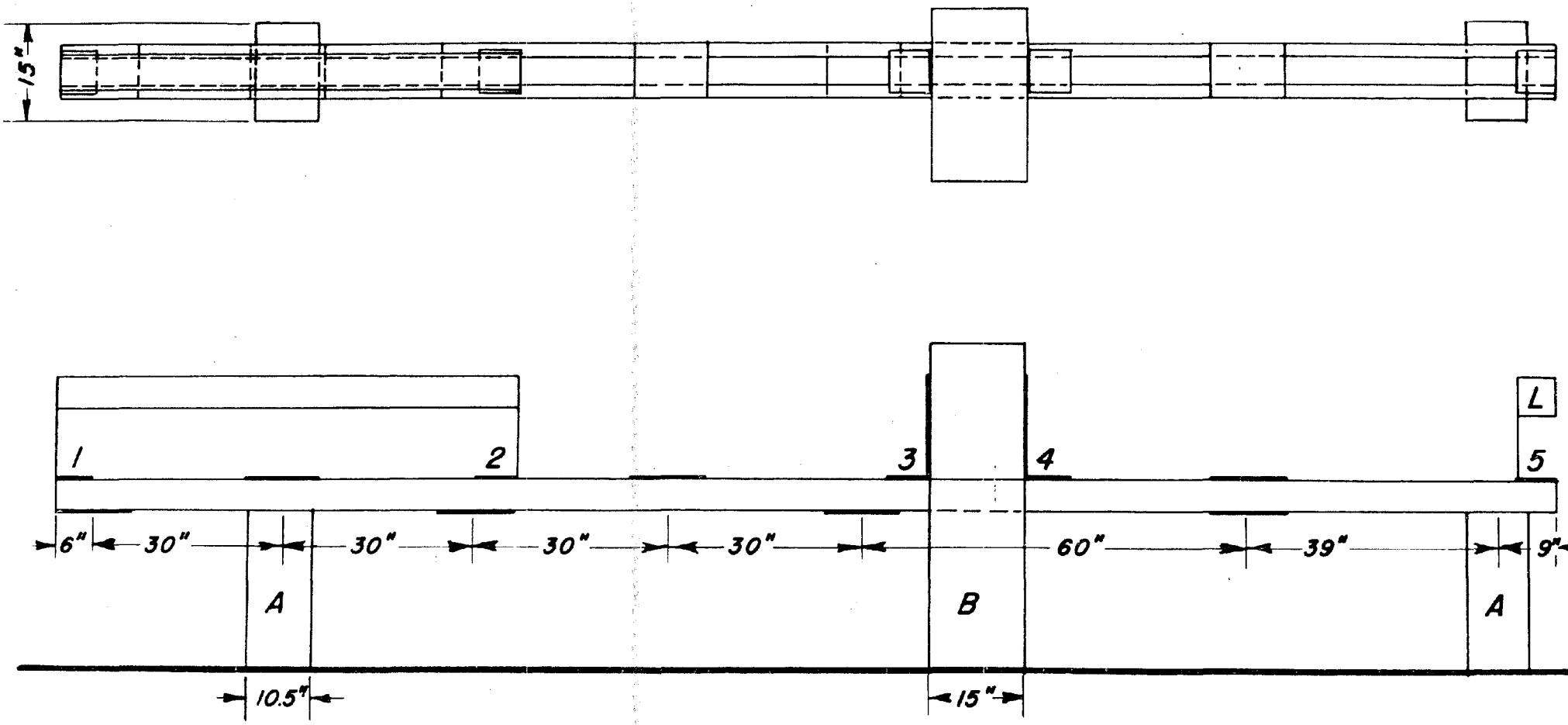


Figure 11

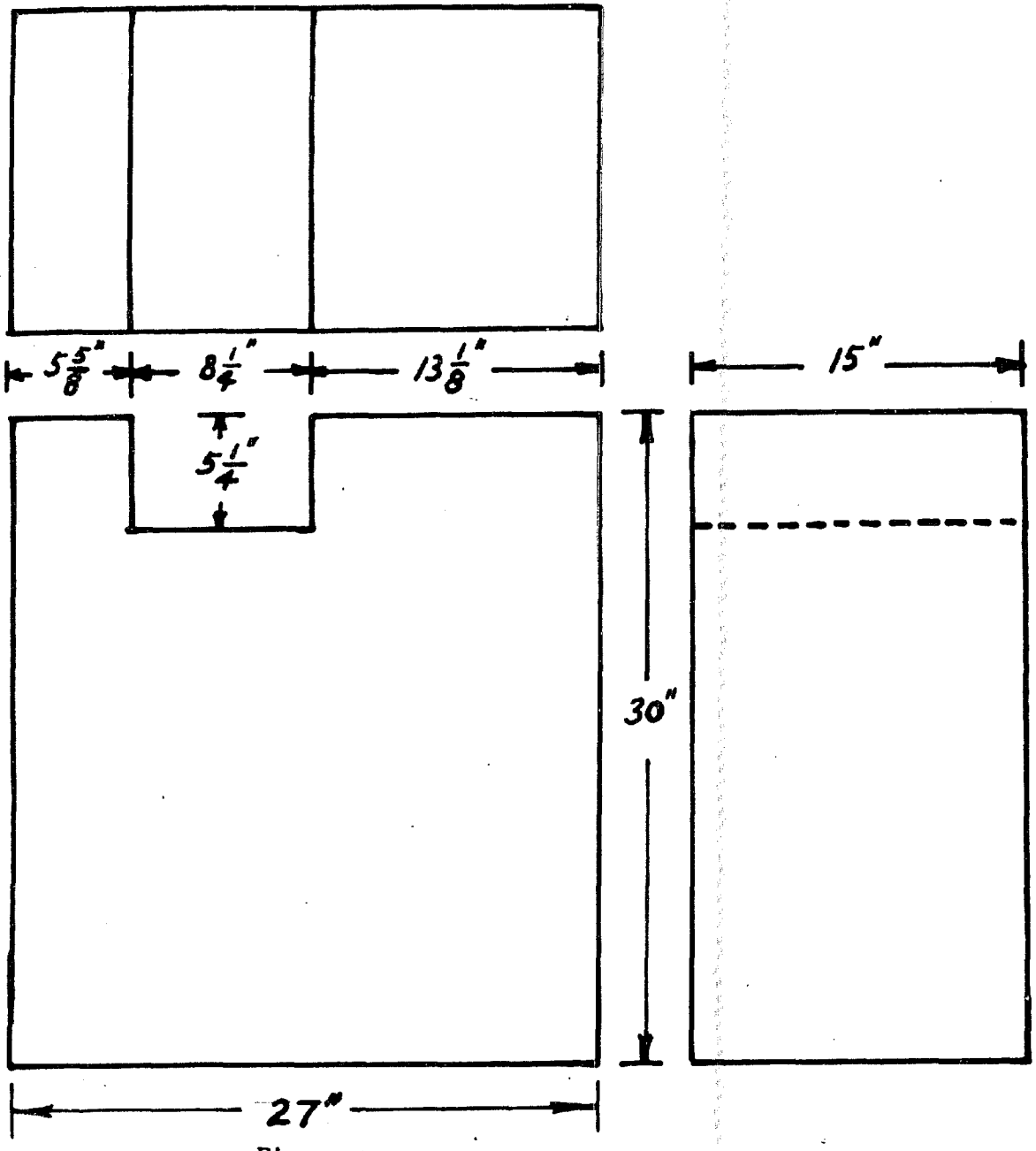


Figure 12

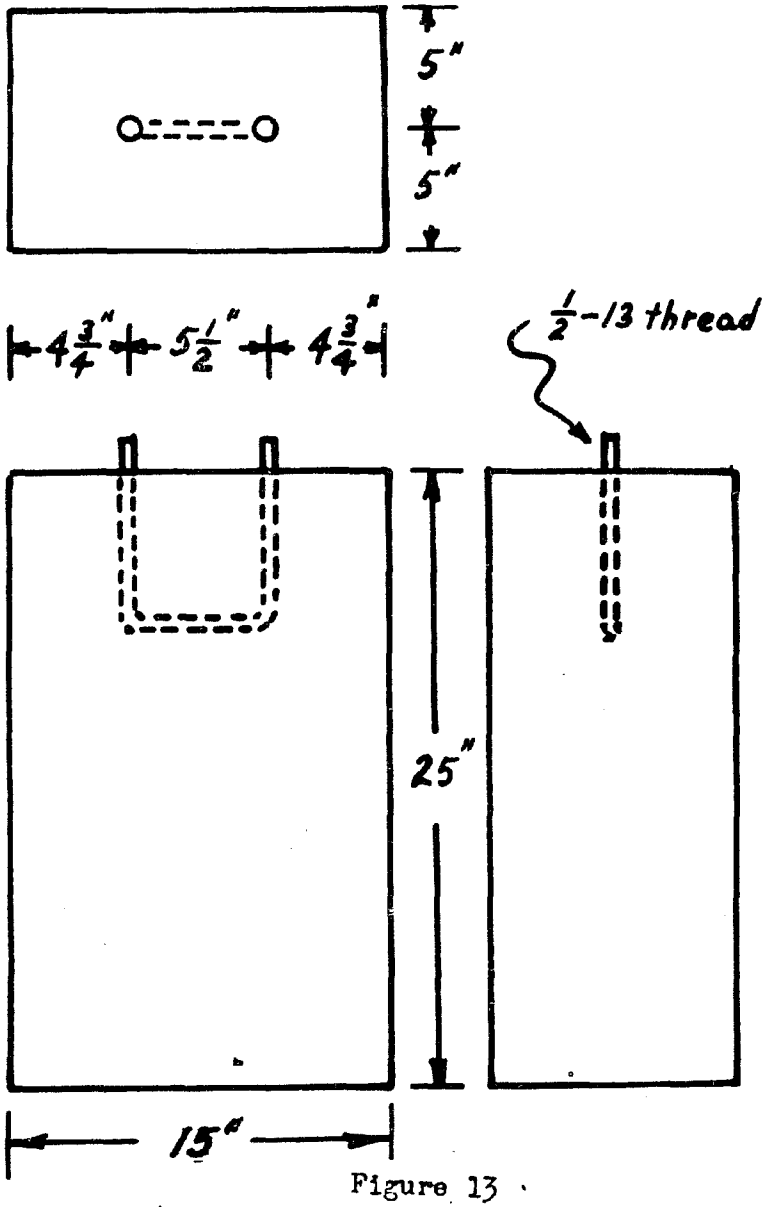


Figure 13

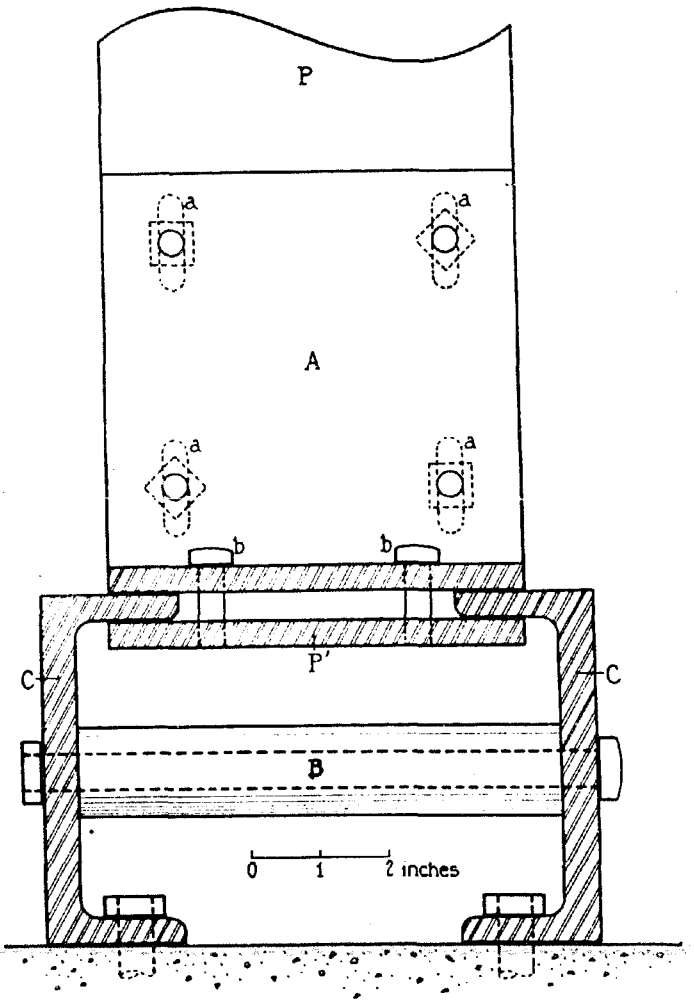


Figure 14. Diagram showing method of attaching optical elements to the optical bench. The steel angle iron, A, is clamped to the upper flanges of the channel, C, with the aid of the plate, P', and the bolts b. The lens or other optical element is carried by a steel plate, P, which is bolted to the angle through slots as shown at a. The iron pipe, B, is used to space the channel irons 8" apart. (Longsworth, 27)

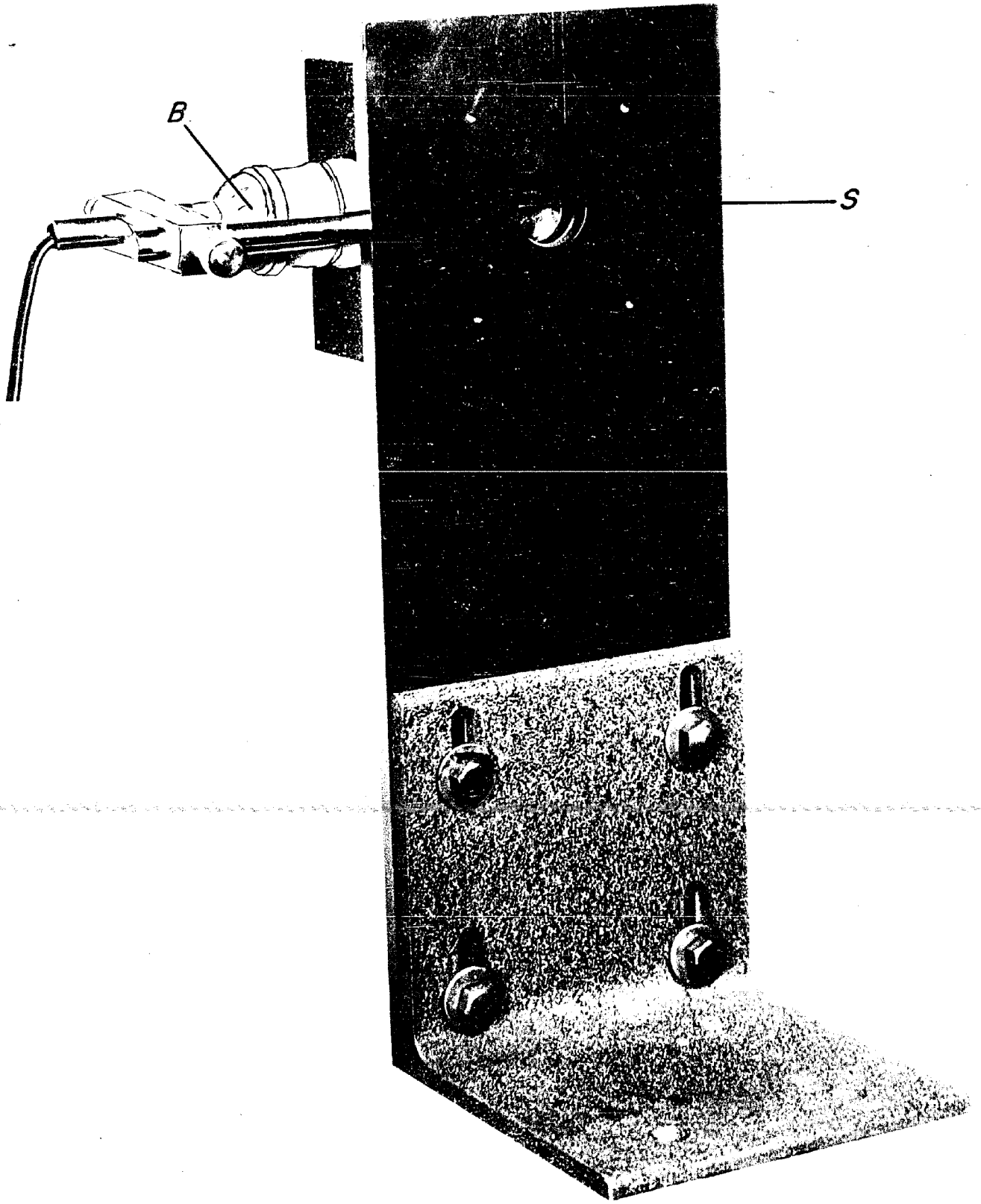


Figure 15. Light source and housing, showing adjustable horizontal slit S, and lamp socket attachment B. (Courtesy Klett Mfg. Co.)

(The H4 type lamp requires a General Electric auto transformer #59G22 with 110 volt AC current.) The vertical dimension of the slit is adjustable by screws. A length of 25 mm. and a width of .5 mm have been used satisfactorily. The width is measured by means of a feeler gauge when the lamp is hot. This has been found necessary as the slit width contracts as the lamp heats up.

In work with cloudy or colored solutions the light waves of a H4 mercury vapor lamp may be absorbed by the solution. Under such conditions a 300 watt tungsten filament lamp and condensor (Spencer Lens Co. Model 367) have been found to furnish satisfactory lighting for most samples of this type. If solutions show a marked turbidity light from a carbon arc lamp (Bausch and Lomb Mechanical feed arc lamp) furnishing light in the red spectrum will often penetrate through the solution and permit a scanning photograph to be made.

Improved patterns are obtained by filtering the light and permitting only a single wave length to pass through the solution. For blood plasma and serum samples a "Wratten #22" filter is satisfactory. With the mercury vapor lamp this permits only the light of 577 and 579 millimicrons through. In addition to such filters, smoked glass filters are often necessary to reduce the intensity of the light reaching the photographic plate. Over exposure of the plate causes a blurring of the edges of the patterns.

Thermostat

A cross section view of the water bath is shown in Figure 16. A 24 gauge tinned copper tank, 20" deep, 24" long, and 12" wide is supported by a wooden box lined with celotex. The horizontal flange on the top of the tank is covered with strips of black linen bakelite.

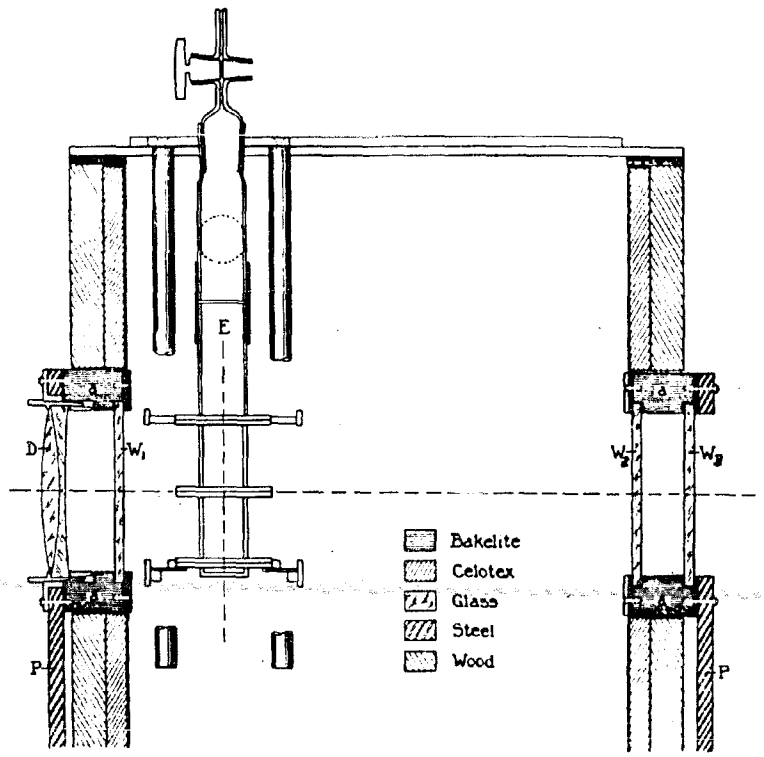


Figure 16. Cross section diagram of water bath with cell, E, in place. D, schlieren lens, W_1, W_2, W_3 windows of optical glass. (Longsworth, 27)

The Schlieren lens, D, Figure 16, forms one of the windows. It is a 4" achromatic lens of 36" focal length. (Perkin, Elmer and Moffit). The chromatic correction is for green and blue. The double window is necessary to prevent moisture condensation in the schlieren lens. The windows W_1, W_2 and W_3 are 4 1/2 x 1/4" discs of optical plate glass. The schlieren lens and glass windows are clamped to the bakelite ring. Moisture in the space between the windows is removed by circulating dry air drawn through calcium chloride. (A portable General Electric vacuum pump #5KH43AB125 is adequate for this purpose). Figure 17 shows the outward appearance of the water bath with the schlieren lens in view. This same figure also shows the stirring motor and standard and the synchronous motor and syringe used to displace the boundaries. It may be remembered from Figure 11 that the water bath rests on the concrete pier B, and is not supported by the optical bench or motor support as might be inferred from Figure 17.

The synchronous motor with syringe attached is shown in more detail in Figure 18. When the motor is in gear and the current on, the plunger is pushed into the syringe through a set of gears and a threaded shaft. The motor is attached in such a manner that its position may be readily inverted and the plunger caused to withdraw from the syringe. The speed of the plunger may be varied by changing the gear ratio between the motor and the driving shaft. The auxillary gears are shown on a spindle in Figure 18.

The water bath is circulated and cooled by the power stirrer and refrigeration coil shown in Figure 19. A mercury thermostat Figure 20 controls the electron tube relay switch, Figure 21, which in turn regulates the power to the refrigeration unit. A one quarter horsepower

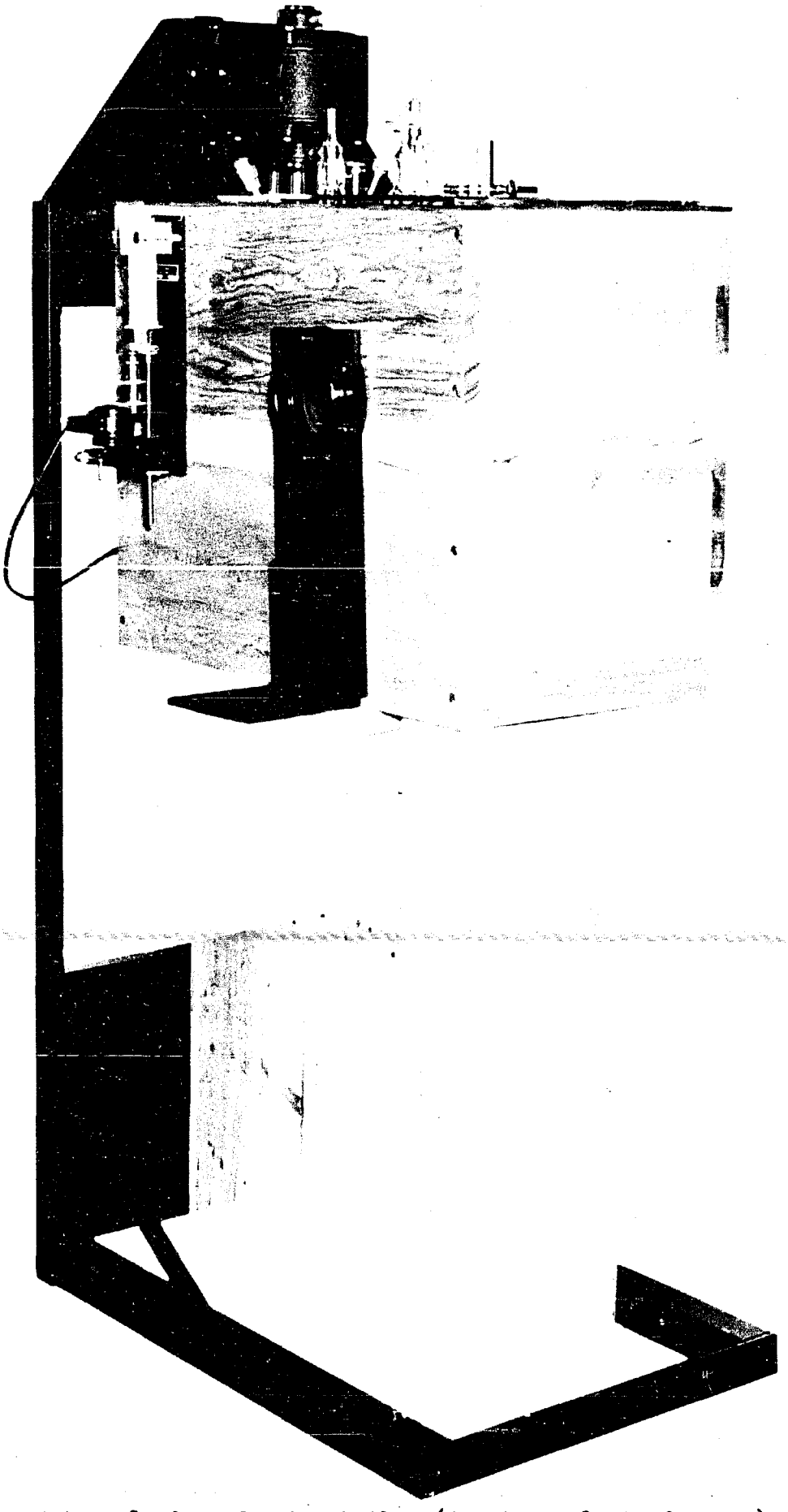


Figure 17. External view of water bath. (Courtesy Klett Mfg. Co.)

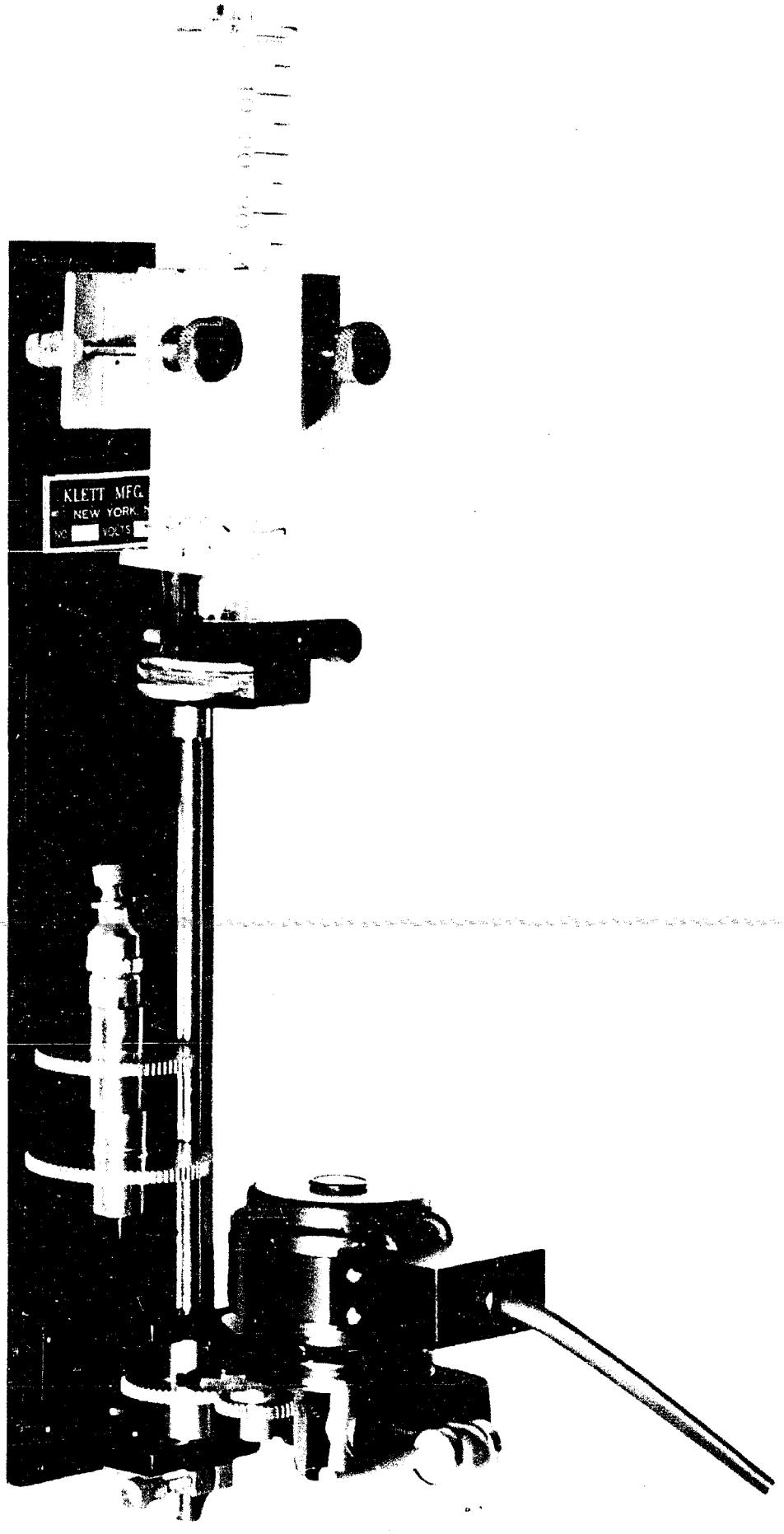


Figure 18. Motor and syringe attachment for displacing boundaries. (Courtesy Klett Mfg. Co.)

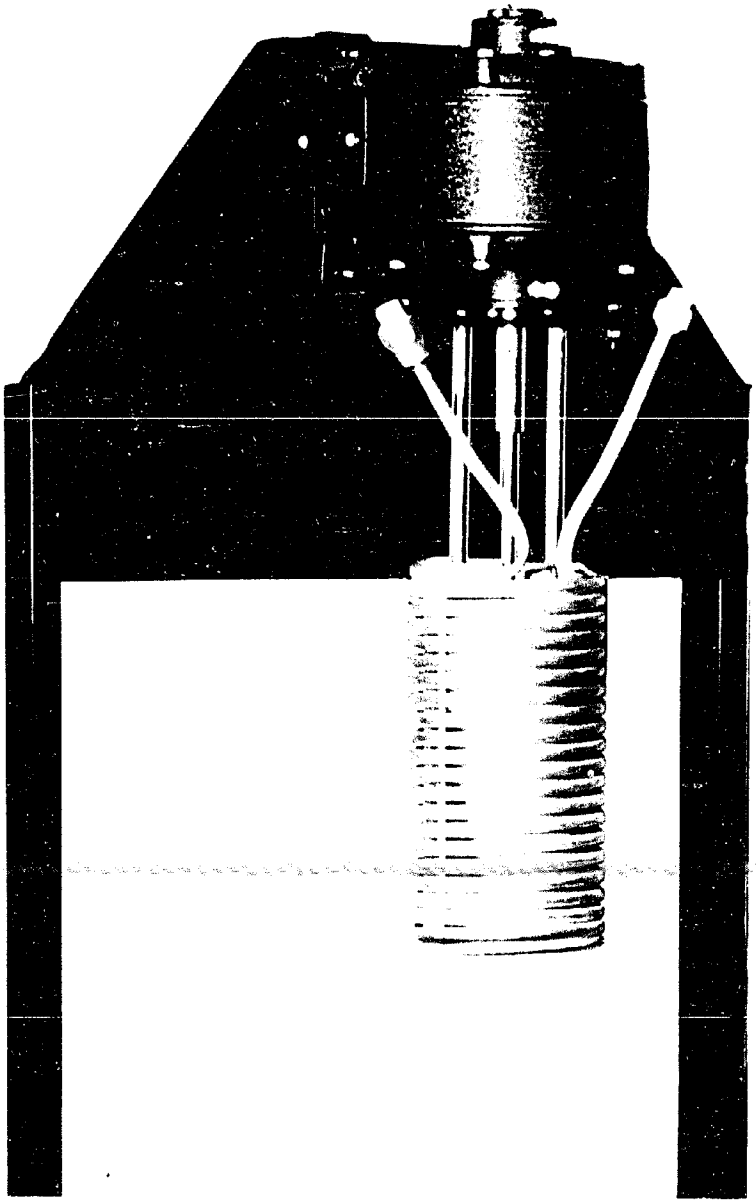


Figure 19. Water bath refrigeration coil and stirring motor. (Courtesy Klett Mfg. Co.)

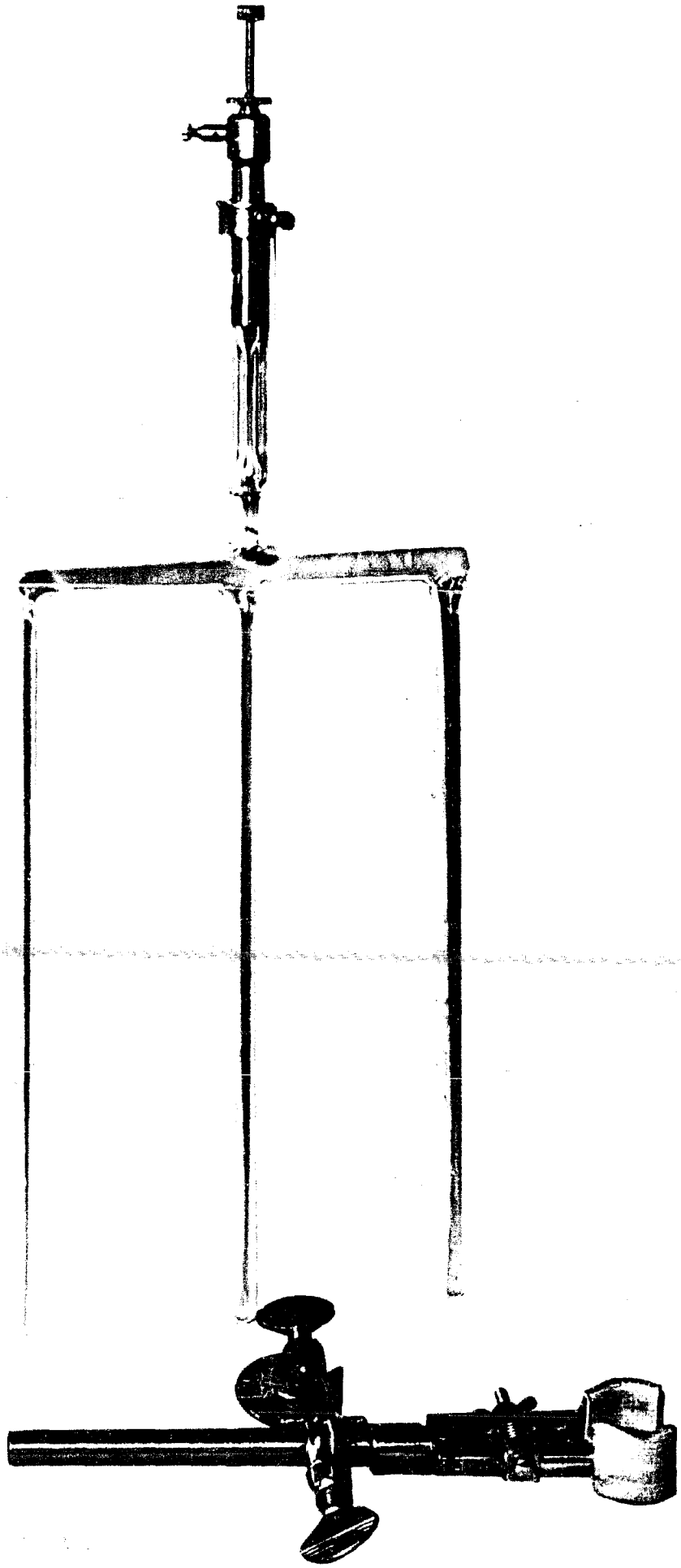


Figure 20. Mercury thermostat for controlling water bath temperature. (Courtesy Klett Mfg. Co.)

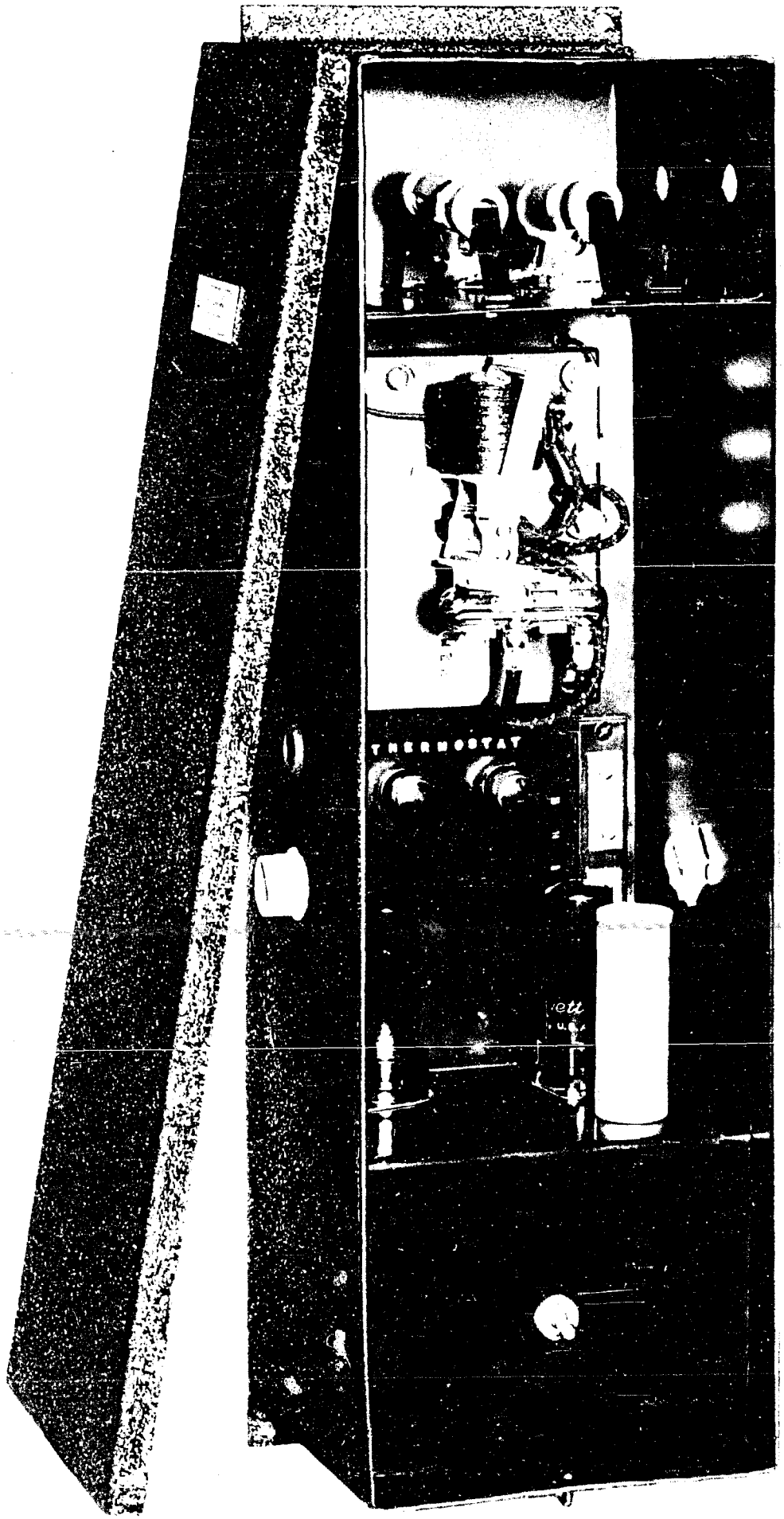


Figure 21. Electron tube relay switch for controlling refrigeration units. (Courtesy Klett Mfg. Co.)

unit has the necessary capacity (Frigidaire-Model AF025). Since the box remains at a low temperature at all times a provision has been made to protect the stirring motor in case the bath freezes up. A 4 amp. fuse is placed in series with the stirring motor. A second thermostat (American Instrument Co. "Quickset") controls a buzzer that rings when the temperature rises above or below the desired setting.

Camera

The camera barrel is shown in Figure 22. The end of the camera bellows facing the water bath supports the camera objective and the schlieren diaphragm Figure 23. The camera objective, O, is a 2" achromat lens of 36" focal length. The circular disc, M, with slots of varying width cut along radii of the disc, is the camera diaphragm. This diaphragm serves to control the amount of light reaching the lens and thus improves its resolving power. It may be rotated manually and once the proper slot has been placed in front of the camera lens no further adjustment is necessary. The width of the slot employed will depend upon the light used, the filter, and the type of photographic plate.

The camera shutter, C, is shown in place behind the camera diaphragm in Figure 23. It is a circular disc with a 2" hole in one quadrant and a sector shaped opening in the opposite quadrant. It is not used in making Schlieren scanning photographs, in which case the disc is set so that the circular opening is directly in front of the lens and is left in this position. It is used, however, in photographing Schlierin bands and opaque boundaries that are occasionally encountered with very turbid suspensions. In the latter case the disc is rotated by means of the electric motor, E, with the plate and plate holder in position.

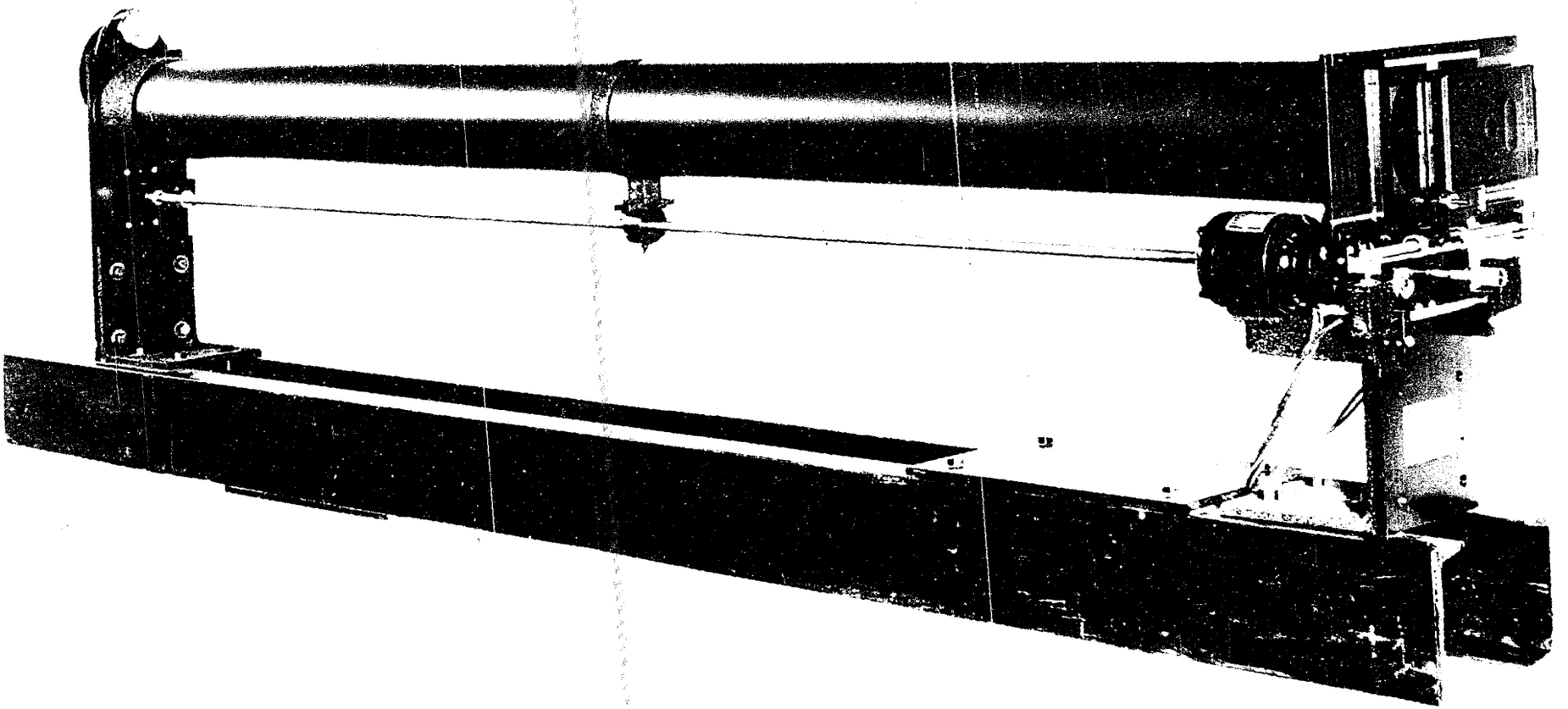


Figure 22. Camera barrel and schlieren scanning attachment.
(Courtesy Klett Mfg. Co.)

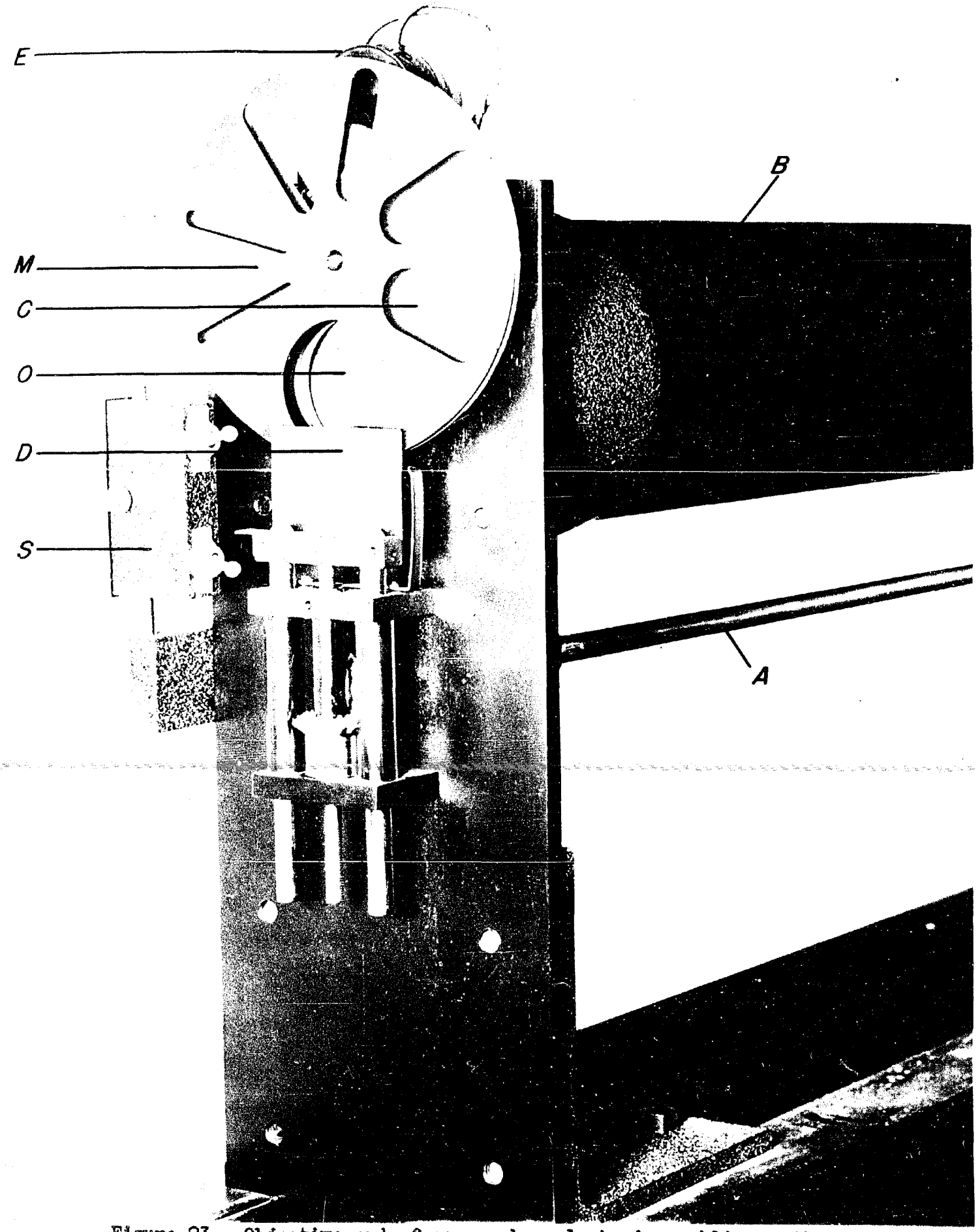


Figure 23. Objective end of camera barrel showing schlieren diaphragm D, camera mask M, and camera shutter C. Description in text. (Courtesy Klett Mfg. Co.)

As soon as the circular opening has rotated from in front of the lens the dark slide is withdrawn from the plate holder and left out until the sector shaped opening in the disc has passed in front of the lens, thus making the exposure, after which the dark slide is inserted before the circular opening again moves across the lens. The exposure can be controlled by loosening the nut holding the two leaves of the disc together and moving the two with respect to each other, thereby adjusting the sector opening. The schlieren diaphragm, D, Figure 23, is moved synchronously with the plate carriage, C, Figure 24, by means of a Bodine gear reduction motor (110 V - 60 cycle - Cat. No. B2246 - 72 R. H. horizontal position) through the shaft, A, Figure 23. The shaft is equipped with a micrometer, D, Figure 24, graduated to 0.01 mm. which records the distance traveled by the schlieren diaphragm. The distance traveled by the plate carriage is read from a millimeter scale, V, Figure 24, directly above it. The ratio of distance traveled by the schlieren diaphragm to that of the plate holder can be calculated from these readings. The ratio can be changed by changing the ratio of the gears shown at G in Figure 24. The plate carriage is locked to the shaft, T, by means of the arm L. The clutch, K, serves to engage the motor, M, with the driveshaft, A. Safety switches, S, are shown in Figures 23 and 24.

The camera mask (E, Figure 24) which controls the area of light striking the photographic plate is shown in more detail in Figure 25. The slit width of the mask is variable and can be adjusted by using a feeler gauge. The mask is so constructed that one-half of the vertical length of the cell may be blocked off if necessary. Thus if all of the protein constituents are migrating as anions and there are no boundaries

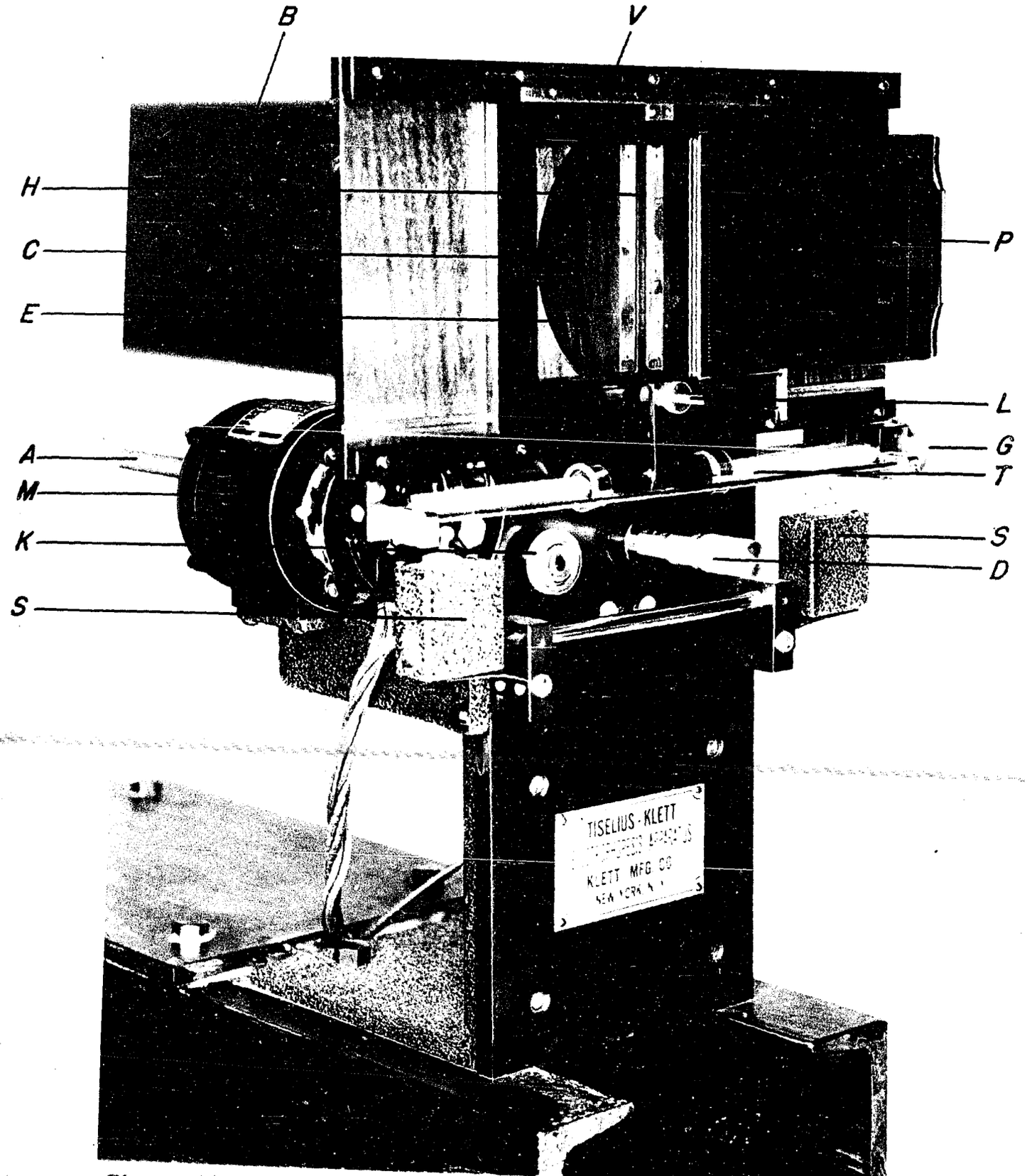
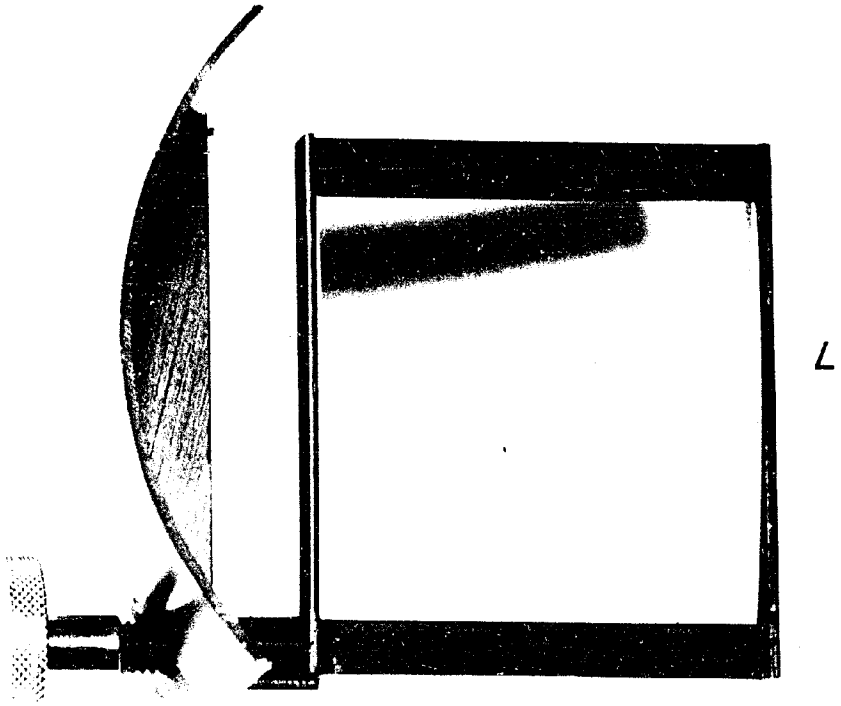


Figure 24. Viewing end of camera barrel and schlieren scanning attachment. Description in text. (Courtesy Klett Mfg. Co.)



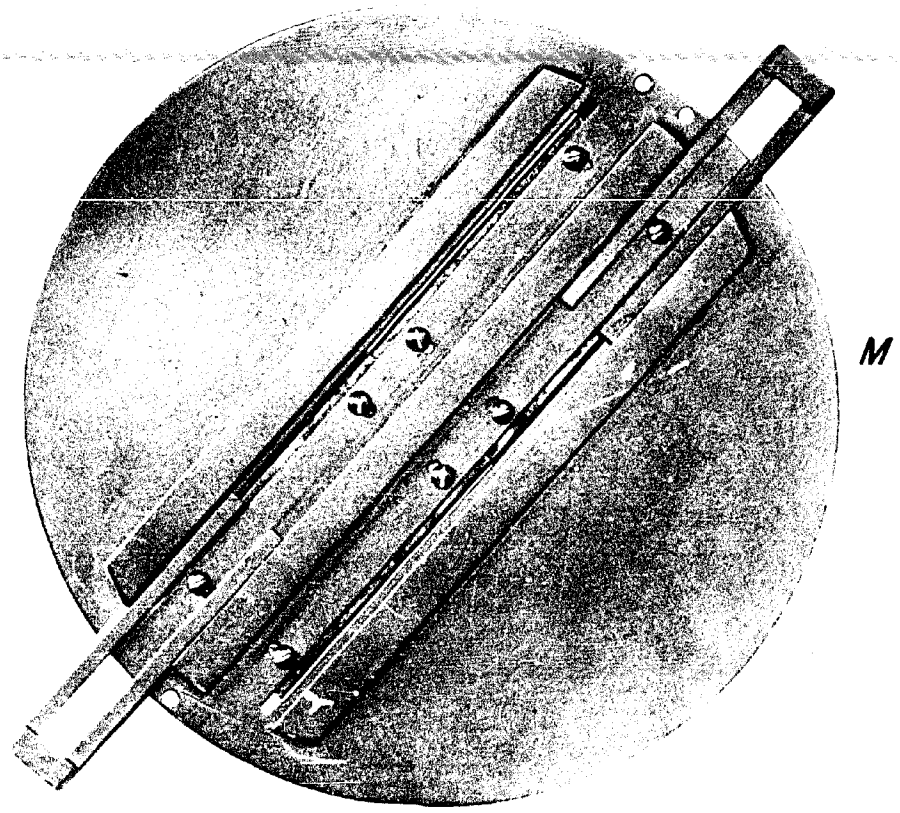


Figure 25. Camera mask, M, and Philpot Svensson cylindrical lens, L. (Courtesy Klett Mfg. Co.)

in the lower anode and upper cathode section, these sections can be masked. The same figure shows the Philpot-Svensson cylindrical lens which is installed in the camera bellows (22" from the camera objective) and can be turned to intercept the light rays striking the photographic plate.

Electrical control Unit

The instrument panel of the electrical control cabinet is shown in Figure 26. (Hopf Apparatus Co.). The wiring diagram of the instrument panel is shown in Figure 27. The source of current for electrophoresis comes from five 45 volt "B" batteries connected in series. A 10,000 ohm resistance is connected in series to control the voltage. If 110 volt A. C. current from a power line is the source of power, a current rectifier and voltage control unit is necessary (Model V-United Transformer Co).

Current passing through the electrophoresis cell is measured roughly by a milliammeter and accurately by means of a potentiometer. In order to increase the range of the potentiometer two step resistances of 100 and 1000 ohms are connected in series with it. A standard cell is used to standardize the bridge. The other essentials of the control equipment are a voltmeter, a de'Arsnoval type galvonometer and an automatic timing recorder, (reading in seconds).

In practice the potentiometer is first standardized against the standard cell. In carrying out this operation the three-way control switch is thrown to the position marked standard cell. The potentiometer is set at the standard cell rating (1.0185V.), the galvonometer is turned on and the galvonometer damp released. Standardization is accomplished by adjusting the coarse and fine resistances until no galvonometer deflection is evident when the key is depressed.

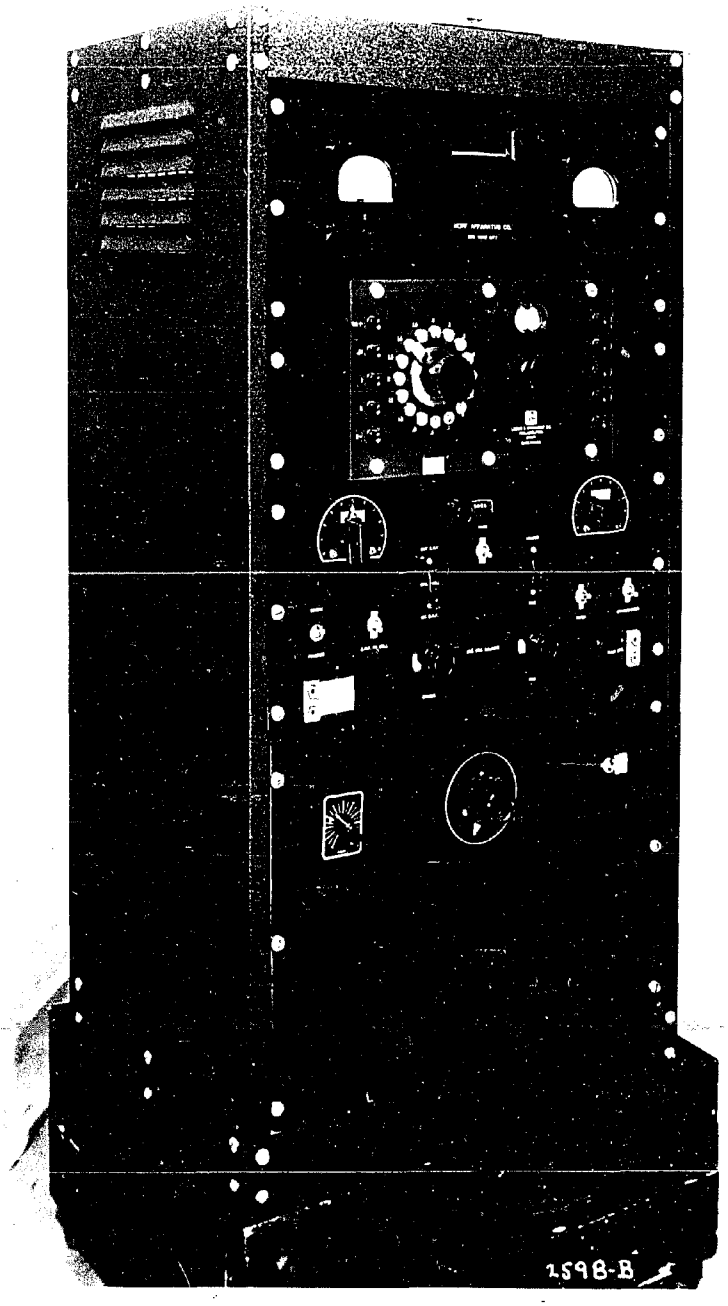


Figure 26. Electrical Control Cabinet.
Description in text.

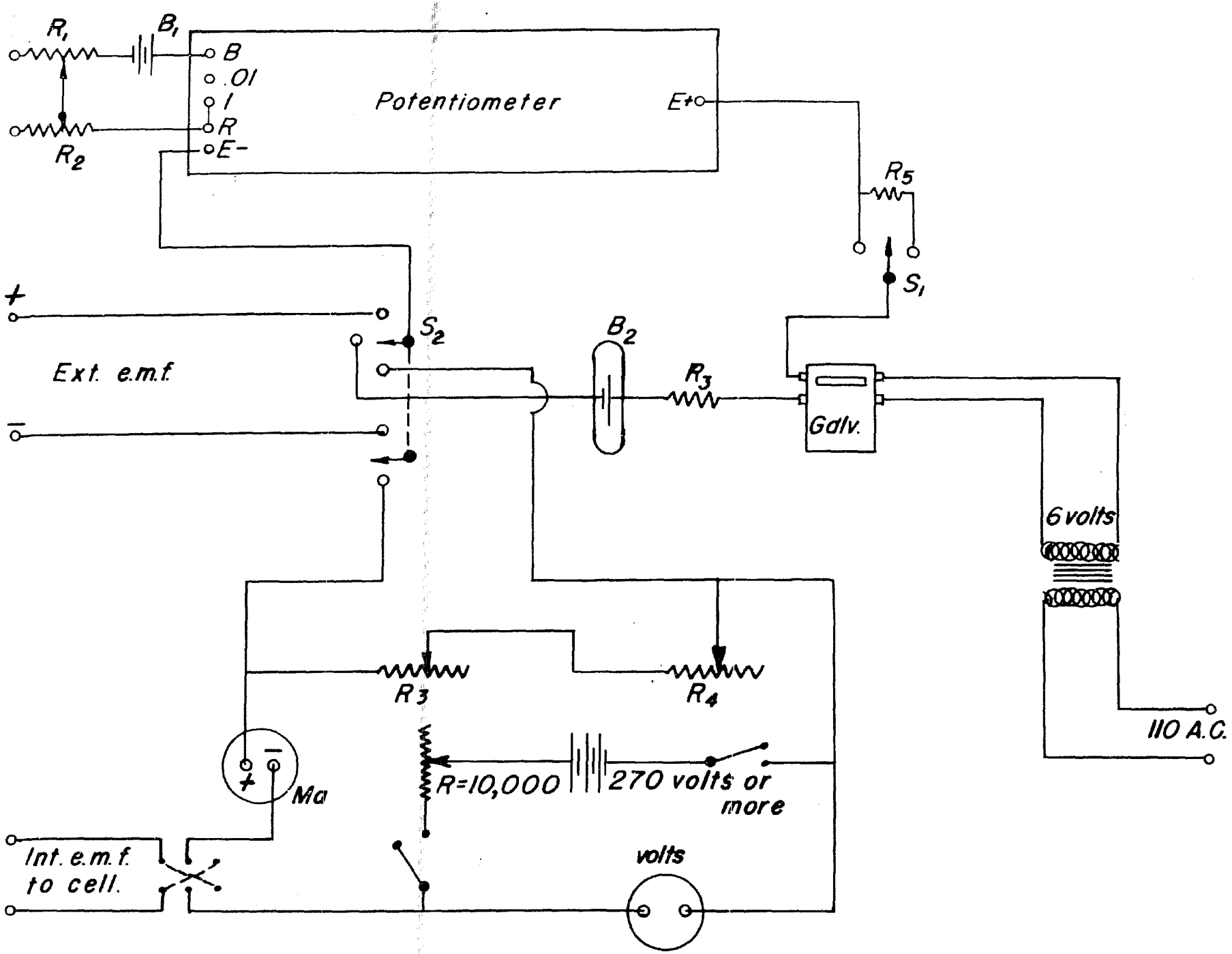

R1
B1
B
.01
1
R
E-
R2
Potentiometer
E+
R5
S1
+
Ext. e.m.f.
−
S2
B2
R3
Galv.
6 volts
110 A.C.
R3
R4
+
−
Ma
R=10,000
270 volts or more
Int. e.m.f. to cell.
volts

Figure 27

An accurate reading of the current flowing through the cell can be made by determining the potential drop across the resistance R_3 R_4 (Figure 27) which is in series with the cell, and applying Ohm's law. This measurement is made on the potentiometer with the three way control switch in the internal E. M. F. position. The value of the resistance to be used can be figured roughly from Ohm's law (E=IR) where E is the E. M. F. that can be measured on the potentiometer (1.6V being the highest) and I is the current reading on the milliammeter. Set shunt R_3 and R_4 to the value figured. For current values under 16 millamperes through the electrophoresis cell, which will cover most operating conditions, a resistance of 100 ohms is convenient. By multiplying the potential reading by 10 the current is read directly in milliamperes.

A jack marked Ext. E. M. F. has been added in case one wishes to make external voltage readings up to 1.6 volts. If this jack is used the shunts R_3 and R_4 are out of the circuit. Therefore, values higher than 1.6 volts cannot be measured. When making such external measurements the control switch should be thrown to the position marked "Ext. E. M. F."

OPTICAL SYSTEMS FOR FOLLOWING AND RECORDING ELECTROPHORETIC SEPARATIONS

Since most protein solutions are colorless the development of the moving boundary technique of electrophoretic analysis followed the development of adequate and simple methods for observing the rate of migration of the boundary. Tiselius (23) (24) used the schlieren or streak method to great advantage (28). Abramson (29) has very clearly explained the principle of this method with the aid of Figure 28. "... light from a distant source is brought to focus by the lens to be tested. A diaphragm is placed so that it just covers the image formed at f, the focal point of the lens. An observer looking through a telescope behind the diaphragm and focusing on the lens observes only the rays which fail to converge to a single point at f. These rays which do not strike the diaphragm come from defective portions of the lens and their position is imaged in the telescope. This procedure (used by Foucault), was reversed by Toepler who by intercepting the deviated rays made the defective portion of the lens appear dark in the telescope. The present method used for detecting concentration gradients in the electrophoresis cell is an adaptation by Tiselius of the Toepler method." If the lens being examined in Figure 28 is replaced by a protein solution and a converging lens (schlieren) placed immediately behind the solution, dark bands will appear at the boundary that have a greater index of refraction than the adjacent solution. It should be recalled that a beam of light is deviated from its origional direction if its velocity changes in passing from one medium into another. If its velocity in the second medium be less than in the first a beam is bent toward the perpendicular, that is the angle of refraction is less than the angle of incidence. When the

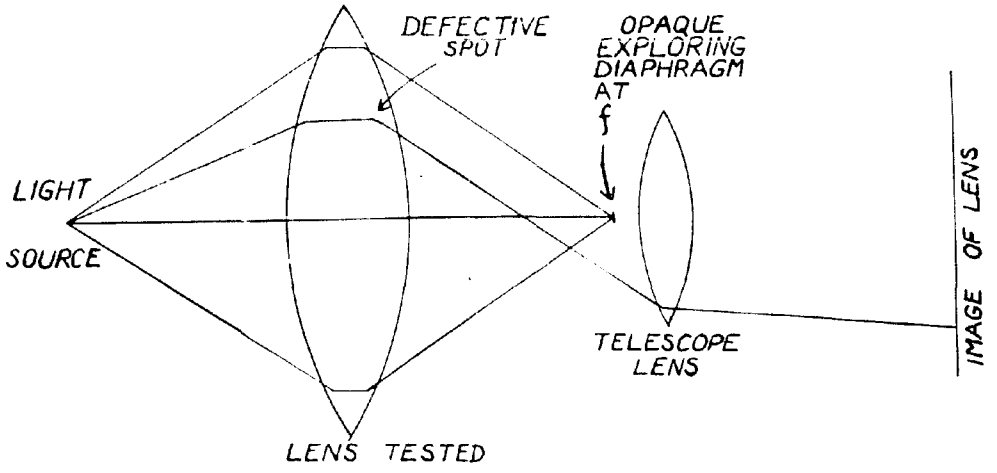


Figure 28. Diagram illustrating method of detecting defective spots in lens by means of an opaque, exploring diaphragm. Explanation in text. (Abramson and Moore, 29)

velocity in the second medium is greater than in the first a beam is bent away from the perpendicular and the angle of refraction is greater than the angle of incidence.

The principles and theory of the schlieren method of optical analysis as first used by Tiselius (23) (24) in electrophoretic work have been so clearly and adequately described by Longsworth (27) that any attempt to alter his description would be foolhardy. For this reason the following discussion will be for the most part verbatim:

"A diagram of the schlieren method is shown in Figure 29. An image of the horizontal slit, S, illuminated by the lamp, L, and condensor, C, is formed in the plane, P, by the schlieren lens D. The schlieren diaphragm, A, a screen with a sharp horizontal upper edge, is placed in the plane P and may be displaced vertically with a micrometer. The electrophoresis cell, E, is placed as near the lens D as the thermostat construction permits. The camera objective, O, placed immediately behind the schlieren diaphragm, is focused on the cell and forms a full size image of this on the ground glass or photographic plate at G.

"In the absence of refraction gradients, i.e., boundaries, in the electrophoresis cell all of the light traversing the cell is brought to focus in the image of the illuminated slit at P and enters the camera objective. If, however, a boundary is present in the tube the refractive index, n, decreases with increasing heighth, x, through the boundary, and the pencils of light through this region are deflected downward. If these deflected pencils are intercepted by the schlieren diaphragm they do not enter the camera objective and the region at G conjugate to the boundary in the cell appears as a dark band on a light background.

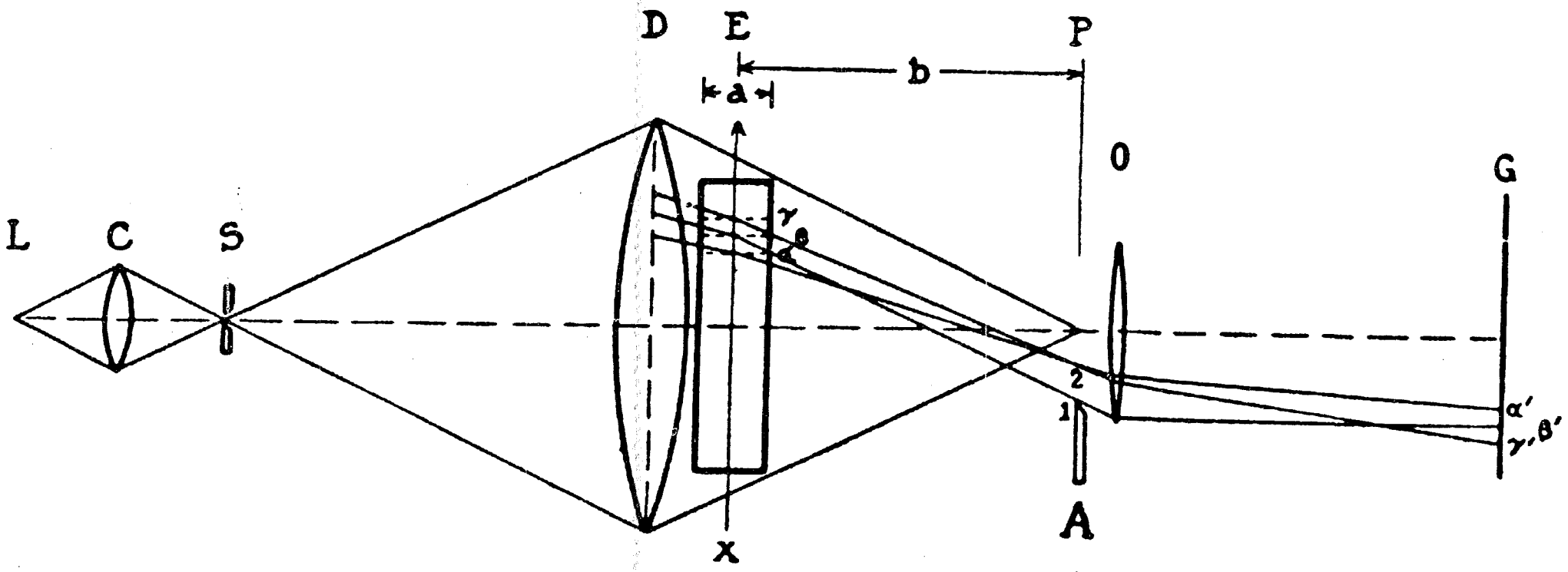


Figure 29. Diagram illustrating the schlieren method of optical analysis. Explanation in text. (Longsworth, 27)

"For boundaries that are not too "sharp" i. e., do not have too large refractive index gradients, the angular deviation of a horizontal pencil of light in a thin horizontal layer of the boundary is proportional (a) to the gradient $\frac{dn}{dx}$, in the layer and (b) to the breadth, a, (Figure 29) of the layer. The displacement, Δ, of the schlieren diaphragm, A, from the position of the undeviated slit image that is necessary to intercept the deflected pencil is also proportional to the optical distance, b, from the center of the cell to the diaphragm. Therefore $\Delta = ab\frac{dn}{dx}$ in which a and b are constants of the apparatus and $\frac{dn}{dx}$ varies vertically through the boundary but is assumed to be constant in any thin horizontal section."

"As the schlieren diaphragm is raised the first pencils of light to be intercepted are those which have passed through the steepest gradients of refractive index. In Figure 29 the paths of three pencils of light through a boundary have been traced. The planes, α, β, and γ in the boundary have been selected such that

$$\frac{dn}{dx}_{\alpha} = \frac{dn}{dx}_{\gamma} \quad \frac{dn}{dx}_{\beta} = \frac{dn}{dx}\ \text{Max.}$$

and the lines conjugates to these in the focal plane of the camera are shown at α', β', and γ'.

"The pencil through β Figure 29 suffers the maximum deflection to the position 1 near the plane of the schlieren diaphragm while those through α and γ are deflected equally to a common position 2 near that plane. With the upper edge of the diaphragm at 1 only the pencil through β is intercepted and the resultant dark band, or line, at β' has a minimum width. With the diaphragm raised to position 2 the pencils through α and γ, together with the pencils through all planes between α and γ

are also intercepted and the band at G has broadened correspondingly. With the proper conditions the displacement of the diaphragm from the position of the undeviated slit image is proportional to the refraction gradient at positions in the cell E conjugate to the edges of the schlieren bands."

Thus if a single component is present in a solution which has been separated electrophoretically, a single dark band will show up on the photographic plate (excluding boundary anomalies). If several components are present in the solution used then a dark band will show up for each component present in a concentration of 0.02 per cent or above. From the distances traveled by these bands from the initial boundary the mobilities of the components may be calculated.

The schlieren method as origionally used by Tiselius yielded no information as to the relative amounts of electrophoretically separable proteins present. However, a method was suggested by Tiselius and developed by Lamm (30) that overcame this difficulty. The Lamm "scale" method gives estimates of the quantities of the various constituents, their mobilities and relative homogeneities. Longsworth (27) has explained this method with the aid of Figure 30. In this method the cell is moved toward the camera objective a few centimeters and a transparent ruled scale, having several lines per millimeter is placed in the position formerly occupied by the cell. "In the absence of refractive gradients in the cell, E, a scale line, S, is brought to focus at s' by the lens O. If however there is a gradient on the cell between the planes pp' the rays from S which are collected by the lens have been deflected downward as shown in the figure and intersect at approximately S". Due to the faulty lens action of the gradient the image of s at s" is imperfect. With sufficient depth of focus s" appears on the plate as a line displaced by an amount δ from

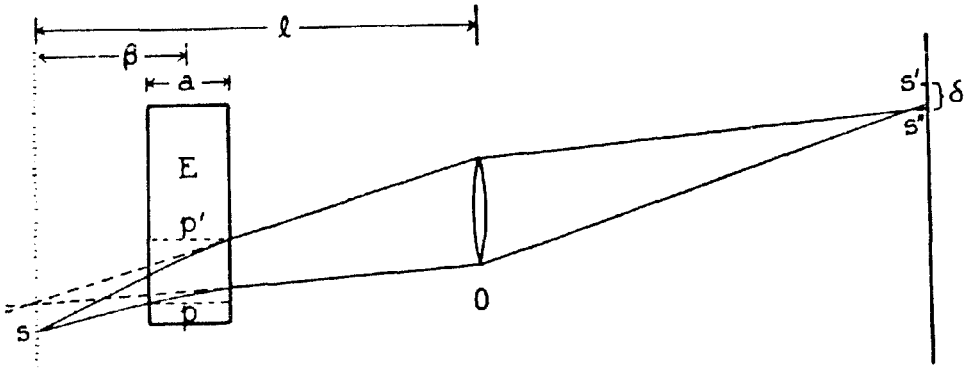


Figure 30. Diagram illustrating the Lamm scale method of optical analysis. Explanation in text. (Longsworth, 27)

its position s' in the absence of gradients in the cell. In order for the displacement of each line to be determined by the gradients in a thin horizontal section of the column the lens O should have a long focal length and be used at a small aperture. The latter condition is also desirable in order to increase the focal length."

In using this method the filled cell is put in place and the scale is photographed through the cell with the schlieren diaphragm removed so that schlieren bands will not obscure certain of the scale lines. After completion of the experiment the scale lines are again photographed through the cell. By using a comparator the positions of corresponding lines on the two photograph in the neighborhood of the boundary are computed.

If the refractive gradients are not too great the displacement δ, of a scale line is proportional to the horizontal breath of the boundary, a, the optical distance, β, from the scale to the center of the cell and the gradient $\frac{dn}{dx}$. For unit magnification

$$\delta = a\beta \frac{dn}{dx}$$

the value of s" must be reduced by a factor since the electrophoresis cell undergoes an apparent enlargement when moved out of the focal plane toward the camera. The factor is

$$\frac{L - \beta}{L}$$

Where L is the optical distance from the scale to the camera.

By plotting scale line displacement on the y axis against distance from the initial boundary on the x axis a pattern is obtained the area of which under certain conditions is proportional to the concentration of the component. The plotted results obtained by KeKwick (31) using this procedure

are shown in Figure 31.

The schlieren method was modified by Longsworth (32), so that graphs of the gradient dn/dx, in a thin horizontal layer of the column as a function of the position, x, of the layer could be recorded automatically. Figure 32 illustrated this "schlieren scanning" method. An image at P of the illuminated slit s-s is formed by the schlieren lens D. The camera objective O is focused on the cell E and forms an image on the screen at G-G. A boundary, B, is shown between a protein solution and a buffer. This boundary does not consist of a single geometric plane but a region in which the composition varies gradually from that of one solution to that of the other as shown by the density of the shading in Figure 32. The refractive index in this region changes with the heighth h, in the cell and the refractive index gradient varies from zero to a maximum and back to zero again as shown by the pencils of light passing through the boundary. The horizontal plate movement of the carriage C, Figure 24, past the narrow slit H, Figure 24, has been synchronized with t he vertical displacement of the schlieren diaphragm D (see also Figure 23). If the plate carriage is connected with the driving mechanism of the schlieren diaphragm when the latter is displaced so that no deflected rays are intercepted the slit at G is entirely illuminated. When the driving motor is turned on the plate moves towards the reader (Figure 24) as the schlieren diaphragm is raised. Figure 32a shows a band in the slit G-G resulting from the interception of a few pencils of light that have suffered maximum refractions. The area e-f-g shows that portion of the photographic plate that would have been exposed up to this point and the darkened area in the slit to the right shows the band as it would appear on a ground glass plate. Figure 32b shows

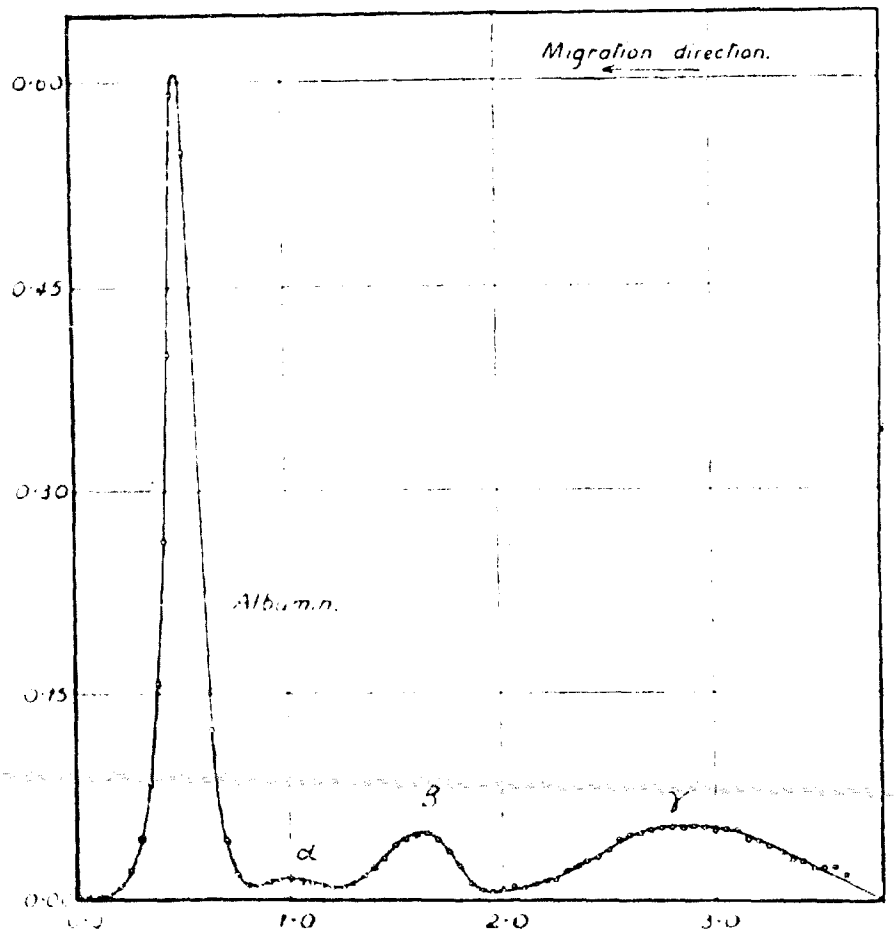
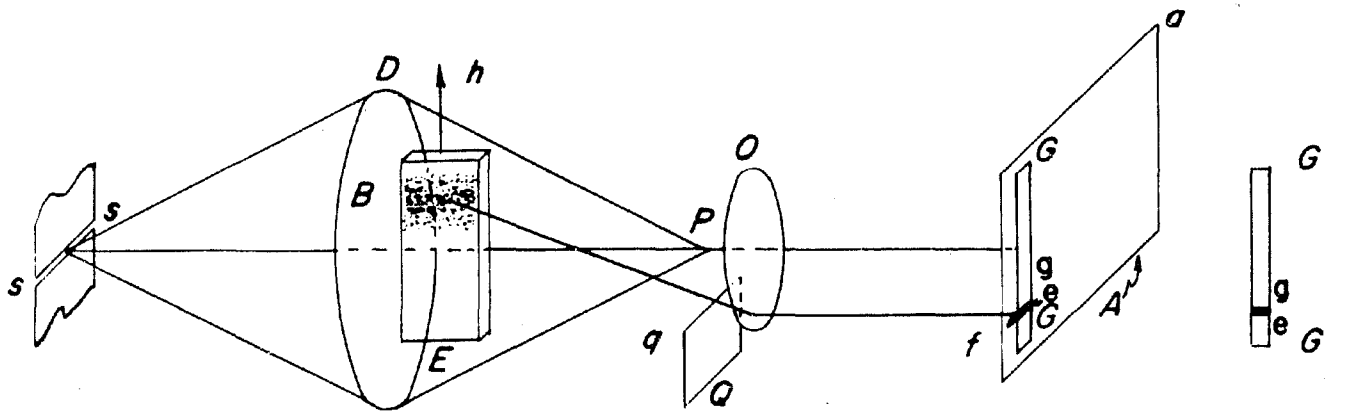
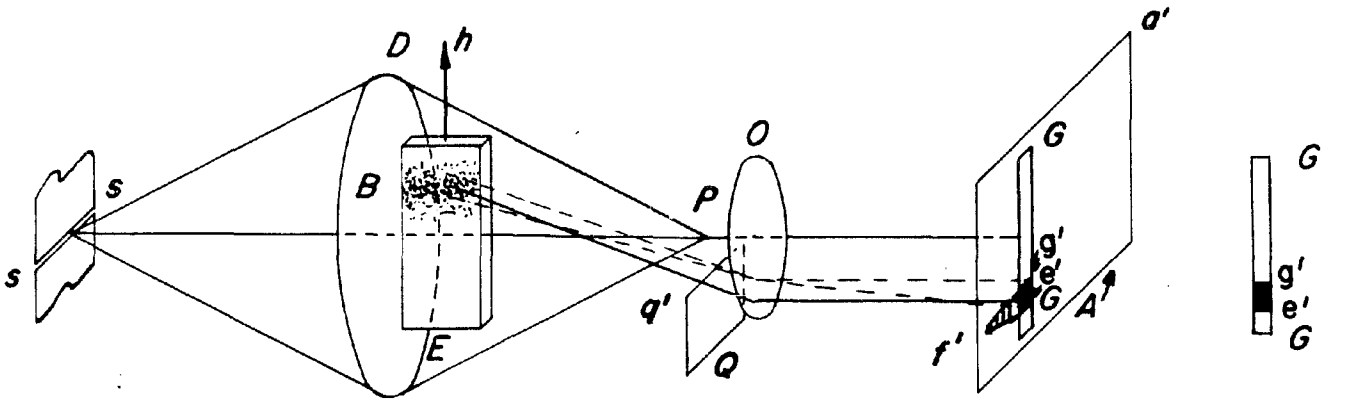


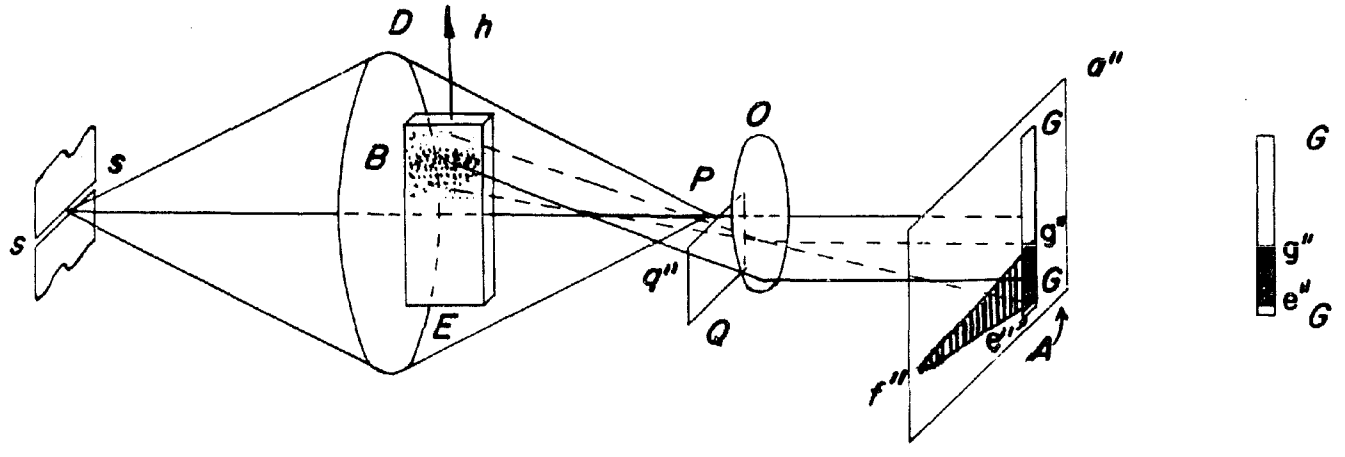
Figure 31. Electrophoresis of normal human serum. Photographed by Lamm scale method. Ordinate: scale line displacement in mm. Abscessa: distance in U tube in cm. (Kekwick, 31)



(a)



(b)



(c)

Figure 32

the schlieren diaphragm at the position q' and more pencils of light have been intercepted causing a widening of the band at G-G. Since the plate has moved over further the exposed area of the plate e'-f'-g' is larger. Figure 32c shows the schlieren diaphragm at the position q" just below the focal point of the lens O. The pencils of light suffering the minimum deflection have been intercepted and the area e"-f"-g" has reached its maximum area. As the diaphragm moves up from the position q" the entire vertical length of the slit at G-G becomes dark. By continuing the operation the entire field has been covered all of the boundaries in the cell image are photographed. The contour of the area e-f-g indicates both the position of the boundary and the magnitude of the refractive index gradients existing in it. If the change in refractive index is proportional to the change in protein concentration in a boundary then the area e-f-g is a measure of the change in protein concentration from one side of a boundary to another. It has been experimentally shown that the schlieren scanning method gives patterns that are comparable to the Lamb scale method (27).

With a gear ratio of 1-1 from the motor drive shaft to the plate carriage shaft the ratio of the plate movement to schlieren diaphragm displacement is 6:1. The distance traveled by the schlieren diaphragm is 4.20 mm per minute making 25.20 mm per minute the rate of travel of the photographic plate in the carriage past the slit. Thus three minutes is the average time necessary to take the complete scanning photograph of the boundaries.

Another simple and adequate method for recording electrophoresis patterns was suggested by Philpot (33) and modified by Svensson (34). The Philpot-Svensson cylindrical lens method has been explained by Longsworth

(35) with the aid of Figure 33. "An illuminated horizontal slit, present on the left of S but not shown in the figure, is focused by means of the schlieren lens, S, in the plane of the schlieren diaphragm, D. The latter contains a diagonal slit, kk, as shown in the front view, D'. The camera objective, C, is focused on the electrophoresis cell, E, and forms, in the absence of the lens H, a normal image of the cell on the ground-glass or photographic plate at G. The cylindrical lens, H, with its axis vertical, is focused on the schlieren diaphragm and also on the plate at G. Viewed from the side (Figure 33a), the cylindrical lens has no effect on the pencils of light forming the cell image. Thus the vertical coordinate of each point in the image is conjugate to the corresponding level in the channel E and, owing to the focusing action of the camera lens, C, this also remains true for pencils that may be deflected by gradients in the channel. Viewed from above however (Figure 33b) the cylindrical lens, in conjunction with the diagnoal slit, causes, as will be shown below, a lateral deviation of a pencil of light that is proportional to the vertical deflection the pencil has suffered in a boundary. The curve to the right of G in Figure 33a represents the pattern of the boundary, B, as it would appear on the screen if the later were hinged at the side and turned toward the reader, where as if it were hinged at the top and turned, the pattern would appear as in Figure 33b.

"If the fluid in the electrophoresis cell is homogenous, all of the light through the channel is concentrated in an image of the illuminated slit at the upper or normal level on the diaphragm, i.e., f' of D' or D. As can be seen from the figure, only the extreme left hand portion of the light in this image passes through the diaphragm to form a straight verticle line, i.e., the base line, on the screen at the position c-f. The width of this line varies with the width of the diagonal slit and, since a wide line is undersirable, Svensson has made the practical suggestion of tapering the end of

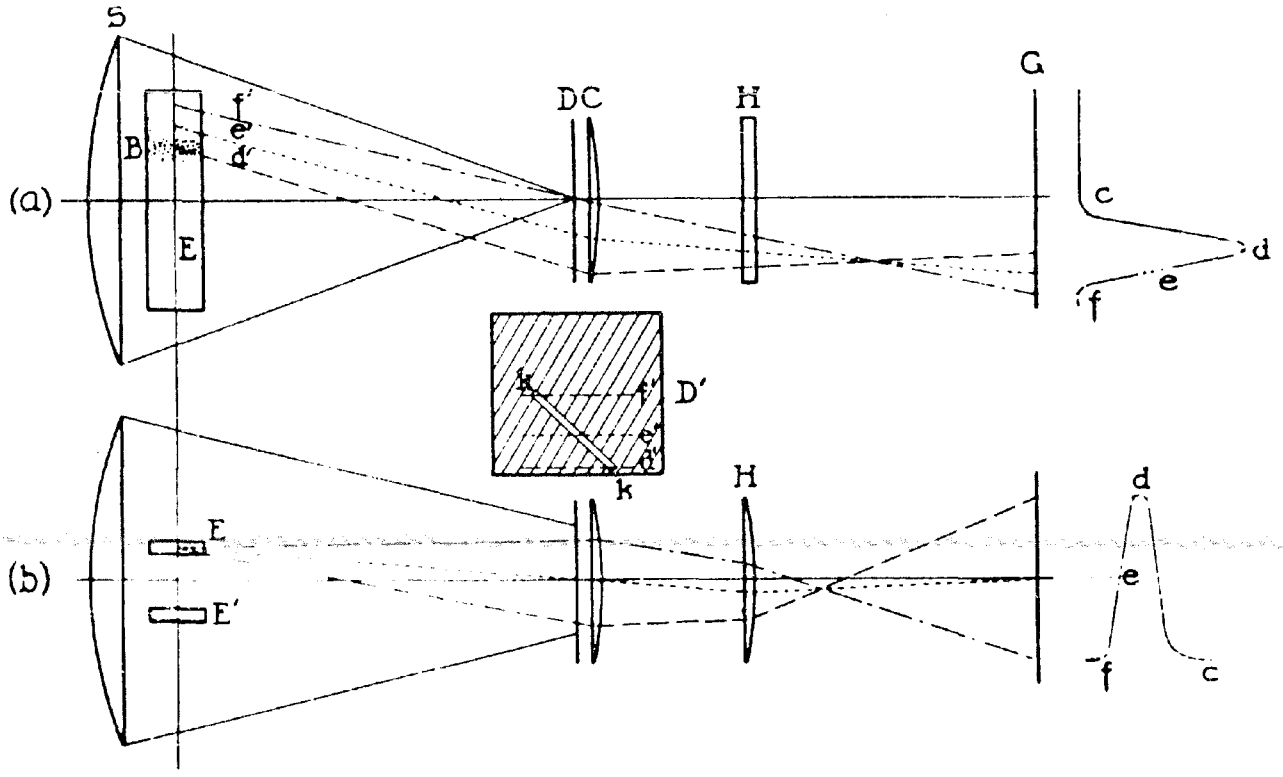


Figure 33. Diagram illustrating cylindrical lens method for the observation of electrophoretic patterns. (Longsworth, 35)

the slit kk to a point. If, on the other hand, a boundary B is present in the cell, a pencil through the layer of solution in the boundary having the maximum gradient, for sample, is deflected downward as indicated by the line d'd and forms an image of the slit at the lower level d' on the diaphragm. Owing to the angle the diaphragm slit makes with the vertical, the portion of the light in the lower image d' that enters the slit is shifted laterally from the position at which the normal pencil enters by an amount proportional to the vertical, deflection in the boundary gradient. The cylindrical lens consequently imparts to this pencil a corresponding lateral shift in the opposite direction to the position d, Figure 33b without affecting its vertical position i.e., d of Figure 33a.

"The path of a pencil through another portion of the boundary is indicated by the line e'-e and forms the corresponding element in the pattern. All other elements in the complete patterware formed similarly."

In a discussion on the cylindrical lens, Longsworth (35) has summarized its use as follows. "The cylindrical lens method is convienient for visual observation during an experiment and for the control of electrophoretic separations, since the pattern may be viewed directly on the screen of the camera. For visual observation a slit diaphragm is preferable to a straight edge, since the greater quantity of light reaching the screen in the latter case causes a decrease in the apparent contrast between the pattern and the background. For photographic work, however, the straight edge is to be preferred, owing, in part, to the superior resolving power and simpler diffraction phenomena characteristic of the diaphragm. Consequently the permanent photographic records of an experiment, on which the analyses are based, are obtained with a straight edge as diaphragm and with the scanning procedure. With the latter method the optical errors inherent in the uncorrected cylindrical lens are eliminated".

As described in the section on apparatus our camera has been modified so that either the scanning or cylindrical lens procedures can be used interchangeably. Figures 34a, 34b and 34c show complete electrophoretic patterns obtained from the electrophoresis of human blood serum and photographed by the schlieren scanning method, the diagonal slit method and the diagonal straight edge method respectively. If a slit is used with the schlieren scanning method patterns of the type shown in Figure 34c are obtained (36). In either method a slit used as a diaphragm produces the pattern as an illuminated line on a dark background whereas in the case of a straight edge the pattern is obtained as the contour between a light and dark field.

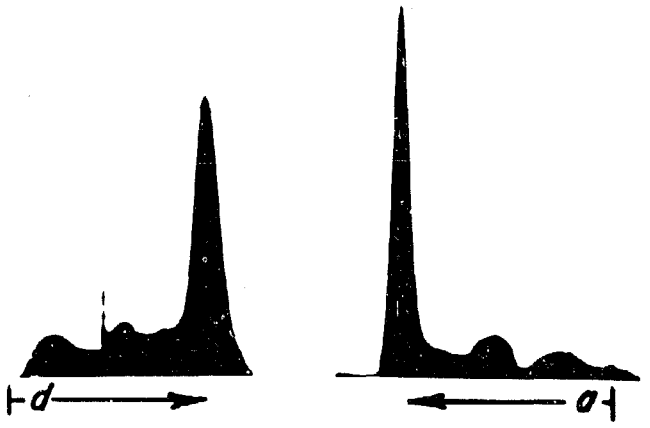


Figure 34a

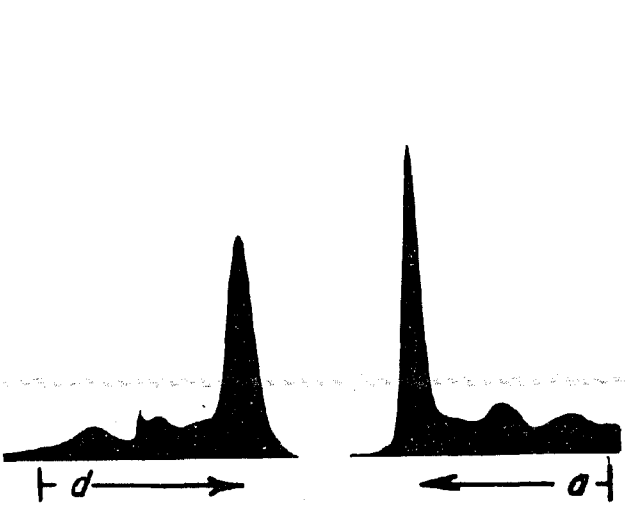


Figure 34b

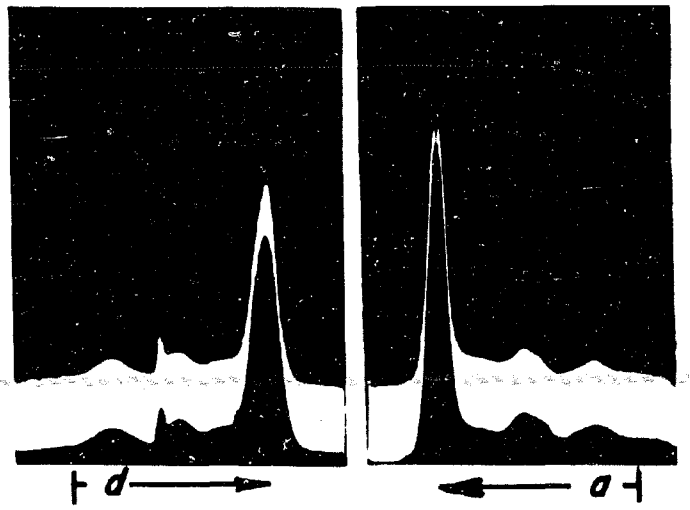


Figure 34c

Electrophoretic boundaries of normal human plasma diluted to one percent in phosphate buffer, pH 7.5, ionic strength 0.2. Electrophoresis carried out for 10,800 seconds at a potential gradient of 3.84 volts per centimeter. Boundaries photographed by the schlieren scanning method, Figure 34a; the cylindrical lens and diagonal straight edge, Figure 34b; and the cylindrical lens and diagonal slit, Figure 34c.

METHODS FOR DETERMINING TOTAL PROTEIN CONCENTRATION

If the total protein concentration in a sample is known the concentrations of the various components separating during an electrophoresis experiment can be caluclated from certain data. Since it is desirable to obtain this information in most cases, several well known methods for determining protein concentration will be discussed in some detail.

Since the schlieren method for photographing boundary positions and areas depends upon differences in refractive index between buffer and protein solution, it is not surprising that refractometric methods are widely used for determining total protein concentration. Siebenmann (37) has modified Reiss's (38) refractometric method for determining serum protein. His so-called "graphic" method is applicable to native serum and protein. This method utilizes a dipping refractometer and the readings are made on the solution in a constant temperature water bath at 20°C. Three to four ml. of serum are required for a determination. A standard graph (Figure 35) is made by plotting refractometric readings against the total protein concentration as determined by the Kjeldahl or some other method. The protein concentration of an unknown serum sample can then be read directly off the graph if the refractometer reading is determined. This method is reliable when the salt and non-protein concentration in the sample are of the same order as the samples used for determining the standard curve. The graphic method cannot be applied to serum dilutions or other protein solutions containing unknown amounts of salt.

In our laboratory the "differential" method of Siebenmann's (37)

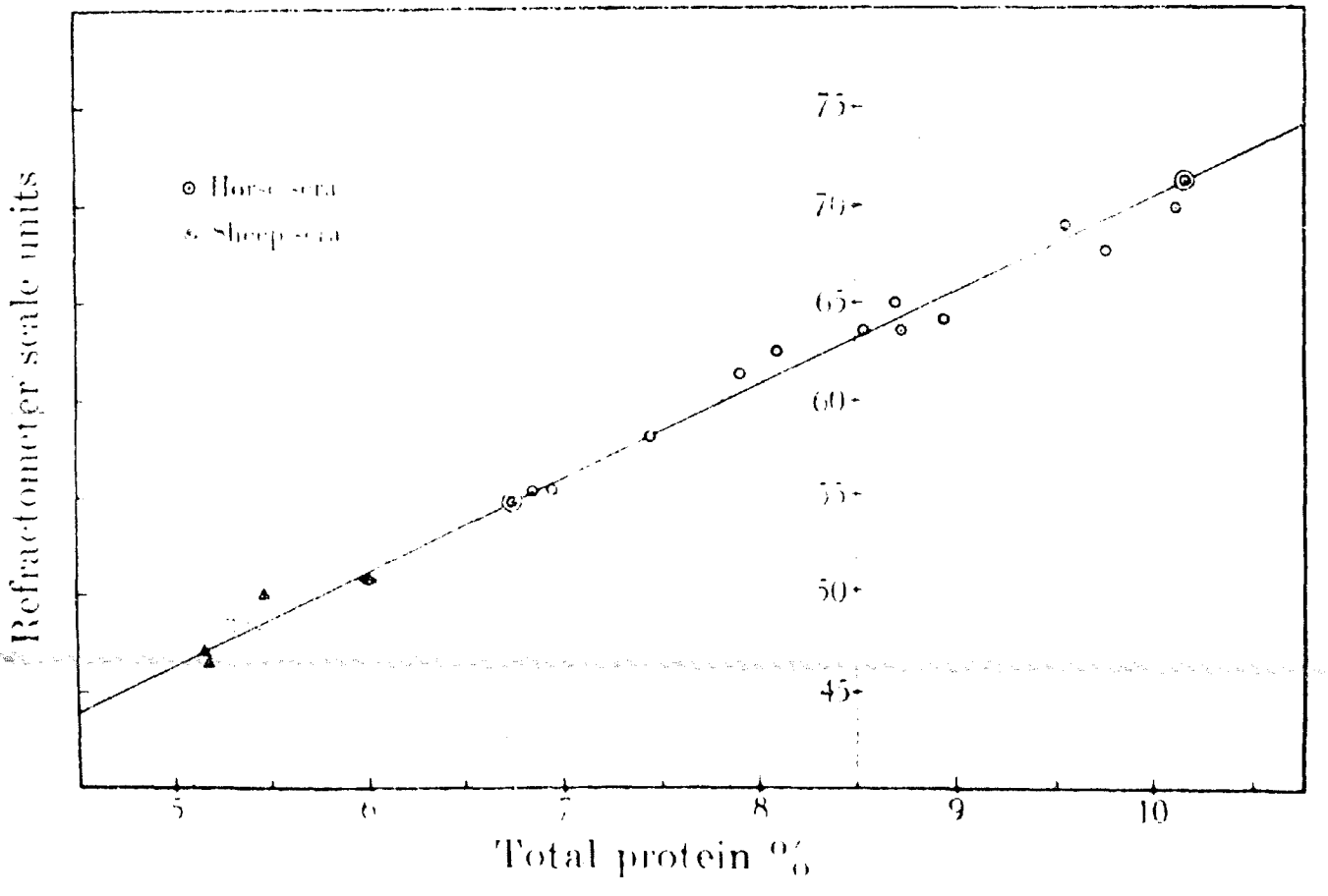


Figure 35. Standard curve showing percent protein (microkjeldahl) against scale readings.(dipping refractometer) (Siebenmann, 37)

is used. This method is based upon the refractometric change taking place when the protein solution is coagulated by heating. The difference between the refractometric reading of the protein in an acetate-acetic acid buffer (pH 4.6) and the same sample after heating at 100°C for 15 min. is taken as the index of protein concentration. For serum and plasma samples the following technique is employed:

To 10 ml. of a sodium acetate-acetic acid buffer (113 gms glacial acetic acid-236 gms. sodium acetate per liter) is added 2 ml. of serum. The refractive index is determined at 20°C by the dipping refractometer on 4 ml. of this mixture. The rest is used for heat coagulation, employing a special double tube shown in Figure 36. By use of the tube arrangement aqueus solutions may be heated at 100°C, without evaporation. The size of the outer tube containing water is 20 x 150 mm., that of the inner tube 15 x 125 mm. (pyrex no. 2370). The double tube is placed in boiling water for fifteen minutes. It is then cooled and the inner tube containing the sample centrifuged. The refractive index on the protein-free supernatant is determined as before.

For the calculation of the percentage of protein from the scale readings, the specific refractive increment of the protein must be known. This represents the change in the refractive index of the protein solution due to a change in protein concentration of one per cent. The specific refractive increments, a, have been carefully determined for horse serum albumin and globulins. The variations in the increments with changing wavelengths of light have been reported by Pederson and Andersson (39).

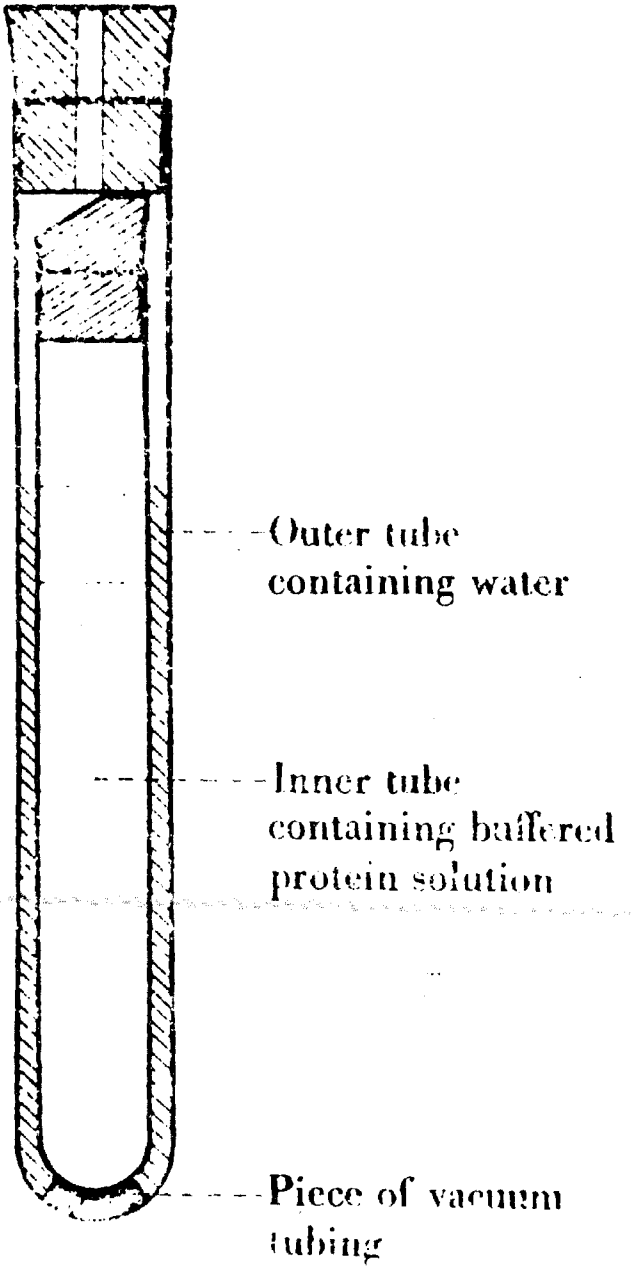


Figure 36. Diagram of test tube arrangement for heat coagulation of protein solutions. (Siebenmann, 37)

Wavelength (mμ)	(a) Albumin	(a) Globulin
366	0.00198	0.00202
436	0.00190	0.00195
546	0.00185	0.00187
579	0.00183	0.00186
589	0.00183	0.00186
656	0.00179	0.00182

The scale readings may be converted into refractive indices and the protein percentage calculated by means of the following equation

$$C = \frac{n_1 - n_2}{a} \cdot \text{dilution}$$

where n_1 is the refractive index before and n_2 the refractive index after heat coagulation of the protein.

The conversion of scale readings to refractive indices may be avoided by using the following equation

$$C = (R_1 - R_2) \cdot k \cdot \text{dilution}$$

here R_1 and R_2 are the scale readings before and after protein coagulation and k is the amount of protein in per cent which on heat coagulation leads to a drop in the dipping refractometer reading of one scale unit. The constant k may be determined as follows: Tables for converting dipping refractometer readings to refractive indices show that in the range of 20 to 30 scale units the constant difference in refractive index for a change of one scale unit is 0.000383. Taking the refractive increment value of horse serum as 0.00185 (value to be used with a tungsten incandescent lamp) the constant k is calculated

$$\frac{.000383}{.00185} = 0.207\%$$

where 0.207 represents the percentage of horse serum protein equal to one scale unit. (0.205 in the range of 30 to 40 scale units). Since very little information is known concerning specific refractive increments,

the values for horse serum are used for other protein solutions.

Example: Serum 616, dilution: 2 ml serum plus 10 ml acetate buffer

$R_1 = 29.0$, $R_2 = 23.5$

$C = (R_1 - R_2)\ K \cdot \text{dilution}$

$C = 5.5 \times 0.207 \times 6 = 6.83\%$

The "Differential" method is the only refractometric method available for determining protein concentrations in samples with an unknown salt content. The method estimates protein concentrations that are not influenced by the amount of non-protein substances present. For determining protein in solutions other than serum and plasma slight dilution modifications are necessary depending upon the protein concentration of the samples. In all cases the ratio of protein solution to buffer should be made so that upon heat coagulation a suitable drop in scale readings is observed (1 to 10 units). Siebenmann (37) gives the following dilutions to be used covering a wide range of protein concentrations.

Protein percentage range	Acetate buffer (ml) pH 4.6		Protein Solution (ml)	Dilution
4-12	10	+	2	6
1-4	5	+	5	2
0.2-1	1	+	10	1.1

For protein solutions containing less than 1 per cent protein a more concentrated buffer is used (56.6 gms. glacial acetic acid-118 gms. sodium acetate per liter).

In our laboratory a Bausch and Lomb dipping refractometer with constant temperature control equipment (No. 33-45-86. Bausch and Lomb) is employed for making refractometric readings. The refractometer and constant temperature water bath are shown in Figures 37 and 38.

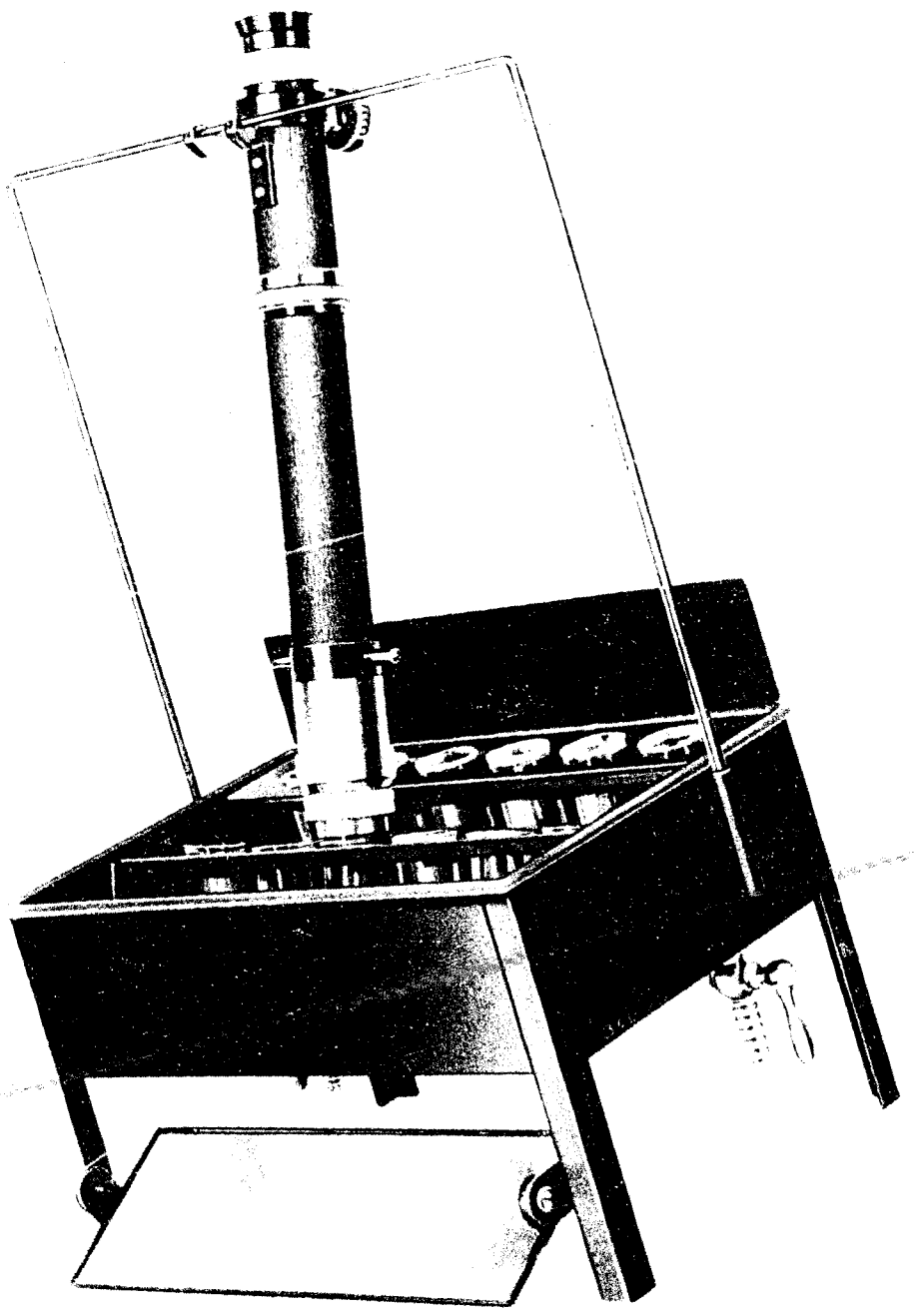


Figure 37. Bausch and Lomb dipping refractometer and water bath. (Courtesy Bausch and Lomb)

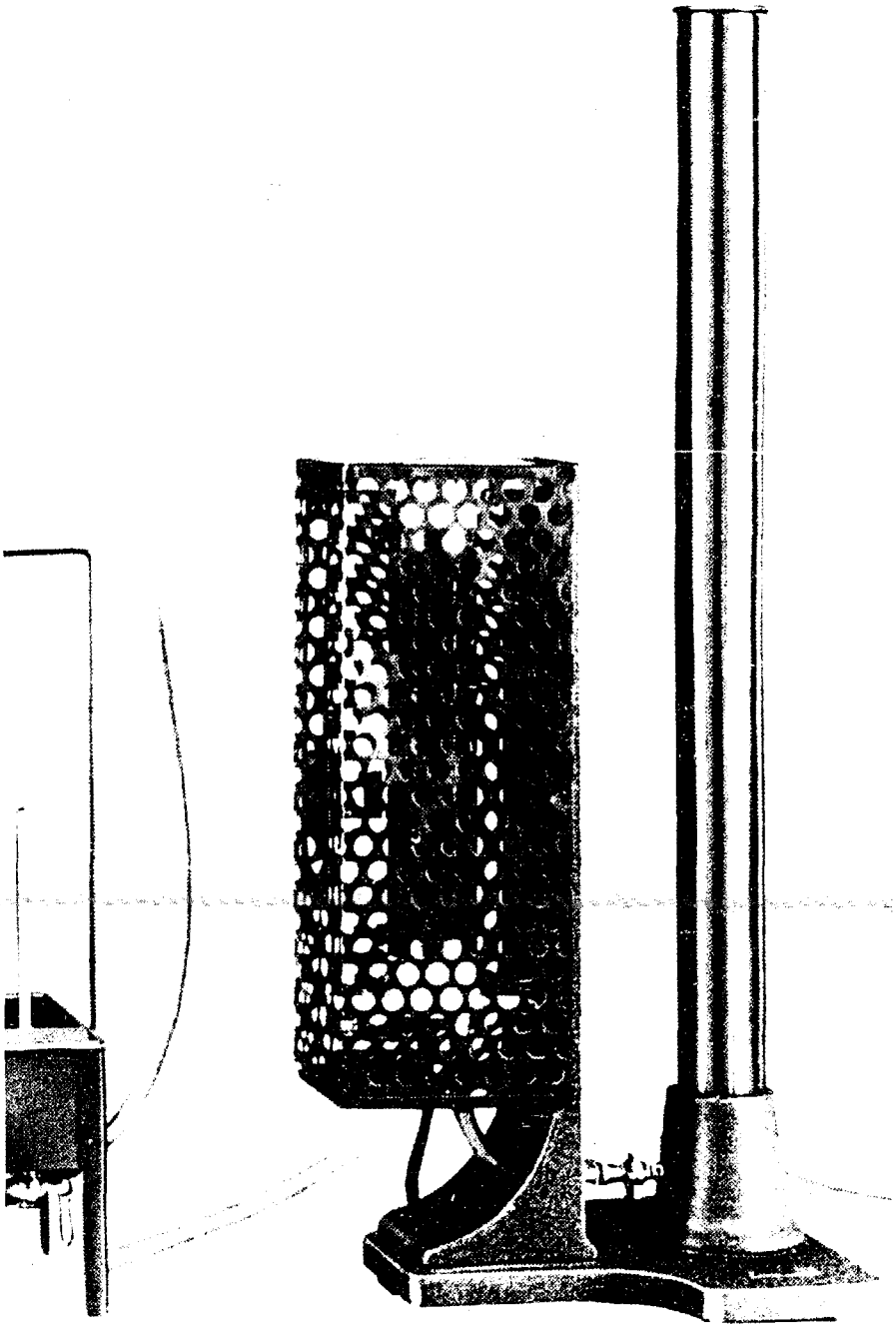


Figure 38. Bausch and Lomb constant temperature control equipment showing attachment to water bath. (Courtesy Bausch and Lomb)

Siebenmann (37) also describes two gravimetric methods for determining protein concentrations. In the heat coagulation method 2 to 5 ml. of serum or protein solution are diluted in a porcelain dish (acetic acid-sodium acetate buffer pH 4.6) so that the dilution contains not more than 1 per cent protein (2 ml serum + 18 ml buffer). This solution is covered and heated for 20 min. on a steam bath. The protein precipitate is then filtered and washed with hot water. The filter paper is then dried in an aluminium dish for 16 hours at 100°C. and weighed.

The acetone gravimetric method of Bierry and Vivarro (40) modified by Guilaumin etal. (41) has been further modified by Siebenmann (37). Two to 3 ml. samples of plasma or serum are pipetted into 10 ml. of acetone in a centrifuge tube. The acetone precipitates the proteins and dissolves some of the lipides, after stirring and standing centrifugation is employed and the supernatent discarded. The precipitate is washed twice with acetone and again centrifuged. 20 ml. of 0.6 per cent sodium chloride solution is added to the precipitate causing partial solution of the precipitated protein. To the suspension is added acetic acid-sodium acetate buffer pH 4.6 and the mixture covered and heated on the steam bath for 20 min. The coagulated precipitate is then filtered, washed and weighed as described under the heat coagulation method.

According to the author, all of these methods give sharply reproducible results and agreement between the various methods, where applicable, is good.

A semi-microkjeldahl nitrogen method for determining protein

concentration may be required for certain types of samples. A modification of two methods (42) (43) has been used with great satisfaction in this laboratory. An all glass distillation apparatus with digestion tube attached. (M-3065 Scientific Glass Apparatus Co.) is shown in Figure 39.

In making a determination the digestion flask is removed and 2-5 ml of the protein solution (depending upon N content) are added. To this is added 10 ml. of a digestion mixture of the following composition per liter.

2 gms. copper sulfate
100 gms. potassium sulfate
200 ml. concentrated sulfuric acid

After the addition of a few glass beads to prevent bumping, the flask is heated in a hood over a micro-burner. Heating may be rather rapid at first if frothing does not occur, but when the white fumes of sulfur trioxide begin to evolve the heating must be cautiously observed to prevent bumping and subsequent loss of material. The mixture must be digested until it is clear. The time required for this may be shortened by using small quantities of perhydrol near the end of the digestion. In adding the perhydrol, remove the flame and allow to cool slightly; add the perhydrol slowly down the side of the flask and then continue heating. Several treatments of this type may be necessary. When the digestion is complete, the flask is allowed to cool and is then attached to the distillation apparatus. The receiving flask contains 10 ml. of saturated boric acid solution (44) and 10 drops of a mixed indicator (45) (methyl red-methylene blue). The indicator mixture as described by the above authors has been found to be unsatisfactory. Ball (46) has found that by adding one drop of aqueus .05% methylene blue to 10 drops of aqueus .02% methyl red at the time of using, the indicator changes from violet

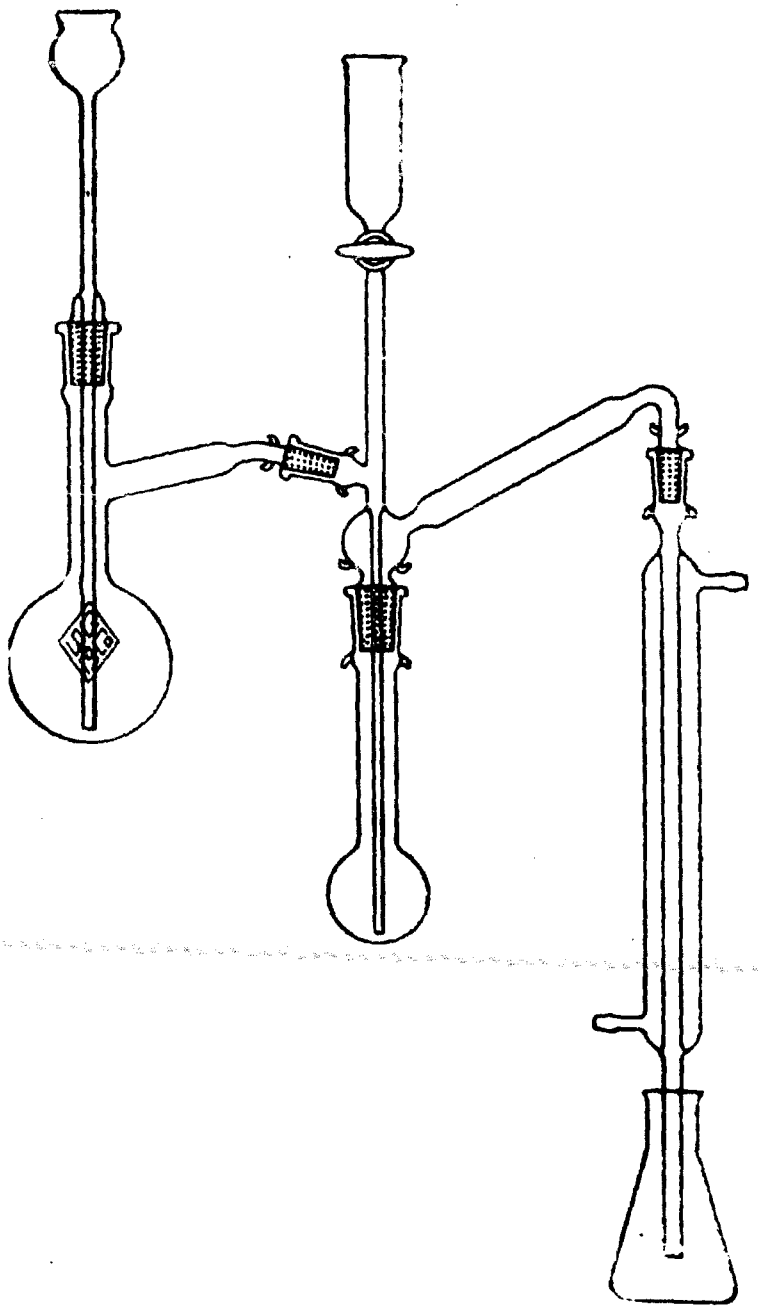


Figure 39. Diagram of an all glass distillation apparatus for micro-kjeldahl determinations. (Courtesy Scientific Glass Apparatus Co.)

(acid) to green (alkaline) with a definite intermediate grey color as the end point. Fifteen ml. of sodium hydroxide solution (500 gms per liter) is then added through the funnel and stopcock above the digestion flask. Steam is then passed through the apparatus until the volume in the receiving flask totals about 250 ml. The contents of the receiving flask are then titrated with standard acid to the grey end point. The amount of protein origionally present is calculated by multiplying the amount of nitrogen found by 6.25.

Another rapid and reliable method for determining total protein in serum was published by Kingsley (47). This method is based upon the blue color developed in the biuret test for proteins (48). The procedure calls for the addition of copper sulfate and sodium hydroxide to the serum sample in such a concentration that precipitation is avoided. A photoelectric colorimeter is used to estimate the amount of color developed and standardization is accomplished by using diluted (0.85 per cent sodium chloride) pooled blood serum and determining the actual protein concentration by the Kjeldahl method. To determine total protein 0.1 ml. of fresh serum (free from cells) is pipetted, from a Folin micropipette, into exactly 4 ml. of 10 per cent sodium hydroxide solution. After mixing by rotation, 0.5 ml. of 1 per cent copper sulfate is added. The solution is shaken vigorously 5 to 6 times. If the serum is jaundiced or lipemic, 2 ml. of ethyl ether is added and the sample is shaken vigorously for about 20 seconds. After standing 25 minutes the solution is read in a photelectric colorimeter. The readings remain stable until opalescence appears, usually about 1 hour. The authors experimental results show that excellent checks can be obtained between this method and the Kjeldahl method. In addition to determining total protein, techniques are reported for determining albumin and globulin in serum.

BUFFERS, BUFFER EFFECTS AND THE PREPARATION OF BUFFERS

Emil Fischer (1889-1918) showed that amino acids are the primary decomposition products of proteins. Adams (49) first suggested that amphoteric compounds such as amino acetic acid exist largely in a special state, called an inner salt

$$NH_3^+ - CH_2 - CO_2^-$$

Bjerrum (50) coined the term "zwitterion" for this type of compound. He pointed out that all of the amino acids existed in the salt-like double ion state.

$$NH_3^+ - R - CO_2^-$$

The zwitter ion theory changes the viewpoint on the reactions of amino acids with acids and bases (51). "Acids react with the acidic groups of amino acids and proteins, and bases react with the basic groups of amino acids and proteins." The reactions between glycine and hydrochloric acid and sodium hydroxide are represented as:

$$NH_3^+ - CH_2 - COO^- + HCl \longrightarrow NH_3^+ - CH_2 - COOH - Cl^- \quad (1)$$

$$NH_3^+ - CH_2 - COO^- + NaOH \longrightarrow NH_2 - CH_2 - COO^- + Na^+ + HOH \quad (2)$$

Proteins are multivalent ampholytes and exist largely as dipolar ions in the neighborhood of their isoelectric point. They are present as salts and possess a net charge at other reactions. The valence of a protein might be expected to influence mobility in two ways (52): (1) "the greater the valence the greater the net charge and therefore the greater the mobility (2) ...the greater the valence the more the activity coefficients deviate from unity at any given concentration and the more the mobility will diminish from that which would obtain in infinitely dilute solution."

Since the sign and net charge of a protein molecule depend upon the surrounding reaction (53) it is necessary that electrophoresis experiments be carried out in suitable buffers in order to make comparable results.

From reactions (1) and (2) it should be evident that there must be some pH value at which the sum and magnitude of the charges will be equal resulting in an electrically neutral molecule. When a molecule is in this state it is said to be isoelectric. The isoelectric point has been defined in terms of electrical transport as: "that hydrogen ion concentration at which there will be a tendency for as many cations to migrate towards the cathode as there are anions migrating toward the anode." (51)

The isoelectric point may be calculated for ampholytes from the apparent acidic, and basic, dissociation constants (54). Brönsted (55) writes all ionization constants in terms of acid constants. By considering an amino acid as a dibasic acid this method can be applied to the ionization of an amino acid. The following equations are essentially those of Schmidt (54).

$$NH_3RCOOH^{+} \rightleftharpoons NH_3RCOO^{+} + H^{+} \text{ or } R^{\pm} \rightleftharpoons R^{-} + H^{+} \quad (3)$$

$$NH_3RCOO^{\pm} \rightleftharpoons NH_2RCOO^{-} + H^{+} \text{ or } R^{\pm} \rightleftharpoons R^{-} + H^{+} \quad (4)$$

$$K_1 = \frac{aH^{+} \cdot aR^{\pm}}{aR^{+}} = \frac{(H^{+})(R^{\pm})(\gamma H^{+})(\gamma R^{\pm})}{(R^{+})(\gamma R^{+})} \quad (5)$$

$$K_2 = \frac{aH^{+}aR^{-}}{aR^{\pm}} = \frac{(H^{+})(R^{-})(\gamma H^{+})(\gamma R^{-})}{(R^{\pm})(\gamma R^{\pm})} \quad (6)$$

where a = activity

γ = activity coefficient

Multiplying equations (5) and (6) together

$$K_1K_2 = \frac{a\,H^{+2}\;a\,R^-}{a\,R^+} = \frac{(H^+)^2\,(R^-)\,\gamma H^{+2}\,\gamma R^-}{(R^+)\,\gamma R^+} \qquad (7)$$

At the isoelectric point (R^+) and (R^-) are equal. Hence, if I is the value of (H^+) at the isoelectric point

$$I^2 = K_1K_2 \quad \frac{\gamma R^+}{\gamma R^- \gamma H^{+2}} \qquad (8)$$

If pI' is used to represent the value of pH at the isoelectric point, we have

$$pI' = 1/2\,(pK_1 + pK_2 - \log \frac{\gamma R^+}{\gamma R^-}) \qquad (9)$$

where pK_1 and pK_2 are the negative logarithms of the acidic ionization constants of the ampholyte. If the ampholyte has more than two ionizable groups all of the ionization constants will be effective in determining the location of the isoelectric point. A complete review of the theory of the isoelectric point has recently been presented by Hill (56).

Several factors must be considered in choosing a buffer to be used as the solvent in an electrophoresis experiment. These factors are: (1) H-ion concentration, (2) total salt concentration, (3) ionic strength, (4) buffer capacity, (5) conductance, (6) solvent power, and (7) resolving power. These factors will be discussed in some detail.

H-ion Concentration

It has been determined by many workers that there is a certain hydrogen ion concentration or range of hydrogen ion concentrations at which an ampholyte in solution has an equal number of positive and negative charges and is electrically neutral (isoelectric point). If an ampholyte is put into solution at a hydrogen ion concentration above its isoelectric point the ampholyte has an excess of negative charges and therefore behaves as an anion in an electric field. Conversely, below its isoelectric point it behaves as a cation.

Equation (9) shows that the net charge on an ampholyte results in part from the sum of its acidic and basic dissociation constants. Therefore any effect of the surrounding medium on the dissociation constants will be reflected by the sign and magnitude of the net charge carried by the molecule.

This effect can be illustrated by means of a general formula for an amino acid.

NH_3 (+)	NH_2	NH_3 (+)
|	|	|
R	R	R
|	|	|
COO (-)	COO (-)	COOH
(a)	(b)	(c)

An amino acid in its isoelectric condition is shown by formula (a). Now let us suppose that this molecule is placed in solution at a pH value above its isoelectric point. Free hydroxyl ions in the solution combine with the coordinated hydrogen ion of the acidic NH_3^+ group to form slightly dissociated water and as a result the amino acid becomes electrically negative (b). Placing this same molecule in solution at a pH value below its isoelectric point results in free hydrogen ions combining with the basic COO^- group leaving the molecule electrically positive (c). The greater the difference in hydrogen ion concentration of the ampholyte in solution from its isoelectric point, the larger the magnitude of the charge. This point is well illustrated in Figure 40 in which the changes in mobilities with varying pH at constant ionic strength and potential gradient for the components of normal bovine serum are shown (57).

Whether a mobility determination should be carried out at a pH above or below the isoelectric point of the sample depends on several

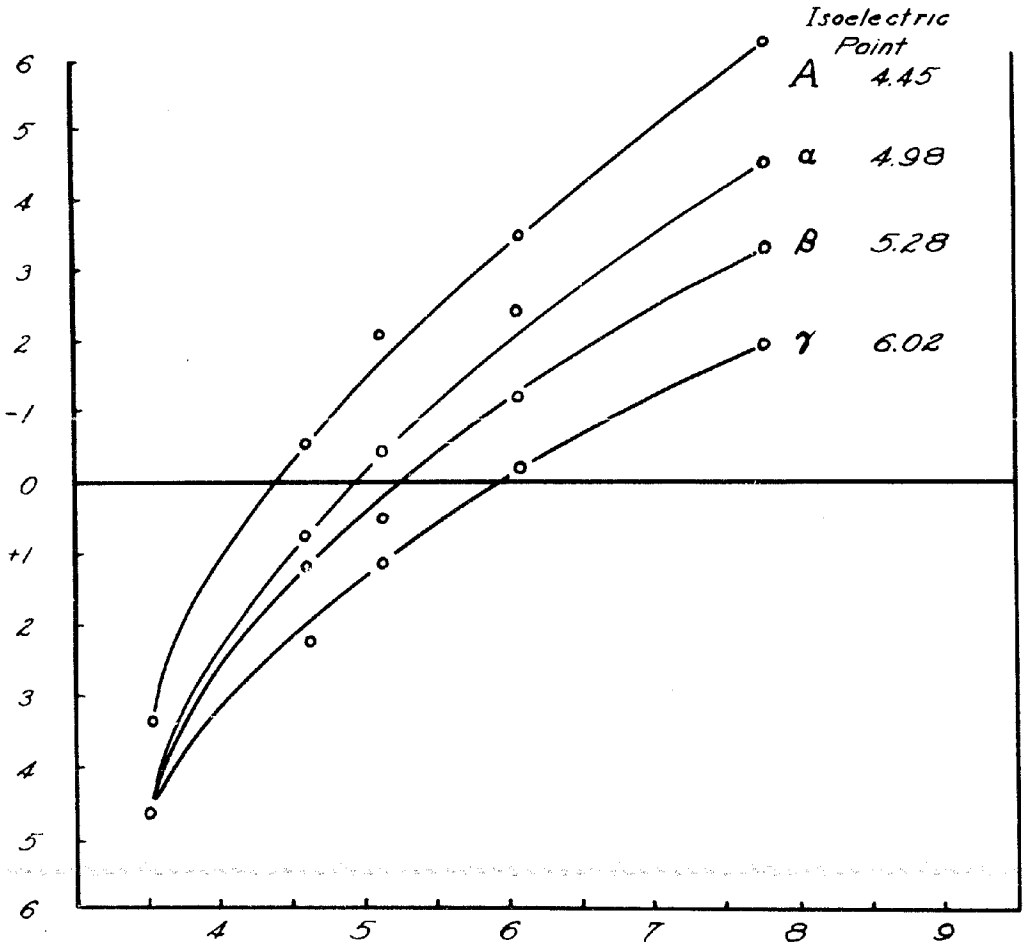


Figure 40. Mobility-pH relationship of cow serum. (San Clemente and Huddleson, 57)

factors: (1) the pH of the isoelectric point (2) the stability of the sample in acidic and basic solutions, and (3) the solvent power of the buffer at a particular pH value.

In addition to this charging process which results from the effect of the solvent on the dissociation constants of the protein, are to be considered the following: (1) polarization effects resulting from a charge approaching a surface and inducing a separation of charges on the surface of the molecule (58), this induced moment in turn may bind the charge to the surface, (2) ion pair formation due to the fact that each charge on a surface may bind a charge of opposite sign due to coulombic forces (59), (3) a specific chemical reaction can determine the charge on protein surfaces (60). Hydrogen bond formation would be of the latter type.

Salt concentration

The effects of salt on the mobilities of proteins are of two kinds (61). The first effect is concerned with the ion atmosphere surrounding the protein. It is non-specific and depends upon the contribution of the salt to the ionic strength. This effect will be discussed in more detail later. The second effect results in an actual change in the charge of the protein. This interaction is highly specific and can be the result of (1) chemical processes, (2) ion pair formation, or (3) adsorption of the ion on the protein.

Figure 41 shows the effect of adding salt at a constant pH to an ideal spherical protein molecule. At each pH value the mobility decreases as salt is added. Abramson points out that with a real protein the net charge and isoelectric point would usually be affected by the addition of salt. In addition to these two effects the solubility of the protein in salt solutions should be considered.

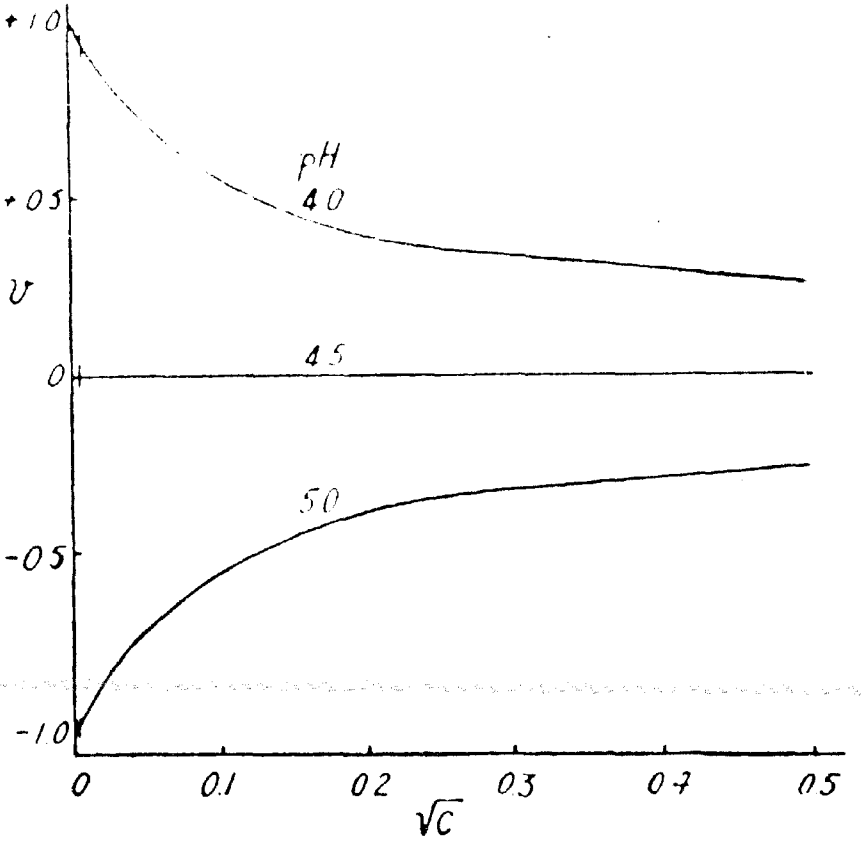


Figure 41. The electric mobility of an ideal spherical protein in various concentrations of salt. (Abramson, Moyer, and Gorin, 1)

Ionic Strength

X-ray studies have shown that a crystal of sodium chloride does not contain molecules of the salt, but in contrast the crystals are composed of sodium and chloride ions arranged in a definite order called the ion lattice. If a crystal is placed in water the ions are separated from the definite lattice form and are distributed throughout the water. According to this evidence, sodium chloride is 100 per cent dissociated in any solution. However, the apparent effects of various concentrations as measured by colligative properties, vary widely. Lewis (62) has developed his activity theory to account for such variations of strong electrolytes. The activity theory applied to strong electrolytes considers these electrolytes to be 100 per cent dissociated in all cases, but that each ion possesses an activity or capacity to function as an ion according to the concentration of ions surrounding it. From known behaviours of particles possessing like and unlike charges it would appear that an ion, surrounded by other ions of the same and opposite charges, would possess various activities according to variations in the concentration of the surrounding ions. The apparent or effective concentration of a substance in solution as shown by its properties, is termed its "activity." If a 0.1 molal solution of a strong electrolyte appears to have an activity in lowering the freezing point corresponding to only 0.08 molal as based on theoretical activity (concentration = 0), the value of the activity a is 0.08. In general the activity of an ion decreases as the ionic concentration about it increases.

Lewis defines the activity coefficient as the ratio of activity a to molal concentration m:

$$\gamma = \frac{a}{m} \qquad (10)$$

If 0.1 molal electrolyte shows an activity of 0.08 the activity coefficient at this concentration is,

$$\gamma = \frac{0.08}{0.1} = 0.8 \qquad (11)$$

The activity coefficient equals unity when the concentration equals 0 (theoretical).

Equation (10) may be rewritten:

$$a = \gamma m \qquad (12)$$

which shows that the activity equals activity coefficient multiplied by the concentration.

The activity coefficient depends upon the total ion concentration regardless of source. This effect is an electrostatic one and the charges carried by the ions are a determining factor. Lewis has used the term "ionic strength", u, to include all of these ionic effects. His equation for determining the ionic strength of a solution is:

$u = 1/2 \ \Sigma CV^2$ where Σ = sum, C = concentration and V = valence of the ion. (13)

In dilute solutions the activity coefficient of a given strong electrolyte is the same in all solutions of the same ionic strength.

Gorin (63) states that variations in the ionic strength at constant pH can effect the electric mobility of proteins in two ways, (1) a change in ion atmosphere will occur, and (2) a change in the charge of the protein might occur due to specific interactions with the ions of the buffer. However the influence of ionic strength on mobility is determined by the type of buffer employed. Cannan (64) has shown that with uni-univalent

electrolytes the influence of ionic strength on the net charge pH curve is not very great. Buffers made of salts of other higher valence show very marked differences in mobilities with change in ionic strength at constant pH (52). "At an ionic strength of 0.02 and a pH of 5.65 a mobility of 10.3×10^{-5} cm ^{2}/ sec/volt was observed for carboxyhemoglobin, in phosphate buffers at 25°C. At the same pH this mobility was reduced to 2.7×10^{-5} at an ionic strength of 0.15. At the lower ionic strength this mobility was observed at pH 6.7. A change from 0.15 to 0.02 in ionic strength thus produces a change in mobility in these systems equivalent to a change of over one pH unit."

Tiselius and Svensson (26) have investigated the effect of phosphate buffers of constant pH and varying ionic strength on the mobility of egg albumin. The results of their experiments are shown graphically in Figure 42.

Not only the observed mobility but the apparent isoelectric point is a function of the ionic strength. Smith (65) has summarized the results (see Figure 43) of the effect of various acetate buffers on the isoelectric point of egg albumin adsorbed on collodion particles.

Longsworth (35) has found that although ascending and descending pattern asymmetries are reduced by increased ionic strengths, improvement is generally more marked if the increase is due to a buffer salt, which simultaneously raises the buffer capacity, than to a neutral salt. Substituting one monovalent salt for another or neutral sodium chloride at constant pH and ionic strength, does not result in appreciable changes of mobility. However, changing from a univalent buffer salt mixture to one containing the divalent $HPO_4^=$ ion has a marked effect on the mobilities (66).

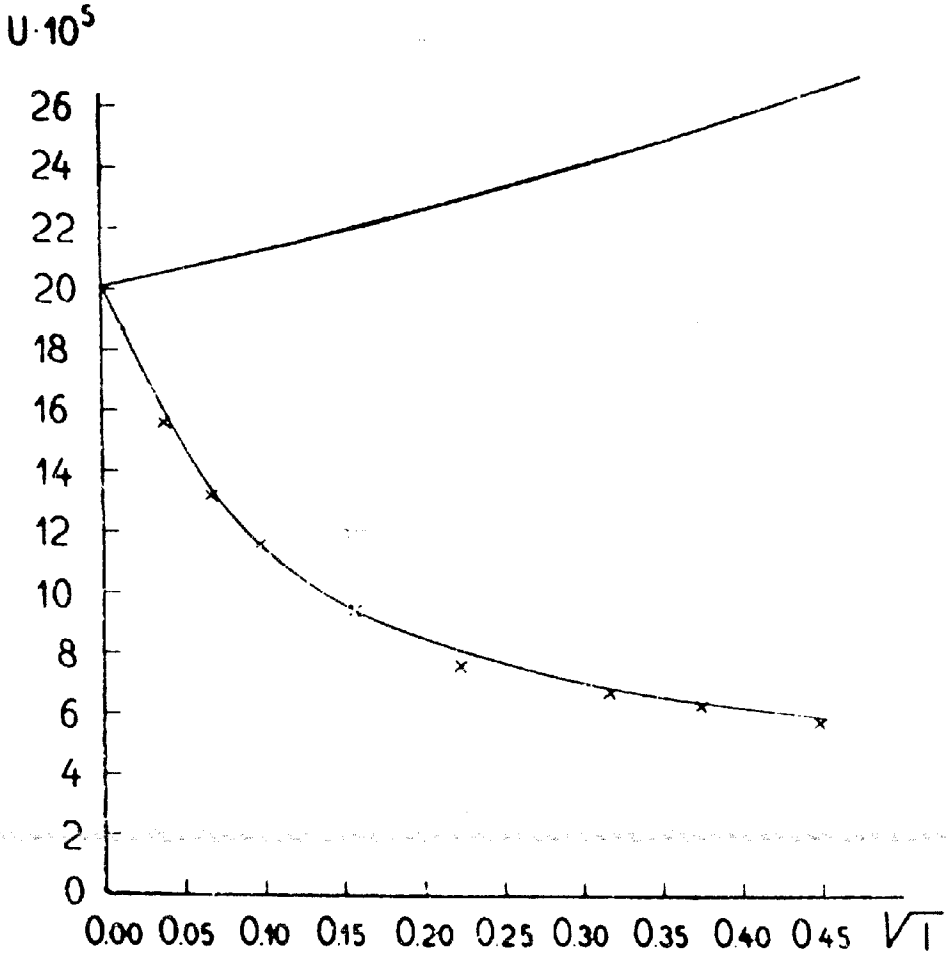


Figure 42. Mobility of egg albumin at pH 7.10, T = 0.5°, and varying ionic strengths. Upper curve: ideal mobility, calculated on the assumption of free ionic migration. Lower curve: calculated mobilities on basis of the Debye-Hückel-Henry theory. The crosses represent the observed values. (Tiselius and Svensson, 26)

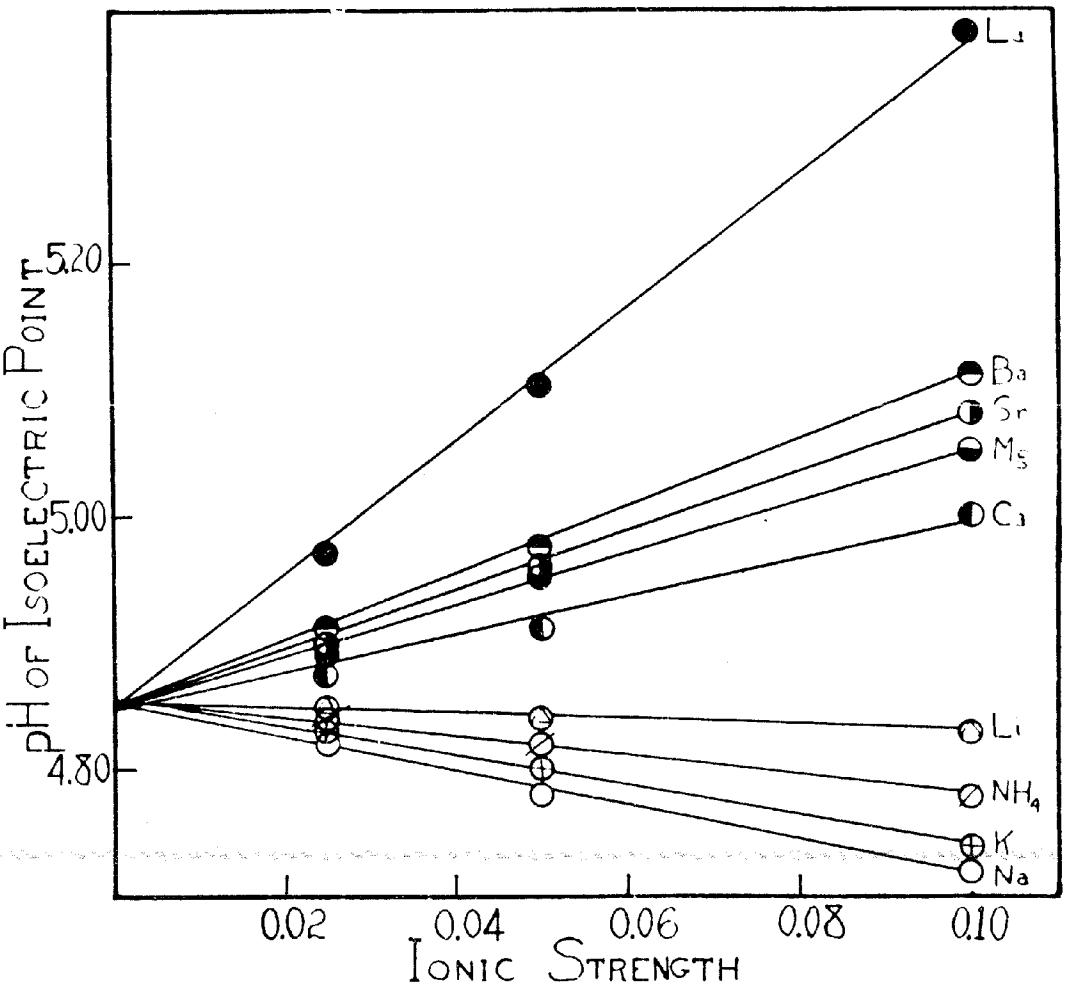


Figure 43. The effect of various acetate buffers of varying ionic strength on the isoelectric point of egg albumin adsorbed on collodion particles. (Smith, 65)

Buffer Capacity and Conductance

The buffer chosen as a solvent should have a high buffer capacity itself so that the protein buffer capacity is relatively reduced. This will result in fewer boundary anomalies (67). A buffer with a low specific conductance is desirable in order to reduce the disturbances due to the heating effect of the current. The generation of heat results from the friction of the ions passing through the solution and is related to the speed of migration of the ions. The speed of migration of ions (mobilites) can be caluclated by combining conductivity and transference number measurements. "The anion transference of an electrolyte expresses the fraction of current that is carried through the solution by the anions, so that multiplying the equivalent conductivity of an electrolyte by this quantity gives the mobility of the anion" (68). Table (1) shows the mobilities of a number of ions in water at 18°C. Since the lithium ion has a lower mobility than either the sodium or potassium ion its salts are preferred in making up buffers. This allows relatively more current to be carried by the protein ions and is desirable since there is an upper limit to the total current which can be used to avoid convection artifacts due to electrical heat (69). Both buffer capacity and conductance increase with the concentration of buffer salts, and it has been pointed out that because of this incompatibility a compromise must be made (67). Since buffer capacity does not depend upon ionic mobilities, buffer salts and ions which have low mobilities should be selected wherever possible.

Solvent Power and Resolution

The sample to be used in an electrophoresis experiment should remain clear when adjusted with the buffer solvent. If a precipitate

TABLE 1

Mobilities in Water at 18°C

(cm./sec. x 10^{-5})

Ion.	Mobility	Ion.	Mobility
H	316.55	NO_3	61.74
		CNS	56.58
Li	33.28	Picrate	25.4
Na	43.39	ClO_4	56
K	64.44	$N(C_2H_5)_4$	28.1
Rb	67.5	HCOO	47.0
Cs	67.65	HCO_3	40.1
Ag	53.77	Ca	51.3
Tl	65.7	Sr	53.5
OH	176.6	Ba	55.1
F	46.65	Mg	45.52
Cl	65.41	Zn	45.5
Br	67.4	Cd	45.9
I	67.4	Cu	45.9
ClO_3	54.99	Pb	60.5
BrO_3	47.9	SO_4	68.0
IO_3	34.02	C_2O_4	61.5

develops and represents a quantity that can be detected in the electrophoretic patterns, then the pattern is not a true one. Buffer solvents for use in the electrophoretic analysis of human plasma and serum have been studied at length by Longsworth (35). Table 2 summarizes his results.

The results of these experiments show that in resolving power none of the buffers are superior to the diethylbarbiturate solution at pH 8.6. Column 8 gives the ratio of the maximum refractive index gradient in the descending albumin boundary to that in the rising albumin boundary. It furnishes an index of the symmetry of the patterns, the value approaching unity in the ideal case.

Figures 44, 45 and 46 show the differences in resolving power of three different buffers at the same ionic strength and potential gradient on normal bovine plasma. The patterns shown in Figure 44 were obtained in a barbital buffer of pH 8.6 and u 1. The various components are well separated from each other and the peaks are sharp and well defined. In addition the descending and ascending patterns approach each other symmetrically. Figure 45 obtained from an experiment carried out in a phosphate buffer of pH 7.7 and u 1. shows a marked asymmetry between ascending and descending patterns and the separation of the alpha globulins is incomplete. Figure 46 shows the same sample separated in a carbonate buffer of pH 9.9 and u 1. The descending pattern shows a poor separation of the various components and the globulin has not separated from the albumin. The peaks are not sharp and well defined. In contrast the ascending albumin peak is so steep that it could not be completely recorded on the plate.

Preparation of buffers of known pH and ionic strength

Henderson (70) and Washburn (71) simultaneously characterized the equilibrium of a buffer solution in terms of the law of mass action, assuming that the concentration of the negative ion approached the total concentration of salt and that the concentration of the undissociated acid is equal to the

Buffer solutions used as solvents for the electrophoretic analysis of human plasmas and sera

(1) BUFFER	(2) μ	(3) pH AT 25°C.	(4) $\kappa_B \times 10^4$	(5) SERUM OR PLASMA	(6) DILUTION*	(7) NUMBER OF PEAKS	(8) SYMMETRY	(9) γ SEPARATION
0.1 *N* NaCac†-0.02 *N* HCac	0.1	6.8	3.10	P	1:2	‡	0.65	Yes
0.008 *M* NaH_2PO_4-0.064 *M* Na_2HPO_4	0.2	7.7	4.81	P	1:2	6§	0.39	Yes
0.004 *M* NaH_2PO_4-0.032 *M* Na_2HPO_4	0.1	7.7	2.62	S	1:4	5	0.52	Yes¶
0.025 *N* LiV‖-0.025 *N* HV-0.025 *N* LiCl	0.05	7.9	1.96	P	1:4	5	0.75	No
0.025 *N* LiV-0.025 *N* HV-0.075 *N* LiCl	0.1	7.9	3.97	S	1:4	4	0.75	No
0.025 *N* LiV-0.025 *N* HV-0.0675 *N* LiCl-0.0025 *M* $CaCl_2$	0.1	7.9	4.00	S	1:4	4	0.70	No
0.02 *N* NaV-0.02 *N* HV-0.08 *N* NaCl	0.1	7.9	5.13	P	1:2	5	0.54	No
0.04 *N* NaV-0.02 *N* HV-0.06 *N* NaCl	0.1	8.2	4.58	P	1:2	7	0.56	Yes¶
0.025 *N* $NaHCO_3$-0.1 *N* NaCl	0.125	8.2	6.64	S	1:4	4§	0.54	No
0.1 *N* NaV-0.02 *N* HV	0.1	8.6	3.03	P	1:2	7	0.81	Yes
0.1 *N* LiV-0.02 *N* HV	0.1	8.6	2.41	P	1:2	7	0.81	Yes¶
0.1 *N* NaOH-0.6 *N* glycine	0.1	9.0	3.48	P	1:2	6§	0.44	Yes¶

* With a dilution of 1:2 the new tall center section was used, otherwise the old short section.

† Cac = cacodylate.

‡ Partial precipitation of the proteins occurred in this buffer solvent.

§ Incomplete separation of α_1 from albumin.

¶ Separation was less complete than in 0.1 *N* NaV at pH 8.6.

‖ V = diethylbarbiturate.

Table 2. A summary of the electrophoretic analysis of human serum and plasma in a wide range of different buffers. (Longsworth, 35)

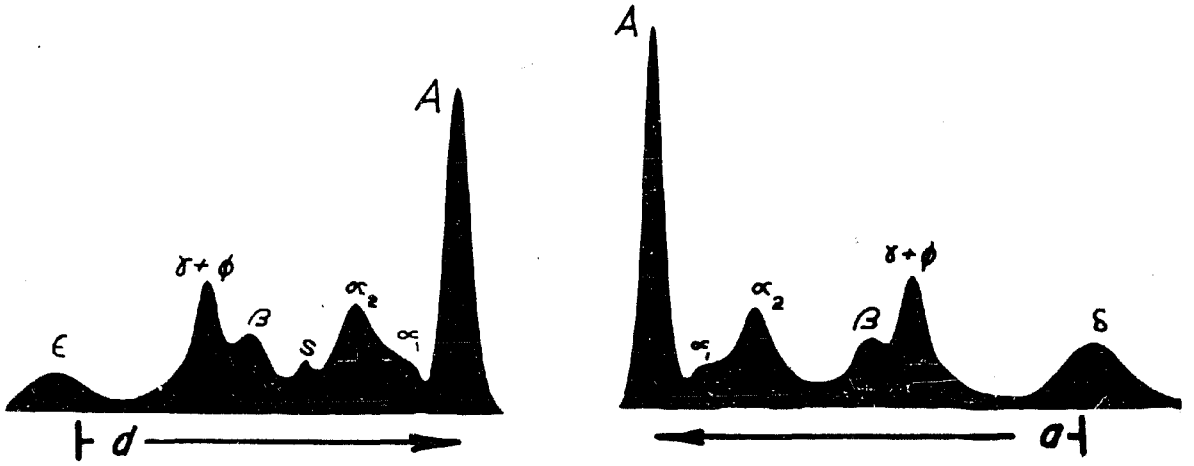


Figure 44. Electrophoretic boundaries of normal bovine plasma diluted to 1.5 per cent in barbiturate buffer of pH 8.6 and ionic strength 0.1. Electrophoresis carried out for 10,000 seconds at a potential gradient of 6.10 volts per centimeter. Boundaries photographed by the schlieren scanning method.

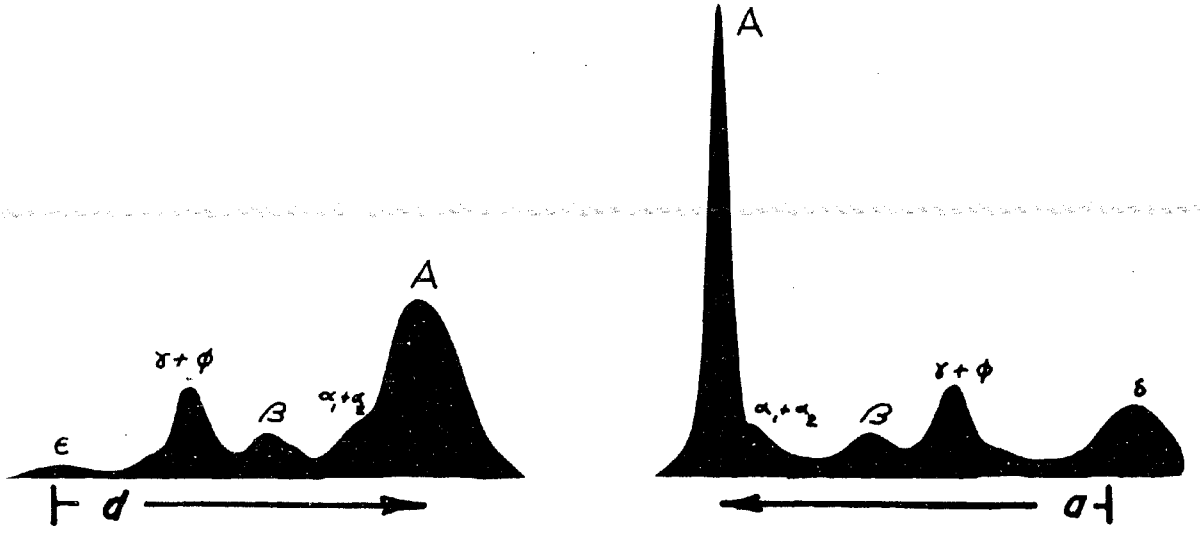


Figure 45. Electrophoretic boundaries of normal bovine plasma diluted to 1.5 per cent in phosphate buffer pH 7.7 and ionic strength 0.1. Electrophoresis carried out for 10,000 seconds at a potential gradient of 6.14 volts per centimeter. Boundaries photographed by the schlieren scanning method.

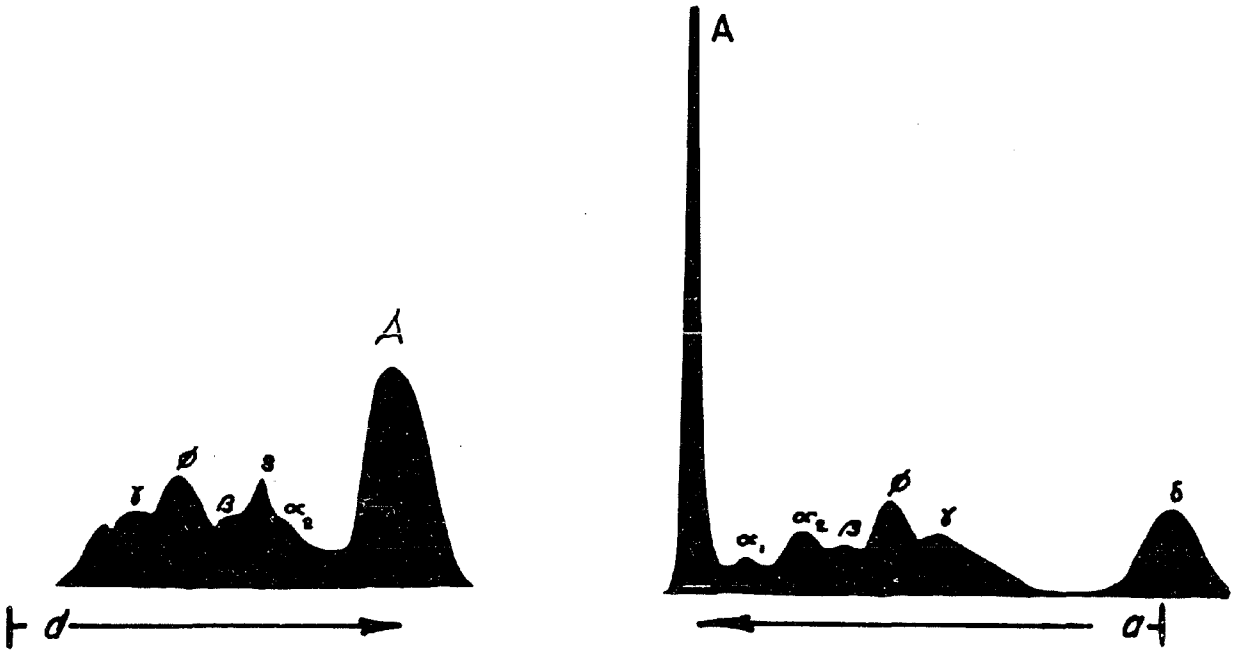


Figure 46. Electrophoretic boundaries of normal bovine plasma diluted to 1.5 per cent in carbonate buffer pH 9.9 and ionic strength 0.1. Electrophoresis carried out for 10,000 seconds at a potential gradient 6.08 volts per centimeter. Boundaries photographed by the schlieren scanning method.

total concentration of the acid. This is expressed as:

$$(H^{+}) = K \frac{(Salt)}{(Acid)} \qquad (14)$$

where salt and acid refer to concentrations and K is the apparent dissociation constant.

Hasselbach used Sörensens term pH and used the above relationship in its logarithmic form.

$$pH = pK - \log \frac{(salt)}{(acid)} \qquad (15)$$

Michaelis and Krüger (72) found that diluting phosphate buffers with water caused the reaction to become more alkaline which indicates that pK does not remain constant. Cohn (73) made an extensive study of this phenomena and found that pK varies with the ionic strength and is constant only at infinite dilution. He introduced the activity coefficients of the salt (γ^2) and acid (γ^1) into the Henderson Hasselbach equation, and accounted for these deviations. In place of $\log \frac{\gamma^2}{\gamma^1}$ a new value pK' can be introducted for pK and this new value is such that it corrects for the activity coefficients. (74)

$$pH = pK - \log \frac{salt}{acid} - \log \frac{\gamma^2}{\gamma^1} \qquad (16)$$

That neutral salts may have a marked effect on the pH of buffer mixtures has been demonstrated by Robinson (75). Tables have been prepared by Cohn (73) (76) and extended by Green (77) from which the molecular ratios of salt to acid can be obtained in preparing phosphate and acetate buffers of constant ionic strength and varying pH or constant pH and varying ionic strength.

For routine laboratory purposes the small correction due to the change in activity coefficients is neglected and the Henderson Hasselbach equation is used with the pK value to determine buffer

compositions. A list of the pK values of the more common acids used for buffer mixtures in electrophoresis work is as follows:

Acetic	- 4.64
Barbituric	- 7.90
Cacodylic	- 6.20
Glycine	- 2.35, 9.77
Phosphoric	- 6.77 (second dissociation)

Following are several examples of the type of buffer problems that arise in practice.

Example . Prepare a sodium acetate-acetic acid buffer of pH 4.34 and u 0.1 (pK acetic acid 4.64).

$$pH = pK + \log \frac{(salt)}{(acid)}$$

$$4.34 = 4.64 + \log \frac{(salt)}{(acid)}$$

$$4.34 - 4.64 = -.30$$

$$\text{logarithm } -.30 = \bar{1}.70$$

$$\therefore \text{ ratio of } \frac{(salt)}{(acid)} = .501$$

Since weak acids do not contribute appreciably to the ionic strength (69) the salt concentration has to be 0.1 M in order to give an ionic strength of .1

$$\frac{.1}{x} = .501$$

X = .1997-molar concentration of acetic acid. Therefore a mixture being .1 molar with respect to sodium acetate and .1997 molar tc acetic acid will have a pH of 4.34 and ionic strength 0.1.

The ionic strength of this solution may be checked using equation (13).

$$u = 1/2 \sum (.1) \times (1)^2 + (.1) \times (1)^2 = 0.1$$

Example 2. Prepare a sodium phosphate buffer of pH 5.59

and u 0.2. (pK second dissociation constant phosphoric acid 6.77)

$$5.59 = 6.77 + \log \frac{(salt)}{(acid)}$$

$$5.59 - 6.77 = \log \frac{(salt)}{(acid)}$$

$$5.59 - 6.77 = -1.18$$

$$\text{logarithm } -1.18 = \bar{2}.82$$

$$\therefore \text{ ratio of } \frac{(salt)}{(acid)} = .066$$

By substituting the value x (acid concentration) and .066 x (salt concentration) in equation 13 the molecular concentrations of acid and salt to be used may be calculated. Equation 13 must be used in making this calculation since the acid in this case contributes to the ionic strength. It will be recalled that the ions contributing to the ionic strength are.

$$Na_2HPO_4 \rightleftharpoons Na^+ + NaHPO_4^- \rightleftharpoons Na^+ + HPO_4^=$$

and

$$NaH_2PO_4 \rightleftharpoons Na^+ + H_2PO_4$$

Then:

$$0.2 = 1/2\ (x) \times (1)^2 + (x) \times (1)^2 + (.066x) \times (1)^2 + (.066x) \times (1)^2 + (.006x) \times (2)^2$$

$$0.4 = x + x + 0.066x + 0.066x + 0.264x$$

$$0.4 = 2.396x$$

$$x = 1.669 = \text{molar concentration of acid}$$

$$0.066 \times 1.669 = 0.0110 \text{ molar concentration of salt}$$

Example 3. Prepare a diethylbarbituric acid-sodium diethylbarbiturate buffer of pH 8.6 and ionic strength 0.2.

$$8.6 = 7.9 + \log \frac{(salt)}{(acid)}$$

$$8.6 - 7.9 = \log \frac{(salt)}{(acid)}$$

$$8.6 - 7.9 = 0.7$$

$$\text{logarithm of ratio} = 0.7$$

$$\therefore \text{ ratio of } \frac{(\text{salt})}{(\text{acid})} = 5.01$$

Since the acid does not contribute to the ionic strength the salt concentration has to be 0.2 M.

$$\frac{.2}{x} = 5.01$$

$$x = .0399 = \text{molar concentration of acid.}$$

However the salt is not soluble to the extent of 0.2 M. In a case of this type the buffer salt and acid concentration may be decreased in the same ratio and the ionic strength may be increased by using a neutral salt such as sodium chloride. In the above example we can use 0.1 M salt and .0199 M acid (the same ratio) and make the solution 0.1 M with respect to sodium chloride. This gives the desired buffer composition. The salt may be prepared by adding an equivalent of sodium hydroxide to an equivalent of the acid.

The calculation of ionic strengths is facilitated by the following generalizations. The ionic strength of any uni-univalent compound is equal to the concentration.

$$0.1\text{M } AB \rightleftharpoons A^+ + B^-$$

$$u = \frac{(.1) \times (1)^2 + (.1) \times (1)^2}{2} = .1$$

Likewise for a uni-divalent compound.

$$.1\text{M } A_2B \rightleftharpoons A^+ + A^+ + B^{--}$$

$$u = \frac{(.1) \times (1)^2 + (.1) \times (1) \times (1)^2 + (.1) \times (2) \times (2)^2}{2} = .3$$

A di-divalent compound has an ionic strength equal to 4 times the concentration

$$.1M \quad AB \rightleftharpoons A^{++} + B^{--}$$

$$u = \frac{(1) \times (2)^2 + (.1) \times (2)^2}{2} = .4$$

Table (3) shows the compositions of several different buffer mixtures that have been used by Longsworth and his associates. The barbital buffer of pH 8.6 and ionic strength 0.1 has been highly recommended as superior to other buffers for use with human serum. It resolves the various electrophoretic components appearing in the ascending and descending patterns into more symmetrical patterns of human serum than do other buffers, (78). Equally good results have been obtained in our laboratory on bovine, guinea pig, and fowl serum. The symmetry and asymmetry of patterns obtained with the various buffers under similar conditions of electrophoresis are illustrated in Figures 44, 45 and 46.

TABLE 3

BUFFER SOLUTIONS

Buffer Composition	pH	Ionic Strength	Gms/1000 ml.	Gms/1000ml.	Gms/1000 ml.
0.02 N HCl - 0.08 N NaCl	1.78	.1	HCl-.7294	NaCl-4.676	
0.1 N HCl - 0.5 N Glycine	3.05	.1	HCl-3.647	Glycine-37.535	
0.02 N NaAc-0.2N HAc - 0.08 N NaCl	3.62	.1	NaAc-16408	HaC-12.010	NaCl 4.676
0.02 N NaAc-0.1N HAc - 0.08 N NaCl	3.91	.1	NaAc-1.6408	HaC-6.005	NaCl 4.676
0.1 N NaAc - 0.2 N HAc	4.34	.1	NaAc-8.204	HaC-12.010	
0.1 N NaAc - 0.15 N HAc	4.47	.1	NaAc-8.204	HaC-9.0075	
0.1 N NaAc - 0.1 N HAc	4.64	.1	NaAc-8.204	HaC-6.005	
0.1 N NaAc - 0.02 N HAc	5.33	.1	NaAc-8.2404	HaC-1.2010	
0.02 N NaCac - 0.1 N HCac-0.08 N NaCl	5.42	.1	HCac-16.5588	NaOH-.8002	NaCl 4.676
0.1 N NaAc - 0.01 N HAc	5.65	.1	NaAc-8.204	HAC-.6005	
0.02 N NaCac - 0.02 N Hcac-0.08 N NaCl	6.12	.1	HCac-5.5196	NaOH-.8002	NaCl 4.676
0.02 N NaCac - 0.004 N HCac-0.08 N NaCl	6.79	.1	HCac-3.3117	NaOH-.8002	NaCl 4.676
0.02 N NaV - 0.02 N H V - 0.08 N Na Cl	7.83	.1	HV - 7.3676	NaOH-.8002	NaCl 4.676
0.1 N NaV - 0.02 N H V	8.6	.1	HV - 22.1028	NaOH-4.001	
0.1 N NaOH - 0.02 N Glycine	10.28	.1	NaOH-4.001	Glycine 1.5014	
0.1 N NaOH - 0.125 N Glycine	10.88	.1	NaOH-4.001	Glycine 9.3837	
0.1 N NaOH - 0.1 N Glycine	11.81	.1	NaOH-4.001	Glycine 7.507	

Ac = Acetate
Cac = Cacodylate
V = Diethyl Barbiturate

CONDUCTANCE MEASUREMENTS

In order to calculate the mobilities of the various components in an electrophoretic pattern it is necessary to first calculate the potential gradient maintained throughout the cell during the electrophoresis experiment (see section on calculation of mobilities). In order to make this calculation the specific conductance of the protein solution and buffer must be known. In this connection a brief summary on the theory and measurement of conductance is presented.

"...The passage of electricity in electrolytic conductors is characterized by the movement of matter, that is to say, by particles larger than electrons, in contrast to metallic conductance in which the movement of electrons alone is involved. The carriers of electricity have both positive and negative charges. In a solution of sodium chloride in water, a portion of the current is maintained by the movement of sodium ions in a positive direction, and another portion is due to chloride ions traveling in a negative direction" (79).

The specific resistance of an electrolyte may be defined as the resistance in ohms of a column of solution 1 cm. long and 1 sq. cm. in cross section.

Specific conductance L is the reciprocal of specific resistance (80).

$$L = \frac{1}{R} \qquad (17)$$

Since specific conductance is the reciprocal of specific resistance and is expressed as reciprocal ohms, indirect methods may be employed to measure the conductivity of a solution. If the resistance of a solution to the passage of an electric current between two electrodes immersed in the solution is determined, the specific conductance can be

calculated. A diagram of a Wheatstone bridge apparatus adaptable to such measurements is shown in Figure 47. The current passes from S to A where part of it is allowed to pass through the unknown resistance R_1 (solution in cell) and the known adjustable resistance R_2. The rest of the current passes through the slide wire resistances R_3 and R_4. D is the current detector (earphones or some suitable device) and when it shows that no current is passing between C and F the following relationship holds.

$$\frac{R_1}{R_2} = \frac{R_3}{R_4}$$

$$R_1 = R_2 \cdot \frac{R_3}{R_4} \qquad (18)$$

Since any current that passes from an electrode to an electrolyte causes a chemical reaction, special precautions have to be taken or the current used in making the resistance measurement will cause a chemical change in the solution. These changes cause variations in the conductance and new potentials are developed at the electrodes. The term used to describe these changes is polarization. To overcome this polarization effect a high frequency oscillating alternating current is employed. In this manner any change that occurs in the positive direction tends to be immediately reversed in the negative direction.

In our laboratory the current source of the conductivity apparatus is maintained from three dry cells connected in series. The current passes through an audio oscillator (Type 213-General Radio Co) which gives a high frequency alternating current of a pure sinewave type.

The conductance cell itself Figure 48 is of the Shedlovsky type (41), (82) (Hopf Apparatus Co). "The electrodes c-c are hollow

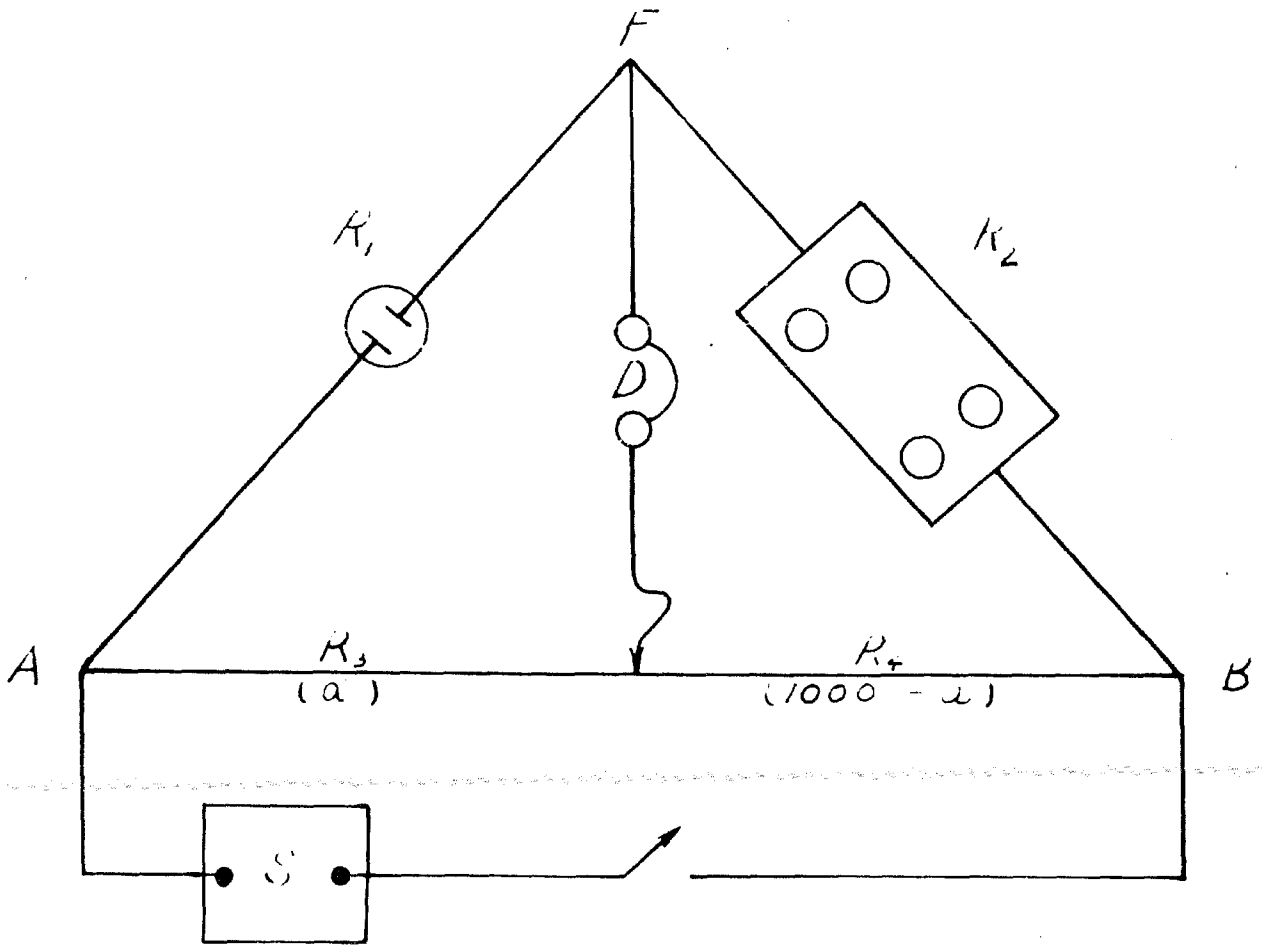


Figure 47. Diagram of a Wheatstone bridge apparatus used for conductance measurements. Explanation in text.

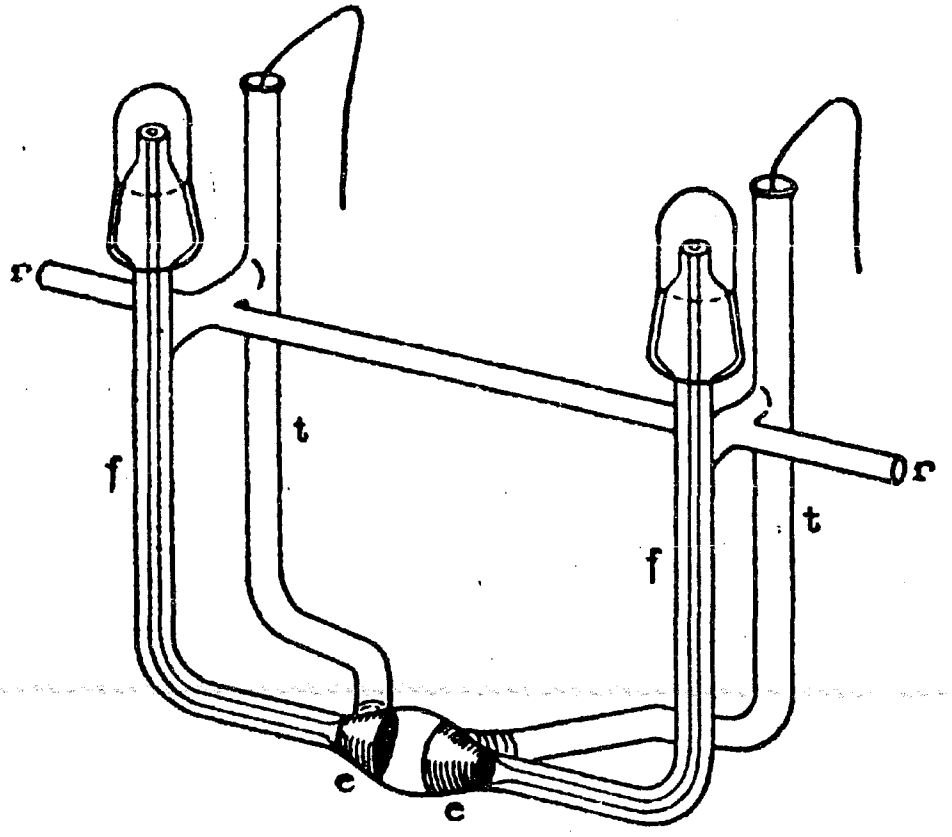


Figure 48. Shedlovsky type conductance cell. Description in text. (Longsworth, Shedlovsky and MacInnes, 82)

platinum cones and are sealed to the glass walls of the cell, and make contact with mercury in the tubes t-t by means of platinum wire fused into the glass. Due to the relatively unimpeded flow when the cell is filled by means of the tubes f-f the protein solutions do not foam. The glass rod r-r is used for strengthening and the projections are used for supporting the cell."

A cell of this type is easily filled by means of a glass syringe and stainless steel needle.

Since it is not practicle to prepare a conductivity cell whose electrodes are exactly 1 sq. cm. in area and exactly 1 cm. apart, equation (18) cannot be used to calculate the specific conductance. However the cell can be standardized using a solution whose specific conductance is known and the equation is then applicable. Potassium chloride is well suited for a standard since, (1) it is easily purified, (2) is non hygroscopic, (3) sufficiently soluble, (4) stable both in solid form and in solution, and (5) is non poisionous to the electrode. Unfortunately the experimental results reported by various workers for the specific conductances of potassium chloride standard solutions vary widely. Jones and Bradshaw (83) have very carefully and painstakingly redetermined the values of several concentrations of potassium chloride. They point out that since atomic weight values change from time to time, and are very apt to change in the future, molar, normal etc. solutions should not be used as standards. They define their standards in terms of weight of potassium chloride per kilogram of solution corrected to vacuum. Their one "demal" solution contains 71.1352 grams of potassium chloride per 1000 grams of solution in vacuum. In preparing standards these authors recommend that the use of "ultra-pure" conductance water be avoided and

that conductivity water prepared in contact with the laboratory air be used. They prepared suitable conductance water by sweeping out the conductance water container with air feed from carbon dioxide and ammonia and by bubbling the air through the water. In practice it has been found that if distilled water is redistilled in a seasoned glass vessel and condenser with ground-glass joints water can be obtained with a specific conductance of about 1×10^{-6} reciprocal ohms if a small amount of potassium permanganate is added to the distilling flask (84). Carbon dioxide can be removed as previously suggested. The constant values for several of their standard solutions are shown in table 4.

Table 4

Specific Conductance of Standard Potassium Chloride Solutions in Ohms $^{-1}$ cm. $^{-1}$

Concentration (Demal)	Grams KCl per 1000 g. of soln. in vac.	Specific Conductance 0°C	18°C	25°C
1D	71.1352	0.065176	0.097838	0.111342
0.1D	7.41913	.0071379	.0111667	.0128560
0.01D	.745263	.00077364	.00122052	.00140877

The specific conductance of the water was determined beforehand and subtracted from that of the standard solution.

The following equation may be used for correcting weights to vacuum (85) (86)

$$M = m + mda \left(\frac{1}{dm} - \frac{1}{dw} \right) \qquad (19)$$

Where M = wt. corrected to vacuum
m = apparent mass
da = density of the air
dm = density of the mass (m)
dw = density of the weights

Having determined the resistance of a known standard solution, at a definite temperature, the cell constant K can be calculated by means

of the equation.

$$K = LR \qquad (20)$$

or

$$K = R\left(\frac{a}{1000-a}\right) L \qquad (21)$$

Assuming the slide wire Figure 47 to be divided into 1000 divisions the specific conductance of an unknown solution can now be determined from its observed resistance, by the following equation.

$$L = \left(\frac{1000-a}{a \cdot R}\right) K \qquad (22)$$

The cell constant should be rechecked occasionally, using a standard solution.

It has been found advisable to clean the platinum electrodes thoroughly before restandardizing. Concentrated sodium hydroxide or concentrated sulfuric acid plus a few drops of perhydrol have been used satisfactorily as cleansing agents.

Considerable difficulty is often encountered in duplicating results with the earphone method of determining the null point. This is due to the fact that the null point appears to cover several scale units on the slide wire. An electronic null point indicator using a J5 electron tube (87) (88) has been modified and adapted (89) for determining the point at which the bridge is balanced. The wiring diagram of this apparatus is shown in Figure 49. The point of balance is indicated visually by means of a fluorescent target. This method of determining the null point is very accurate and varying the resistance R_2 as little as 0.1 ohm causes a visible change on the target.

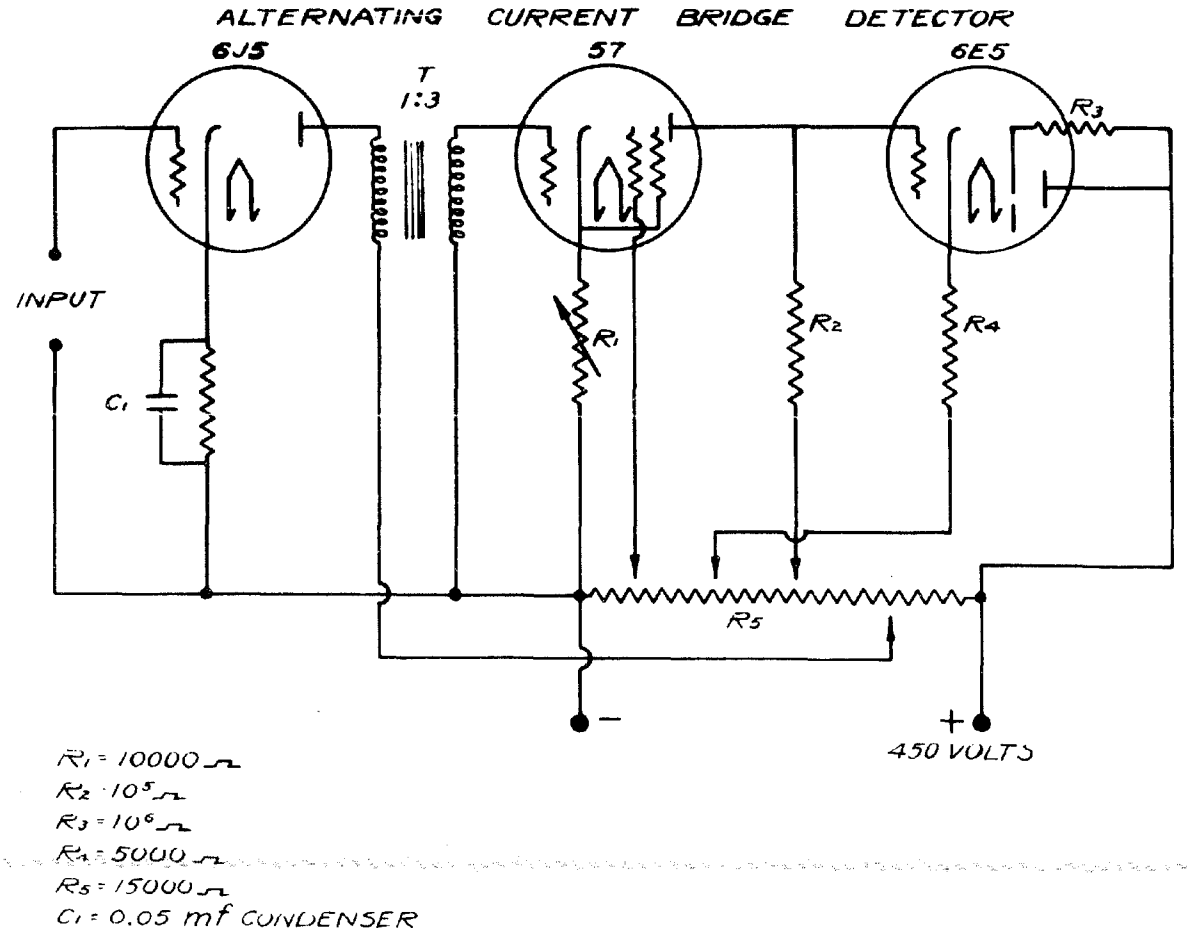


Figure 49. Diagram of an electronic null point indicator using a J5 electron tube. (Clark 89)

In order to improve this accuracy of the measurement the slide wire may always be set at 500 and the null point adjusted by means of the resistance box. With this ratio of 1 - 1 for the slidewire the specific conductance is calculated from the equation.

$$L = \frac{K}{R} \qquad (23)$$

A Kohlraush slide wire bridge (Leeds and Northrup #404669) in conjunction with a 1000 ohm variable resistance box (Leeds and Northrup #167699) completes our conductance equipment.

A consideration of the "Donnan equilibrium" will show that the conductance of the two solutions (buffer and protein) will always vary slightly even after prolonged dialysis of the protein solution against the buffer. This is due to the fact that the protein solution contains protein ions which cannot diffuse through the semipermeable membrane.

Two gases separated by a membrane permeable to both will diffuse through the membrane in both directions, so that at equilibrium the mixture will have the same composition on both sides of the membrane. If two solutions of different concentration are separated in the same manner and if the membrane is permeable to both solute and solvent, equilibrium will be attained when the liquid on both sides of the membrane is identical.

Donnan found that very different conditions prevail when the membrane is impermeable to one of the ions (90). Consider a protein in solution above its isoelectric point being dialyzed against a potassium chloride solution. The original state can be represented as

$$\begin{array}{c|c} Na^+ & K^+ \\ P^- & Cl^- \end{array}$$

and the state of equilibrium as.

Na +	K +
K +	Na +
P -	Cl -
Cl -	

The osmotic pressure of the two solutions are equal but the system containing the protein ions will have a lower conductance. Small differences in pH between the two systems can also be explained by in this manner.

Inasmuch as the electrophoresis cell is maintained near 0°C during a run the conductance is measured with the conductance cell in a Dewar flask containing ice and water. The conductance of both the protein solution, K_P, and the buffer solvent, K_B are determined and the two averaged together for purposes of calculation.

TREATMENT OF ELECTRODES AND ASSEMBLING AND FILLING CELLS AND ELECTRODE VESSELS.

Current is supplied to the electrode vessels from the silver-silver chloride electrodes at E and E', Figure 56. These electrodes are shown in detail in Figure 50. They are composed of a flat strip of corrugated silver sheet wound in a tight spiral. The ends of the spiral are anchored into the core with silver screws. A hollow silver tube insulated by a glass tube is also threaded into this core.

When an electric current is passed through the electrodes an oxidation takes place at the anode and a reduction at the cathode. In the presence of potassium chloride, chlorine ions lose an electron and are deposited on the silver anode. While at the cathode, chlorine ions are given off to the surrounding solution as the chlorine atoms in the silver chloride electrode gain an electron and become ions. Because one side of the assembled cell is closed off and the other remains open, the fittings on the electrodes are different and cannot be used interchangably. It is then necessary to occasionally reverse the current through the electrodes to remove chloride from the anode and deposit it on the cathode. In carrying out this operation the electrodes are immersed in N HCl in separate leakers connected with a liquid bridge and the former cathode is now made the anode. A direct current of 10 milliamperes is then passed. Hydrogen will be liberated at the cathode after the electrode becomes dechloridized and an adherent brownish purple deposit of silver chloride forms at the anode. After a time gas begins to be liberated at the anode also. When this occurs the connections are reversed and the silver chloride deposited on what was origionally the cathode until it begins to gas. The connections are then reversed and another cycle begun.

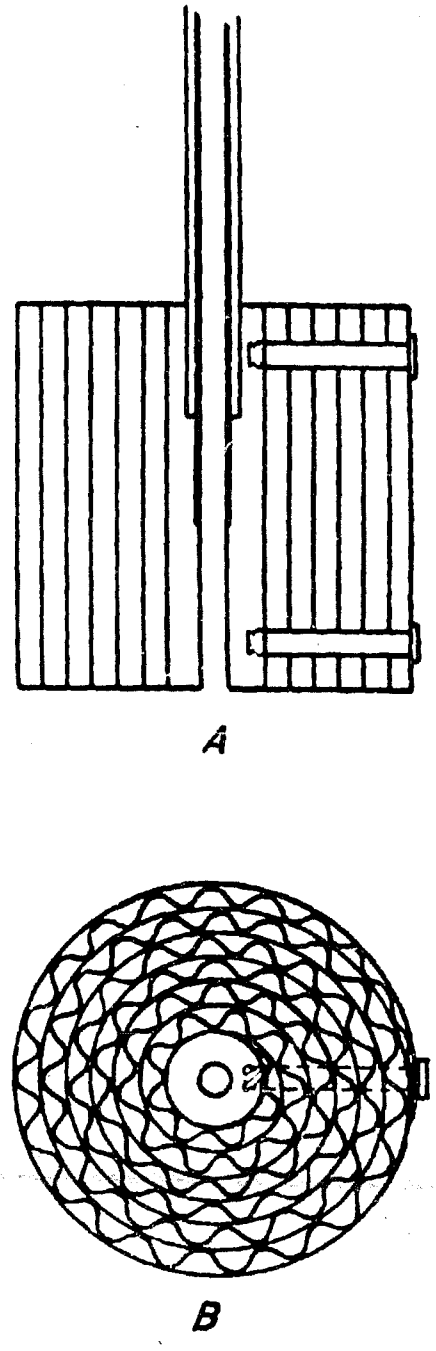


Figure 50. Detailed sketch showing electrode construction. A, side view and B, top view. (Longsworth and MacInnes, 67)

This is repeated until the desired capacity is obtained. It will be observed that with each cycle more silver chloride can be formed before gas evolution begins at the anode than during the previous cycle. Moreover, as the deposit of available silver on the one electrode and available silver chloride on the other are thus built up, heavier currents can be carried by the electrodes without blistering or peeling of the deposit. Longsworth (91) using this method has built up electrodes with a capacity of 500 milliampere hours that are capable of carrying currents of 100 milliamperes without blistering.

In our laboratory electrodes with a capacity as high as this have not been obtained with the above method. However, by using the same arrangement and starting with a low current, say 5 to 10 milliamperes, and reversing the current when gassing occurs at the anode and gradually increasing the current up to 50 to 60 millamperes, capacities of the above order have been built up.

If one is chloridizing new silver electrodes they should be momentarily immersed in concentrated nitric acid followed by thorough rinsing. This cleans the electrodes and etches the silver surface slightly and favors the formation of an adherent deposit of silver chloride (69). Old electrodes can be cleaned, but with loss of silver by dissolving the silver chloride in a concentrated solution of potassium cyanide followed by the nitric acid treatment (69). Grease is easily eliminated from electrode surfaces by dipping in an organic solvent such as ether or acetone followed by distilled water. The electrodes should be immersed in distilled water when not in use.

There are two widely used types of electrophoresis cells. The four piece separation cell designed by Tiselius and the three piece cell designed by Longsworth. These cells are diagramatically shown in Figure 51.

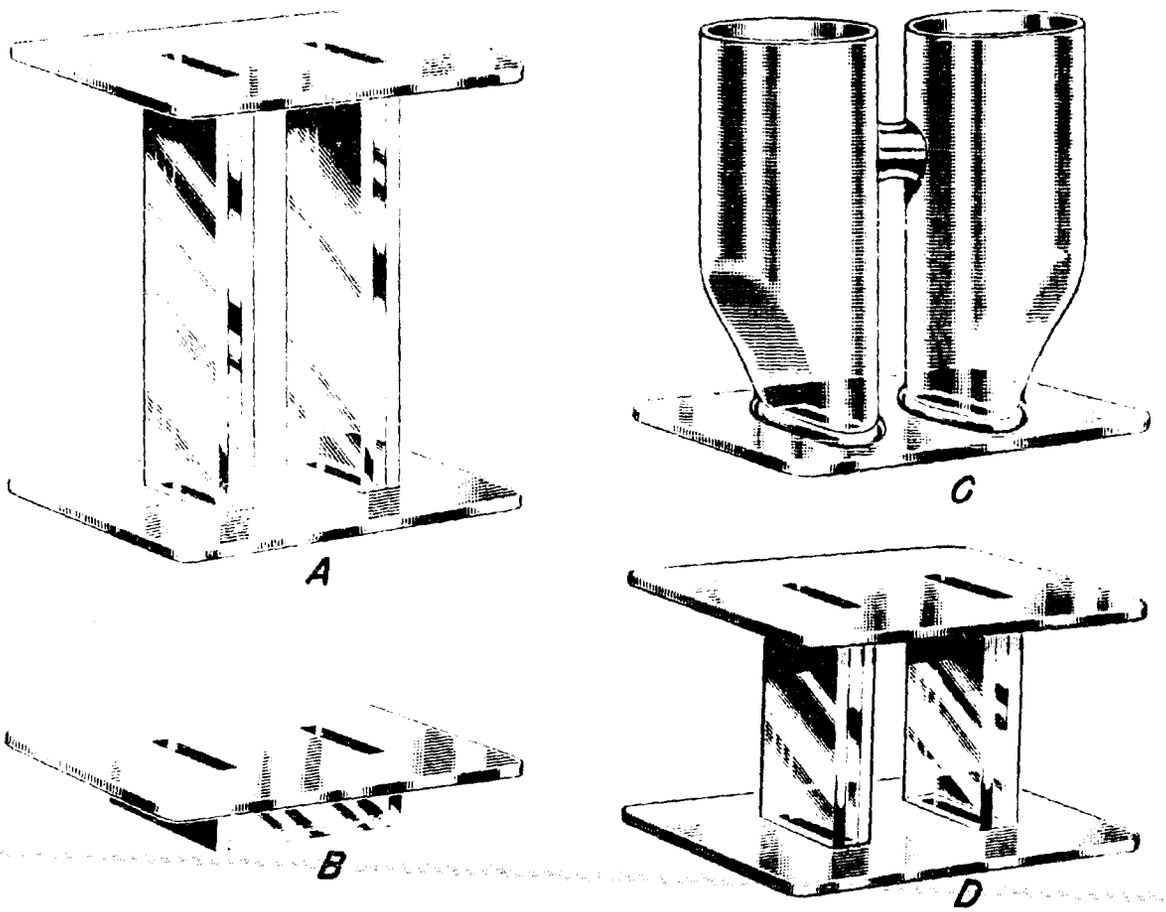


Figure 51. Diagramatic drawing of electrophoresis cells. B, bottom section, and C, top section used with A, single center section or D, double center section for separation studies. (Courtesy Klett Mfg. Co.)

The top and bottom sections are the same for either cell, but with the separation cell the single middle section is replaced with two smaller sections. Both types of middle sections are shown in the above figure.

Figure 52 shows a diagramatic drawing of the assembled four section cell that was first employed for separation studies and illustrates the method of assembling the cells. Each section is connected to the other by means of flat horizontal ground glass plates that slide from side to side at the planes a-a', b-b', and c-c'. A uniform U shaped channel d-d' of rectangular cross section runs through the cells. A top view of the center section is shown in the lower part of the figure. The other type of cell Figure 53 has the center two sections incorporated into a single section with the elimination of the b-b' plane. The rack an pinion device designed by Longsworth (92) and used to slide the cells from side to side in order to separate the channels is shown in Figure 54. "The rod a, of Figure 54 -a, carries a pinion, p, which engages the rack, r, carried by the collar, c. The rod, a, slides vertically through holes in the supporting frame while a tongue, t, Figure 54 -b, of the collar moves in a groove, g, in the bar, b, and prevents the collar from rotating when the rod is turned. The rod a', Figure 54 -a, carries a similiar rack and pinion arrangement for displacement of the cell sections to the left. The racks, r, and r', are provided with pins to limit their movement."

In assembling the cells some provision has to be made for sealing the plates of the separate cells together and at the same time permit the cells to slide from one side to the other. Tiselius recommends the use of vaseline thinned with paraffin oil (2 parts to 1) for this purpose. However, this proportion has to be varied depending upon the viscosity of

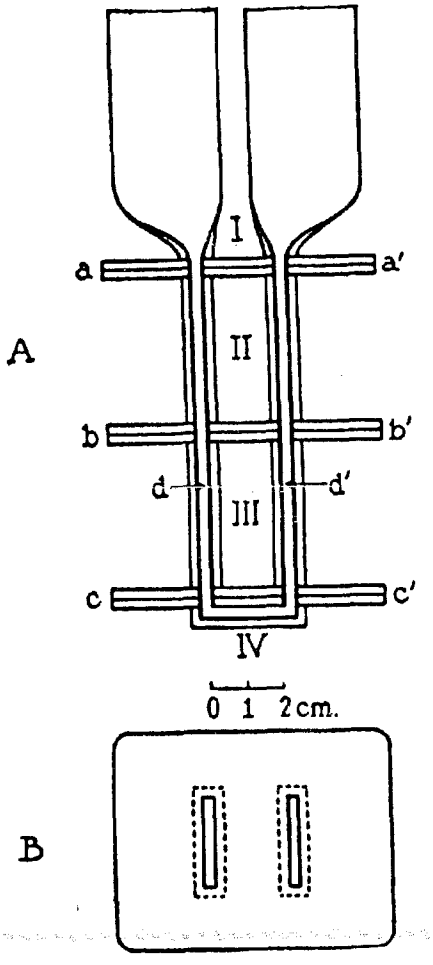


Figure 52. Diagramatic view of 4 piece separation cell, I, top section showing arms used to connect cell with electrode vessels. II, and III center sections of cells attached at planes a-a', b-b', and c-c'. IV, bottom section of cell. B, top view of bottom section. (Longsworth and MacInnes, 67)

Figure 53. Electrode vessels and cell in supporting carriage. (Courtesy Klett Mfg. Co.

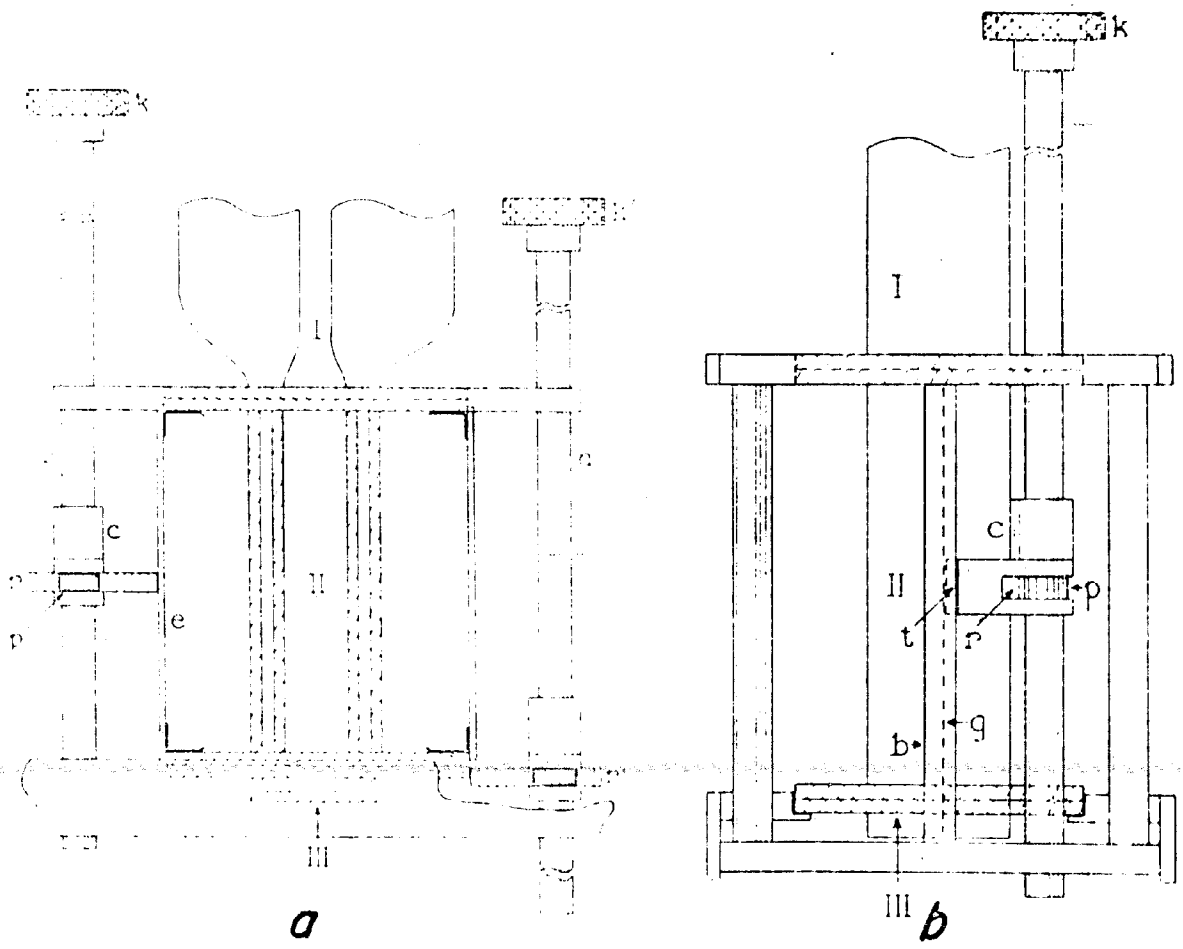


Figure 54. Section of cell support illustrating the rack and pinion device used to displace cell. (Longsworth, Cannan, and MacInnes, 92)

the particular lot of vaseline used. If 2 parts of medicinal vaseline are melted and one part of paraffin oil added, and the mixture allowed to cool a suitable lubricant may result. On the other hand the preparation may not be viscous enough to seal the plates of the cell together or the mixture may be so viscous at zero degrees that it does not permit the various parts of the cell to be slid over each other. In either case a suitable viscosity may be obtained by remelting and adding either vaseline or paraffin oil. Stern (7) recommends the use of "celloseal" for this purpose.

In assembling the cell, the upper surface of the lower section is covered with a thin film of the lubricant. This is worked around the surface with a finger, being careful to keep it out of the channel. The lower surface of the center section is then treated in the same manner. These two surface are then superimposed and gently slid over each other until all air bubbles are removed and the two sections are sealed together. The top section of the cell is then attached in the same manner. The two metal clips used in sliding the cell sections over are then inserted in the position shown in Figure 54. These clips serve to evenly distribute the pressure from the rack and pinion device. The assembled cell is then placed in the supporting frame, Figure 55 and secured in position by spring clips. The electrode vessels are then fastened in their respective positions in the frame. Figure 56 shows the cells and electrode vessels in place in the supporting carriage. The top section outlets of the cell are connected to the arm of the electrode vessels by means of neoprene rubber sleeves (Pioneer Rubber Co., Willard, O.). To facilitate slipping the sleeves on, and to insure water tight insulating connections lubricant is smeared on these sections before the sleeves are attached.

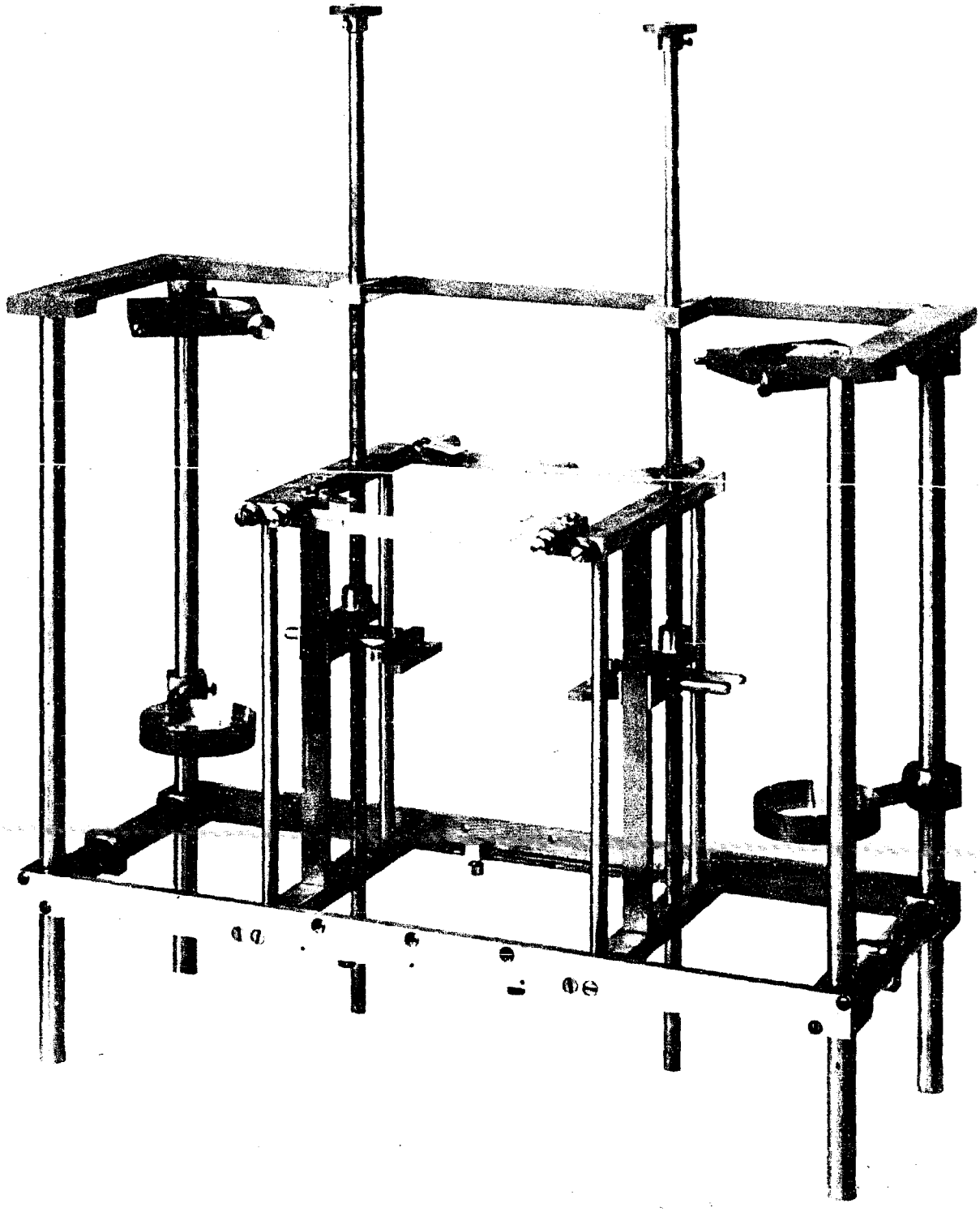


Figure 55. Supporting carriage and rack and pinion device. (Courtesy Klett Mfg. Co.)

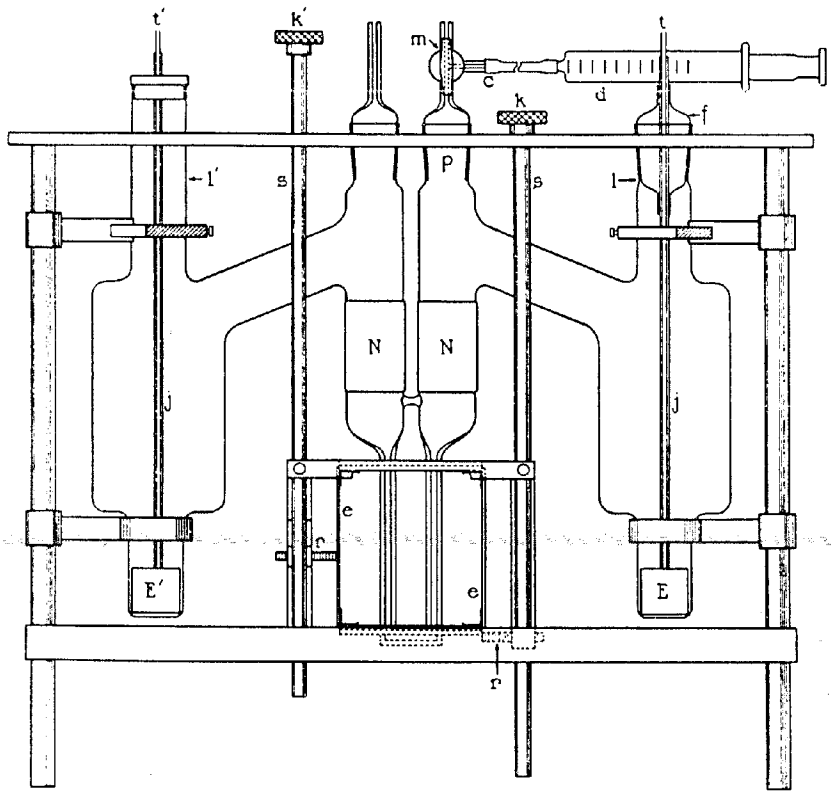


Figure 56. Drawing of carriage support with cells, electrodes, and electrode vessels in position. Neoprene rubber sleves, N, are used to connect the top arms of the cell to the side arms of the electrode vessels. (Longsworth, 35)

The complete assembly is shown in place in the water bath in Figure 57. Milled brass strips placed across the top of the water-bath suspend the supporting frame.

Figure 58 illustrates the method of filling the assembled cells. As shown in Figure 58A, the lower section of the cell is first filled with the sample. A twenty ml. glass syringe attached to a long 18 gauge stainless steel needle (MacGregor Inst. Co.) is very convenient for this purpose. The carriage is then placed in the water bath and left until the sample has reached the temperature of the bath as evidenced by no further contraction of the liquid in the cell. Before removing from the bath, the lower section is shoved to the left until it is isolated from the center section. This is accomplished by the rack and pinion device. A slight positive pressure is applied to the center section in the opposite direction of the pressure being applied to the lower section. This precaution should be observed in all operations of the above type. The carriage is then removed from the bath and the left channel is washed three times with buffer solution to remove traces of the sample. The right channel is filled with the sample to a point above the middle and top section joining plane. Buffer is introduced to the same extent in the left channel. The electrode vessels are now filled up to the arms with buffer. A one liter erylenmeyer flask equipped with a rubber bulb for forcing the buffer out helps to prevent formation of bubbles in the electrode vessels during the filling operation. The carriage is again placed in the water bath until the temperature of the sample and buffer have reached that of the water-bath (0°C). The center section of the cell is now shoved to the right until it is isolated from the top section. The bottom section is then shoved back to its original position as in Figure 58B. The remainder of the sample in the upper section

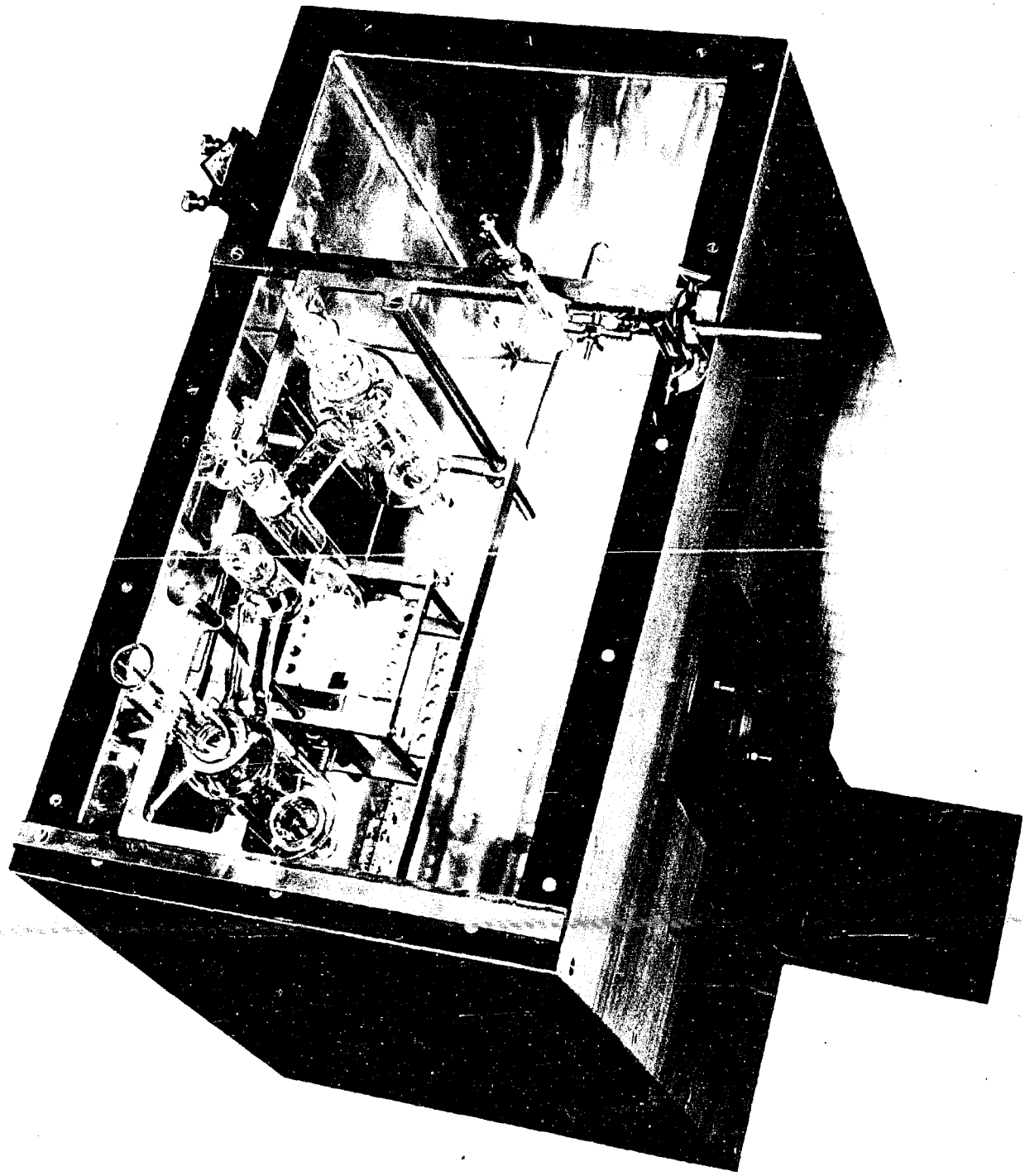


Figure 57. Top view of water bath with thermostat and assembled cells in place. The supporting carriage is suspended from the milled brass strips. (Courtesy Klett Mfg. Co.)

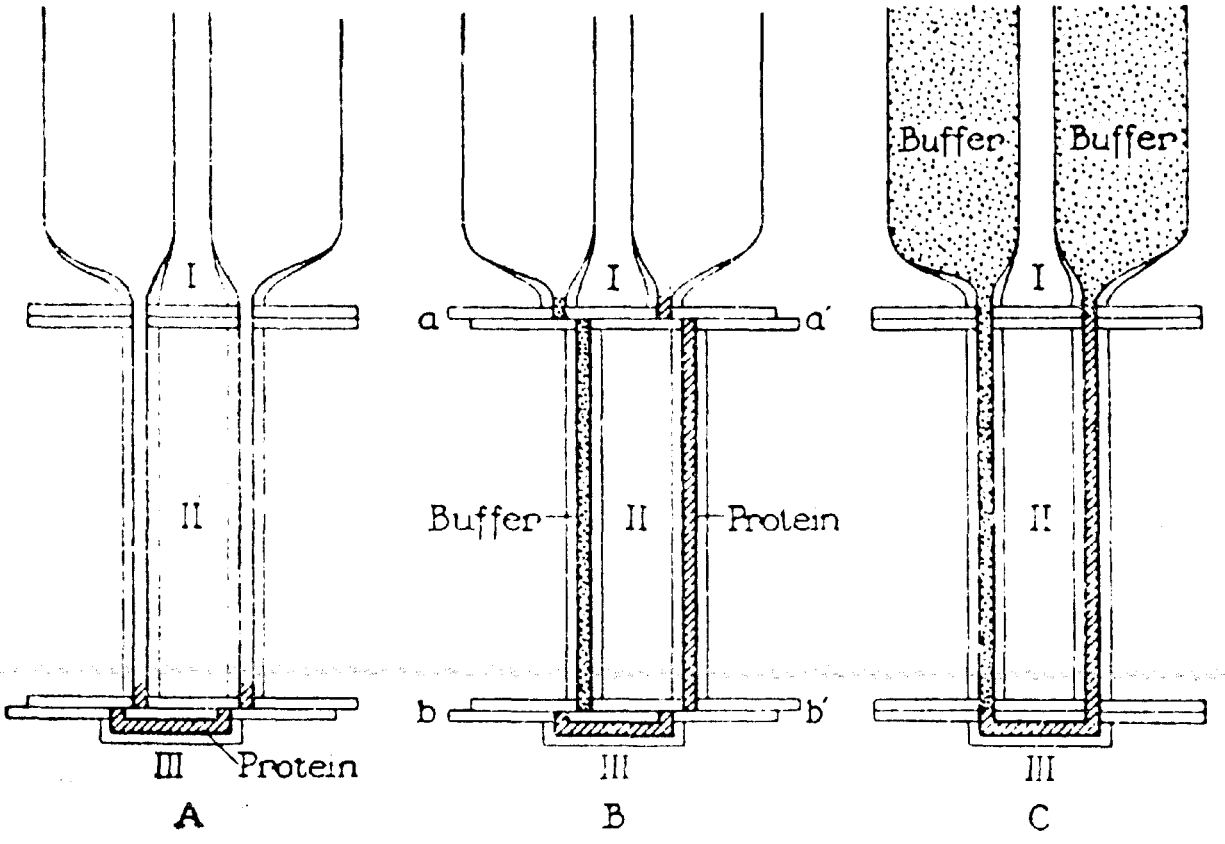


Figure 58. Diagram of assembled electrophoresis cells illustrating the method of filling the cells. Discussion in text. (Longsworth, 35)

of the cell is now washed out with buffer. The electrode vessels are then completely filled with buffer solution. As the vessels fill up buffer runs over into the cell and fills the top sections. The electrodes are then rinsed with buffer and inserted in place after lubricating the ground glass joints. After submerging, the electrode in the filled electrode vessels, they are shaken to remove any air bubbles. The three way stopcock and insert are then lubricated and placed in the electrode arms as shown in Figure 53. The carriage is now placed in the water bath. Any air bubbles in front or behind the cells are removed using a wire with cotton on the end. The carriage is shoved as close to the schlieren lens as possible. The syringe shown in Figure 18 is removed and filled with buffer, and a rubber tubing attached to it. It is then put back into position and the end of the rubber tubing attached to the arm of the three way stopcock. Buffer can then be forced into the electrode from the syringe as the buffer contracts due to cooling. A rubber tubing is attached to the top portion of the three way stopcock to serve as an overflow.

At this stage it is necessary to surround the electrodes with N potassium chloride solution. This is necessary if the buffer is to maintain a constant composition during the run. Any changes resulting from electrolysis will then occur in the potassium chloride solution. Saturated potassium chloride is not desirable for this purpose. In the first place, the electrolysis affects a net transfer of potassium chloride from one electrode to the other so that super-saturation and crystallization may occur. In the second place, saturated potassium chloride has a solvent action on silver chloride.

It will be recalled from the discussion on electrode construction that the silver tube is hollow. A 25 ml. pipette with a stop-cock attached and supported by an arm which can be screwed to the carriage is used to run the cold potassium chloride solution slowly into the electrode vessel through the hollow silver tube. Twenty ml. is sufficient to completely surround each electrode. A rubber stopper is placed over the silver tube on the closed side after adding the potassium chloride to completely seal this side of the apparatus during electrophoresis.

As soon as the sample and buffer have been equilibrated to the temperature of the water-bath the stopcock on the closed side is turned so that the cell on this side is completely closed. The center section of the electrophoresis cell is then pushed over so that the three sections are in alignment with each other (Figure 58C). A mask with two vertical slits, the width and length of the rectangular channels of the center section, is then placed over the schlieren lens and is lined up with the center section of the cell. This mask is so constructed that either of the slits can be covered independently of the other and is used to mask out scattering rays of light from one side of the cell while the other side is being photographed.

The light source is turned on and the schlieren diaphragm is lowered to such a position that no rays of light passing through the camera objective are intercepted. A mask with two vertical slits with dimensions slightly larger than the channels in the center section of the cell is placed in the viewing end of the camera barrel. The cell channels are then viewed through the slits on a ground glass plate inserted in the plate holder carriage. The light coming through the channels should be centered within these slits. $T_h$is alignment is adjusted by sliding the

center section of the cell to the right or left. After the proper alignment has been made the boundaries are shoved out from behind the plated holding the sections of the cell together. This is accomplished by turning the three way stopcock so as to connect it to the compensation syringe. At the same time the motor driving the plunger is turned on and this slowly pushes the boundaries into view. The position of the boundaries can be followed by viewing the ground glass plate. The boundaries are pushed out from 1.5 to 2.0 centimeters. The motor is then turned off and the same time the stop-cock is turned so that the closed side is completely sealed. The initial boundaries are then photographed as described elsewhere. One may now apply the proper potential to the electrodes for electrophoresis.

BOUNDARY COMPENSATION

One of the outstanding features of the new apparatus described by Tiselius was the provision for the so-called "compensation movement," the theory of which was described by him as follows (23):

"When both substances migrate in the same direction the possibility of sufficient separation is limited by the fact that long before the desired separation has been reached, both substances have migrated, out of the electrophoresis tube. This is a somewhat serious limitation, since the absolute differences in mobilities are very often much larger at pH regions where both components have mobilities of the same sign (e.g. the serum proteins). Moreover, lack of solubility often prevents a choice of pH between the isoelectric points. For this reason we arranged for a slow and uniform movement of the solution in the electrophoresis tube at an exactly known rate and in a direction opposite to the migration, by slowly lifting a cylindrical glass tube by clockwork out of the liquid in one electrode tube during the electrophoresis. If the rate at which the tube is lifted is 1 cm. per hour, its cross-section area p cm^2, the free surface of the liquid in each electrode tube Q cm^2, and the cross-section area of the electrophoresis tube q cm^2 than a movement of a given level in this tube will take place, at a rate of

$$\frac{lpQ}{q(2Q-p)} \text{ cm. per hour} \qquad (24)$$

By suitably choosing l and p any desired rate can be obtained; even in the narrow tubes used in our apparatus a rate of several centimeters per hour did not markedly blur the boundaries in the electrophoresis tube.

For fractionation purposes, this "compensation movement" is adjusted so that the observed boundary separating two fractions obtains a suitable "apparent mobility" and consequently, at the end of the run, the column of the solution can be cut off exactly at the right place..."

As an example of the use of this "compensation movement" consider a protein solution containing two types of impurities. One type of impurity has a faster mobility than the protein and the other a slower mobility. If the shift in the level of the boundary of the protein is offset by the change in hydrostatic pressure caused by the compensation drive the boundary will remain stationary in the tube. The impurity with the faster mobility continues migrating in the same direction but at a slower rate and the slower impurity assumes an apparent reversal of sign and migrates in the opposite direction. By using a separation cell the pure protein component can be isolated. Tiselius called attention to another useful application. Thus if two proteins of different mobilities are to be separated the arithmetic mean is calculated and the compensation rate adjusted to this mean. The proteins then migrate in opposite directions. The "compensation movement" extends the separation capacity of the apparatus to its fullest extent.

Figure 59 from Longsworth (67) illustrates the use of "compensation movement" in electrophoretic separations. Three components A, B, and C moving with the (positive) relative mobilities A B C are present. Movement of the lower section of the cell from position a, to position b, brings the protein solution in contact with the buffer solution at the plane, α. Passage of an electric current then causes the components to separate as shown in Figure 59b. However, as illustrated in Figure 59b before any

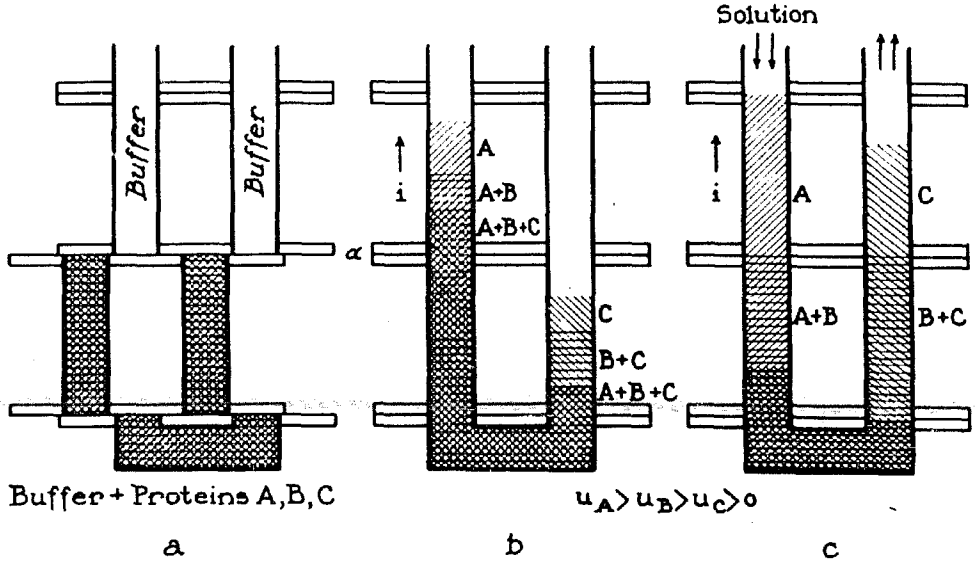


Figure 59. Diagram illustrating the ideal electrophoretic separation of a protein mixture. Explanation in text. (Longsworth and MacInnes, 67)

large proportions of A and C have been separated, the boundaries will have migrated out of the cell in one case and into the bottom section in the other. By applying a compensation movement, as indicated in Figure 59c, the boundaries due to mixtures of A + B and B + C (Figure 59b) can be given an apparent velocity of zero. This leads to the separation of pure A in one side of the cell (cathode). The "compensation movement" gives C an apparent negative mobility and it can be recovered in a pure form from the upper anode section.

Several different devices have been used to bring about this "compensation movement". As previously mentioned Tiselius used a clock-work motor with variable speeds to lift cylindrical rods of glass out of one electrode vessel. Stenhagen (93) slowly injected buffer into one of the electrode vessels to push the boundaries out before the current is turned on. Smith (94) withdrew mercury in such a manner as to accomplish the same. Longsworth has closed one electrode vessel and moves the boundaries out or carries out "compensation movement" by forcing buffer into the closed side of the cell by means of the synchronous motor and syringe (Figure 18). By virtue of the exchangeable transmission gears driving the syringe the rate of compensation can be varied.

HEATING EFFECTS AND CONVECTION CURRENTS

The resolving power of an electrophoresis apparatus is limited by the magnitude of the potential gradient which may be applied without causing appreciable thermal convection currents due to heat developed within the solution in the cell. The heat flows to the thermostat through the walls of the cell. The temperature of the solution along the axis of the tube is thus warmer than at the cell wall. Normally therefore, the solution at the cell wall will be heavier, and in falling will give rise to convection currents. Longsworth and MacInnes (67) have investigated these effects in the following way:

"We shall consider, as a typical example, a current of 0.006 ampere passing through a solution the specific conductance of which is .0038 mhos (0.1N sodium acetate buffer at 0°C) in a cylindrical tube of 5 mm. internal and 10 mm. external diameter. The formula which describes the temperature of the solution, ts, in the steady state as a function of the distance, r, from the axis is

$$t_r = 0 - t_s = \frac{I^2 \epsilon}{4 k K_s} r^2 \qquad (25)$$

The corresponding formula for the temperature of the glass, t_g, is

$$t_g - t_o = \frac{a^2 I^2 \epsilon}{2 k K_g} \ln \frac{b}{r} \qquad (26)$$

In these equation a and b are the inside and outside radii, respectively of the tube, Ks and Kg are the thermal conductivities of the solution and glass, I is the current density, ϵ is the electrical equivalent of heat and t_o is the thermostat temperature. Using equation 25, the solution along the axis of the tube is 0.65° hotter than at the wall and from

equation 26 the drop in the wall is 0.67°C. The computed temperature distribution is given in Figure 60, in which the temperature increase, Δt over that of the thermostat is plotted against the distance from the axis of the tube. If the thermostat is regulating at 25°C., this temperature gradient in the buffer solution is accompanied by the density variations shown in Figure 60 and, as has been stated, it is these differences which cause mixing by convection currents, of the solution in the tube. If on the other hand the thermostat is regulating at 0°C., the density differences in the solution are much less and the variation is in the opposite direction, as shown in Figure 60. The contrast between the curves 60b and 60c arises from the fact that this buffer solution has a maximum density at 2.85°C. If, in this particular example, the thermostat temperature were regulated at 1.85°C., the average temperature in the tube would be 2.85°C., and the density gradient would be a minimum, as indicated by the horizontal line in Figure 60a. Some preliminary measurements of the temperature variations in a rectangular channel indicate that they are of the same order of magnitude as those indicated in the example just given for a cylindrical tube."

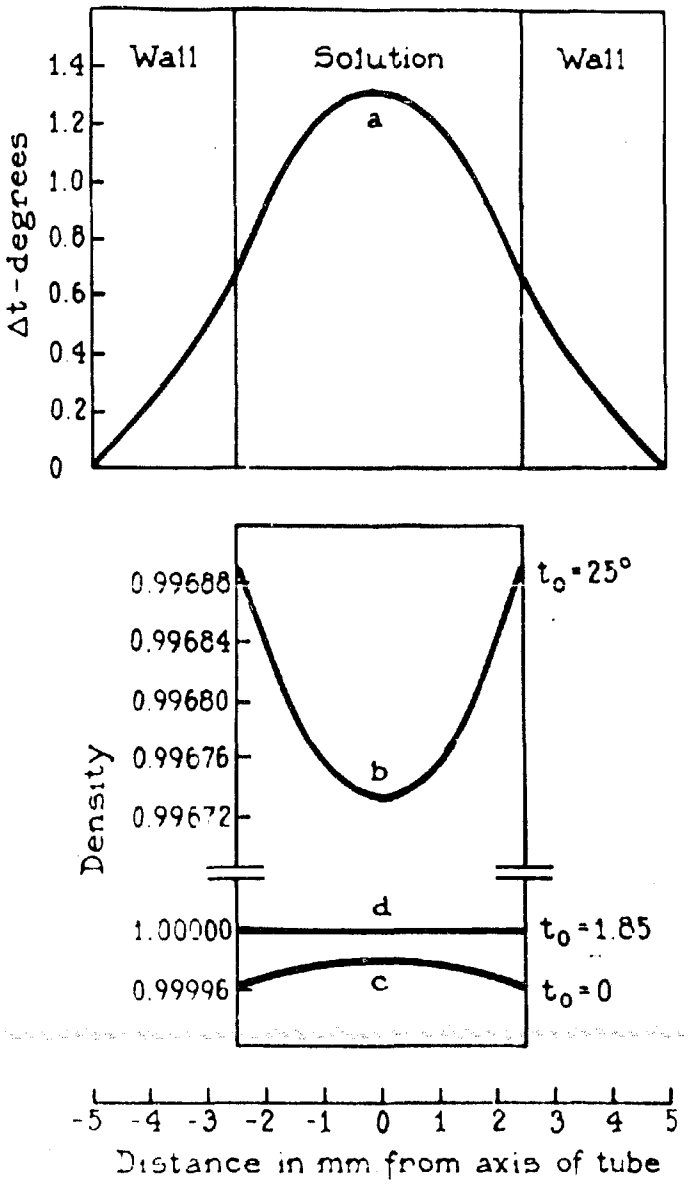


Figure 60. Diagram showing the distribution of temperature and density in a salt solution, in a cylindrical tube during passage of electric current. (Longsworth and MacInnes, 67)

PHOTOGRAPHING THE BOUNDARIES

After the boundaries have been formed but before they are shoved into view it is necessary to record the base line on the photographic plate. The base line is the position on the pattern of undeviated light, including any slight refractions due to irregularaties in the cell walls, the water bath windows or the schlieren lens. In photographing the base line a mask with a 0.2 mm. slit is placed in front of the photographic plate and one side of the cell is blacked out. The schlieren diaphragm is then raised until it nearly intercepts the normal slit image at the camera objective. The plate carriage is then locked to its driving shaft and the spur gears synchronizing the plate carriage and the schlieren diaphragm are engaged. The synchronous motor is then connected to the drive shaft by means of the clutch. A loaded plate holder is placed in the plate carriage and the driving motor is turned on at the same time the plate shutter is pulled over exposing the plate. The motor is left on until the normal slit image has been intercepted and the field has changed from light to dark. It is then turned off and at the same time the plate is covered. In practice the plate is exposed at one end and the diaphragm is placed in such a position that it only has to travel one to two millimeters to intercept all of the undeviated light.

Since the base line recording is due to the manner in which the entire field becomes dark as the schlieren diaphragm intercepts the normal slit image, it will also vary with the focal point of the light source. It will be recalled that the slit image is really a composite of all the images made by each element of the schlieren lens. If the cell image in the focal plane of the camera becomes dark from top to bottom as the

diaphragm is raised it follows that a normal slit image formed by an element at the bottom of the schlieren lens is intercepted before the image formed by an element at the top of the lens is intercepted. This situation arises when the schlieren diaphragm is not situated at the proper focal point of the slit image and can usually be remedied by refocusing the slit. This may be accomplished by moving the light housing backward or forward. If the field becomes dark from bottom to top, the diaphragm is probably behind the focal point of the slit image. The position of the base line can be located with reference to the micrometer scale and since the distance traveled by the plate per unit distance traveled by the diaphragm is known, it is possible to locate it on the enlarged pattern.

In order to measure the distances traveled by the various components in an electrophoretic pattern the initial boundary must be known. For this reason as soon as the boundaries have been pushed out a sufficient distance they are photographed. A mask with a 1/4" horizontal slit superimposed over one side of the cell image is placed in the viewing end of the camera barrel. The schlieren diaphragm is lowered until the boundaries are narrow and sharp. A plate is then placed in the carriage and exposed for a few seconds close to the area previously exposed during the scanning of the cell wall. A separate plate is used for the ascending and descending boundary.

At the completion of an electrophoresis experiment the boundaries in each of the cells are photographed using the Longsworth schlieren scanning method as described previously. The schlieren diaphragm is lowered manually to the point where no refractive gradients are intercepted. The cell field contains no boundary images. The micrometer reading (Figure 24) is recorded.

The schlierin diaphram is now raised manually until the cell field becomes to tally dark. The micrometer reading is again recorded. The difference between the two micrometer readings is the distance in millimeters that the schlieren diaphragm has to travel to intercept all of the deflected gradients of light rays. Since the plate carriage travels six millimeters for each millimeter traveled by the diaphragm, (on our apparatus), the distance that the plate has to travel to record the complete photograph of the boundary images can be calculated by multiplying the above difference by six. The plate holder is placed in the carriage, the carriage and schlieren diaphragm are placed in their proper positions and the driving mechanism thrown in gear (see Figure 24). At the time the plate shutter is removed the motor is turned on. When the plate has traveled the necessary distance as indicated by the vernier scale above the plate carriage, the motor is shut off and the shutter placed over the plate. During the scanning of the boundary images, the narrow slit mask (Figure 25) employed in scanning the base line is used. The same operation is repeated for the other side of the cell using a separate photographic plate.

Figure 61 illustrates the photographic patterns obtained using the previously described technique. The top bands on either side are the cell base lines. The initial boundaries are shown below the base lines, and the schlieren scanning photographs of the boundaries below these.

The electrophoretic patterns shown in Figures 62, 63, 64, and 65 were obtained with the same sample photographed at different time intervals by the Longsworth schlieren scanning method. These figures illustrate the manner in which the course of the separation can be followed on a ground glass plate using the Philpot-Svensson cylindrical

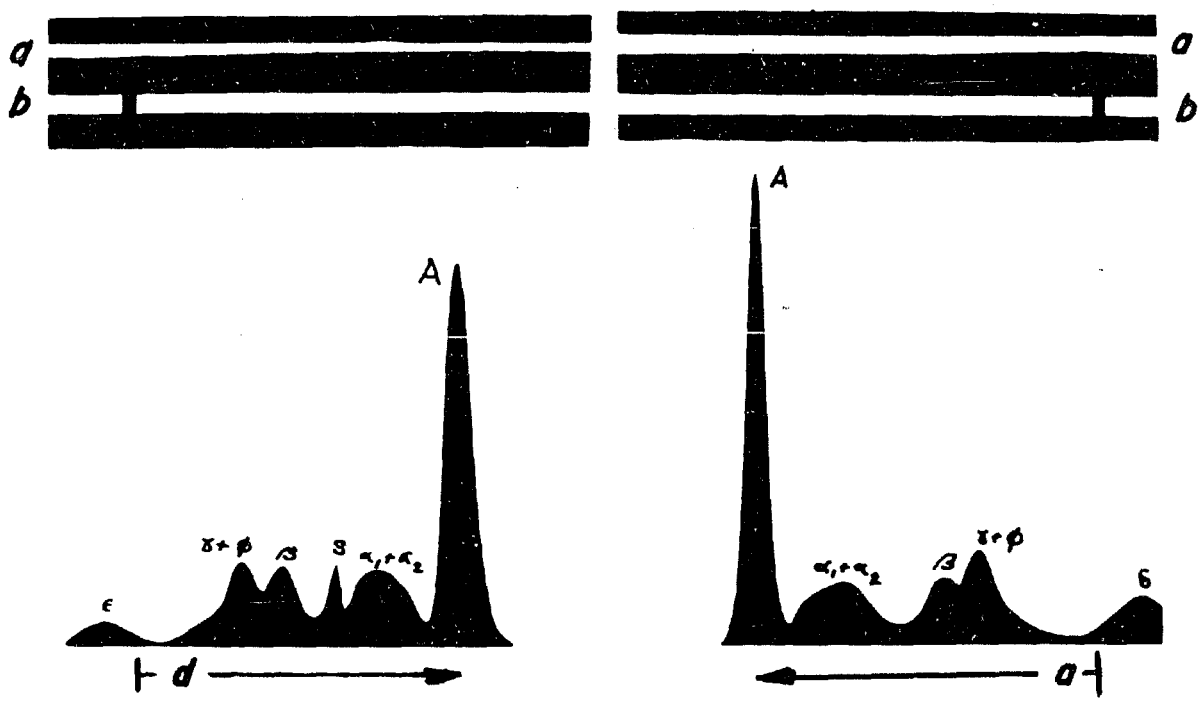


Figure 61. Electrophoretic boundaries of normal bovine plasma diluted to 1.86 per cent in barbiturate buffer of pH 8.6 and ionic strength 0.1. Electrophoresis carried out for 12,000 seconds at a potential gradient of 5.56 volts per centimeter. Boundaries photographed by the schlieren scanning method. The scanning photographs of the baselines are shown at a, and the initial boundaries at b.

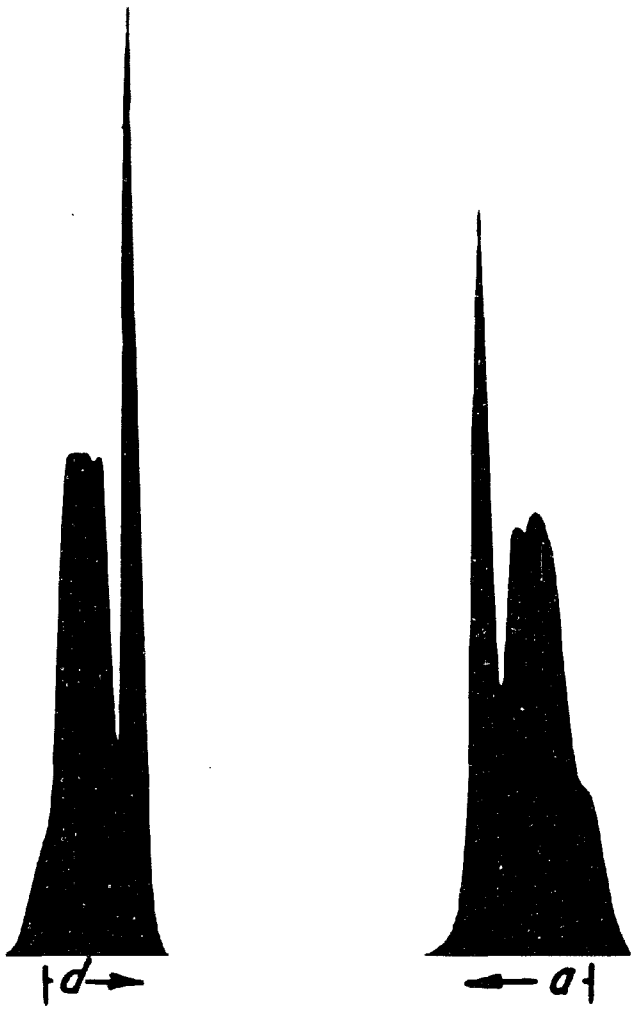


Figure 62

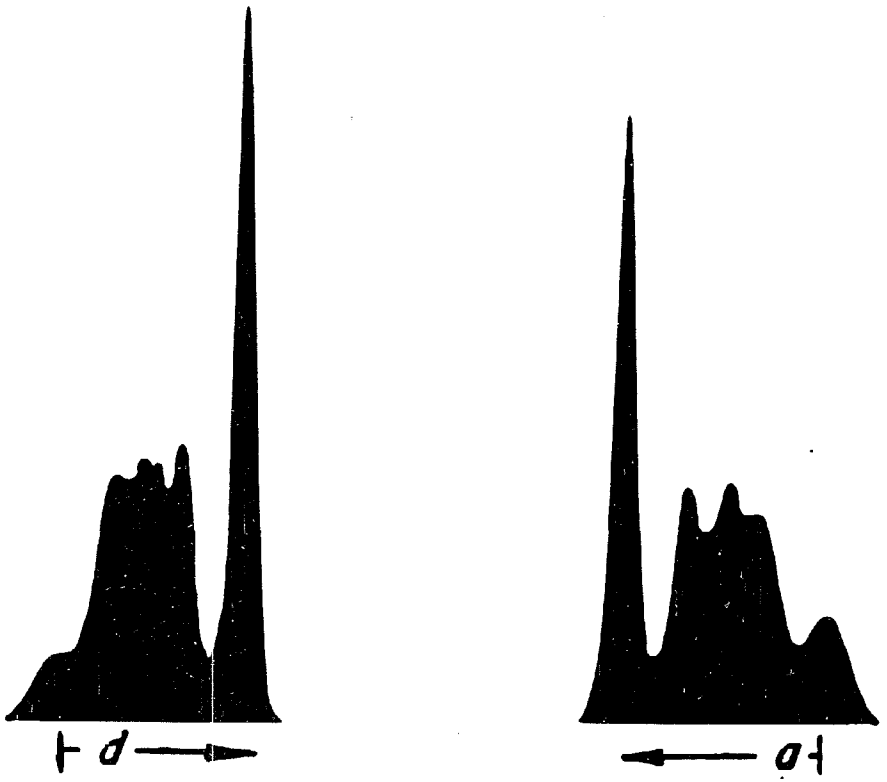


Figure 63

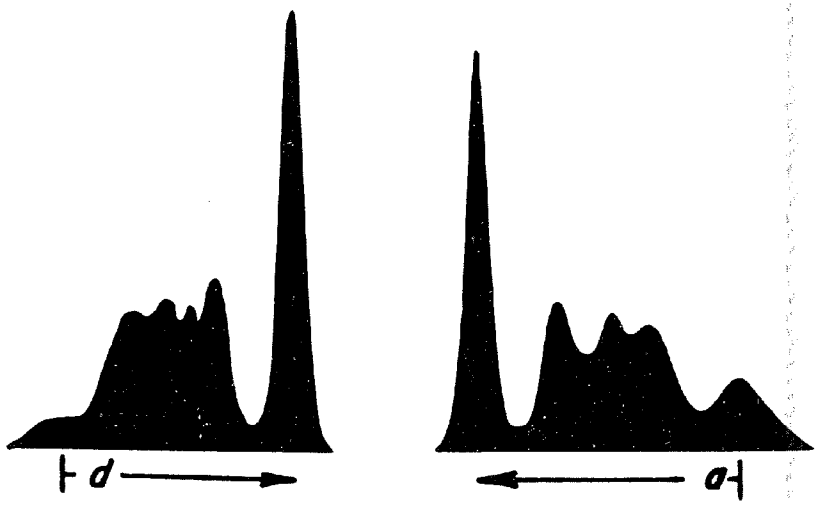


Figure 64

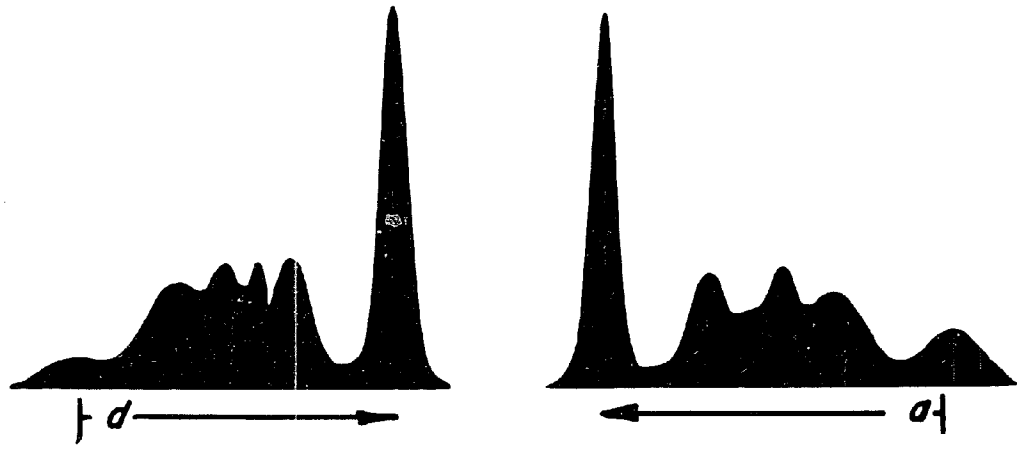


Figure 65

Figures 62, 63, 64, and 65. Electrophoretic boundaries of normal bovine plasma diluted to 2 per cent in barbiturate buffer of pH 8.6 and ionic strength 0.1. Electrophoresis carried out for 10,000 seconds at a potential gradient of 6.41 volts per centimeter. Boundaries photographed at intervals by the schlieren scanning method. Figure 62 photographed after electrophoresis for 2,500 seconds, Figure 63 after 5,000 seconds, Figure 64 after 7,500 seconds and Figure 65 after 10,000 seconds.

lens and diagonal straight edge. Figure 62, taken after electrophoresis for 2,500 seconds shows a separation of the albumin and globulin components. The slower moving globulins cannot be identified due to incomplete separation. Figure 63 photographed after electrophoresis for 5,000 seconds permits identification of the various globulin components. More complete separations are noted in Figures 64 and 65 photographed after electrophoresis for 7,500 and 10,000 seconds respectively.

The selection of the type of photographic plate to be employed depends upon the character of the sample being analyzed. For colored solutions Eastman C.T.C. or Wratten Process Panchromatic 9 x 12 cm. plates are satisfactory.

A Wratten #22 mercury monochromat filter should be placed in the optical system when a panchromatic type of photographic plate is used. This type of filter isolates the yellow line and improves the resolving power of the lens system. In the case of colorless samples 9 x 12 cm contrast lantern slides are adequate. In developing the plates the manufactures recommendations should be followed.

In our laboratory a "Solar" enlarger (Model 45CL) fitted with a 6 1/2 inch F:6.3 Wollensak enlarging velostigmat lens is employed to enlarge the negative 2.5 times. The enlarged tracing is made in pencil on graph paper. All measurements are made from the enlarged pattern.

It is often necessary to touch up the negative for printing. Photographic masking opaque (Eastman Kodak Co.) is used to darken light areas of the negatives. Blurred or ragged edges can be improved by using masking paste and a reducer (Farmer's reducer, Eastman Kodak Co.). The reducer has given excellent results when one tube of the powder is dissolved in 100 ml. of water and the solution flooded on the wet emulsion.

Outline bromide semi-matte paper is satisfactory for making prints.

CALCULATION OF MOBILITIES AND RELATIVE CONCENTRATIONS

From electrophoretic patterns it is possible to calculate mobilities of the various constituents if the following data are available: (1) The distance each element has traveled from the starting position of the boundary between the protein solution and buffer, (2) the specific conductance, K_p, of the protein solution, (3) the current, i, in amperes, (4) the time, t, during which a given boundary has moved the distance, h, from the starting position, and (6) the cross sectional area of the cell. The latter value is a constant and is determined independently for each side of the center section of the cell in the following manner. The length of each channel is accurately measured using a precision caliper square. One connecting plane of the center section is then greased and superimposed on a flat piece of glass. The center section and glass plate are then weighed accurately. One side of the cell is then completely filled with clean mercury. The weight of the center section and mercury is then determined as before. The difference between these two weighings is the weight of mercury necessary to fill one side of the cell. If the temperature of the mercury is known the volume of mercury can be calculated from temperature density tables. Having obtained the volume of mercury and the heighth of the cell the average cross sectional area of the cell can be calculated.

The derivation of the equation for calculating mobilities has been carefully reviewed by Longsworth and MacInnes (95). The following derivation applied to protein solutions is essentially theirs. The movement of an average protein particle in the body of the protein solution

corresponds to the transport of $u\,A\,(P)$ grams of protein through a reference plane. P being the protein concentration, A the average cross sectional area of the cell, and u the mobility. If the transport continues for t, seconds in an electric field differing from unity the total quantity of protein, p, transported through the reference plane is

$$p = u\,A\,(P)\,Ft \tag{27}$$

and

$$u = \frac{p}{FAt\,(P)} \tag{28}$$

The field strength or potential gradient in the body of the protein solution is

$$\frac{i}{K_p A} \tag{29}$$

in which i is the current in amperes and Kp the specific conductance of the protein solution. Equation 28 then becomes

$$u = \frac{pKp}{(P)it} \tag{30}$$

The quantity p is determined by following the movement of the boundary in the moving boundary method. By reference to Figure 66 the amount of protein passing the reference plane can be calculated (95). If a boundary descends from a to d, it sweeps through a volume $A(a\text{-}d) = Vd$. Multiplying by the protein concentration this becomes

$$A(a\text{-}d)\;(P) = Vd\,(P) \tag{31}$$

Equation 3- then becomes

$$ud = \frac{A(a\text{-}d)(P)\,Kp}{(P)\,it} \tag{32}$$

which is the formula used to calculate mobilites from measurements on

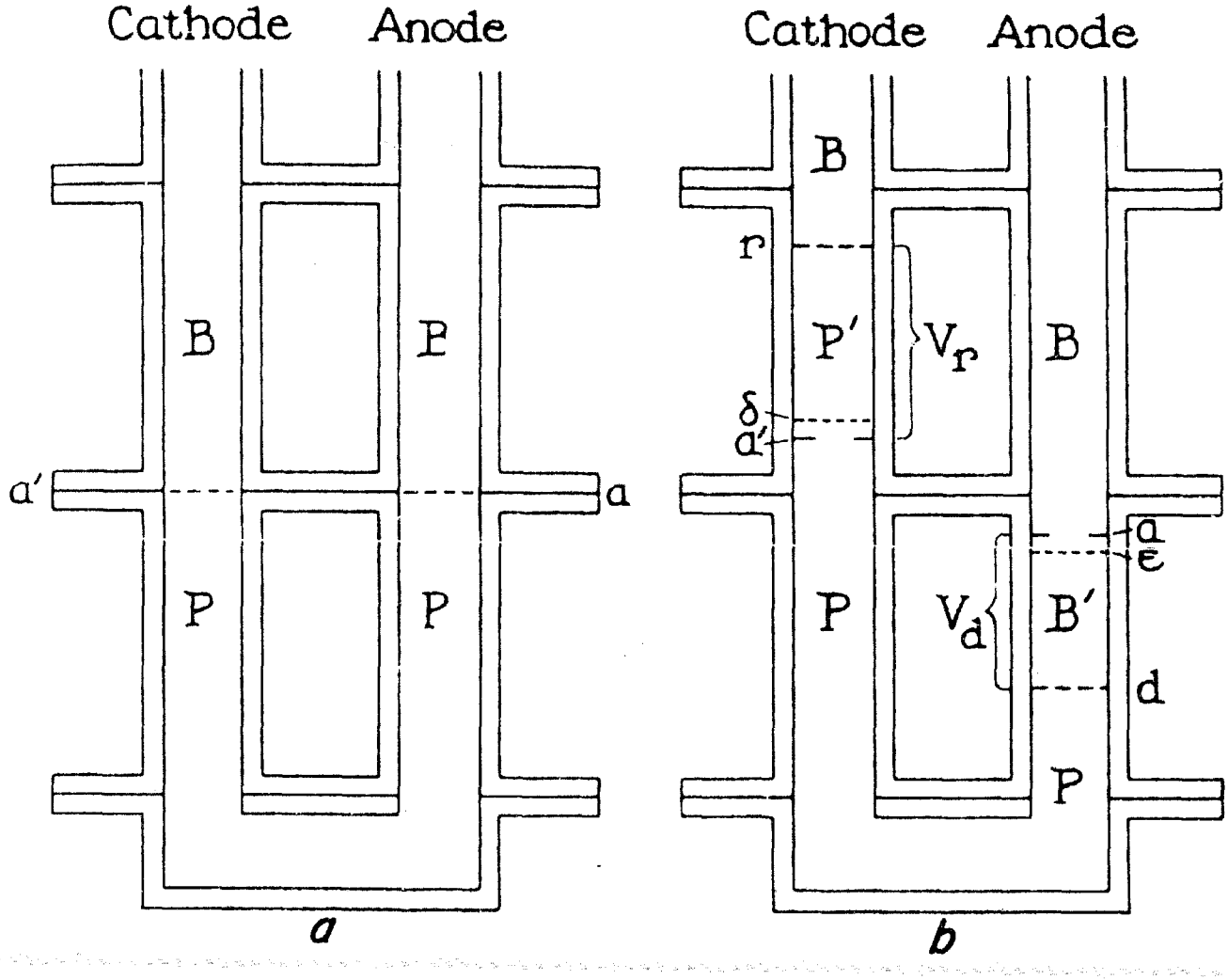


Figure 66. Diagrams of electrophoresis cell illustrating the formation and relative position of the boundaries. (Longsworth and MacInnes, 95)

the descending side. Figure 67 shows the descending pattern obtained from an electrophoresis experiment on normal bovine plasma enlarged 2.5 times. The center of each peak has been located with the aid of a planimeter and the distance between each of these peaks and the center of the initial boundary has been indicated in cm.

In locating the center of each peak it should be recalled that the center of the peak point of a schlieren band photograph does not locate the position of the boundary correctly if the gradients in the latter are not symmetrical about the ordinate passing through the maximum value of the gradient. When the boundaries are first formed they are quite sharp and no difficulty is encountered in determining the center. However, after migrating in an electric field the boundaries may become rather diffuse. Such a boundary is shown in Figure 68. The ordinate d' has been located so that it bisects the area of the gradient curve. The value of d' should be such that when the distance migrated by the plane d' from the initial boundary is multiplied by the cross sectional area of the cell, the same number of grams of protein are obtained as have simultaneously migrated through some reference plane in the body of the solution. By integrating the gradient curve, d may be located with precision. On the curve shown in Figure 68, d' has been located with the aid of a planimeter and it is that ordinate which cancels the shaded areas on either side. It has been found that the determination of d' with the aid of a planimeter gives just as satisfactory results as the laborious determination of d in Figure 69.

From the following data the mobilities on the descending side, Figure 67, may be calculated with the aid of equation 32.

Component	A	α'	α^2	β	$\gamma+\phi$	ε
Distance traveled cm.	11.08	9.63	8.28	5.81	4.16	
Mobility x 10^{-5}	6.91	6.00	5.16	3.60	2.59	
Area	157	26	63	65	64	34
$\frac{Ad}{A_A d}$		.165	.401	.414	.407	
Percentage Concentration	41.86	6.93	16.80	17.33	17.06	

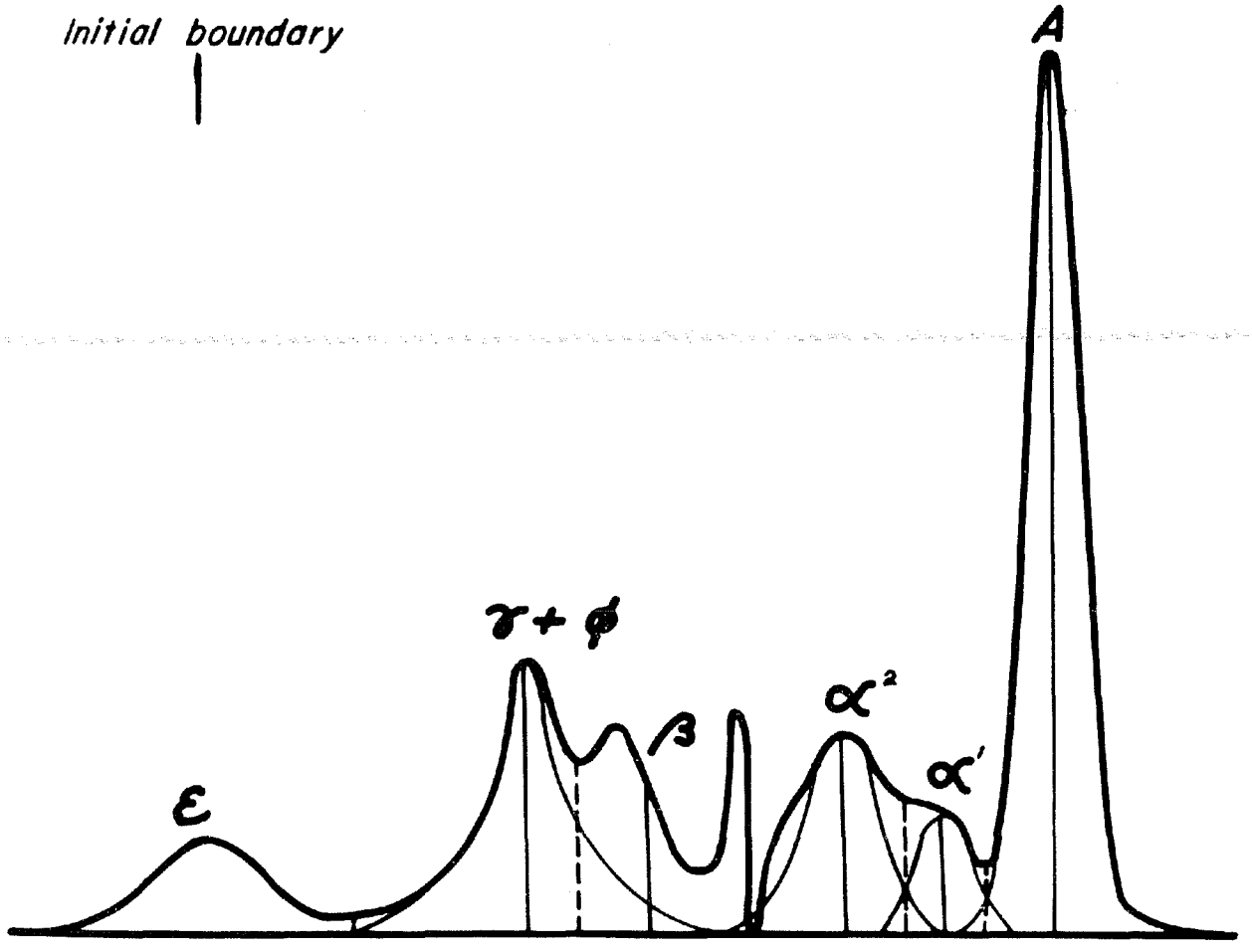


Figure 67

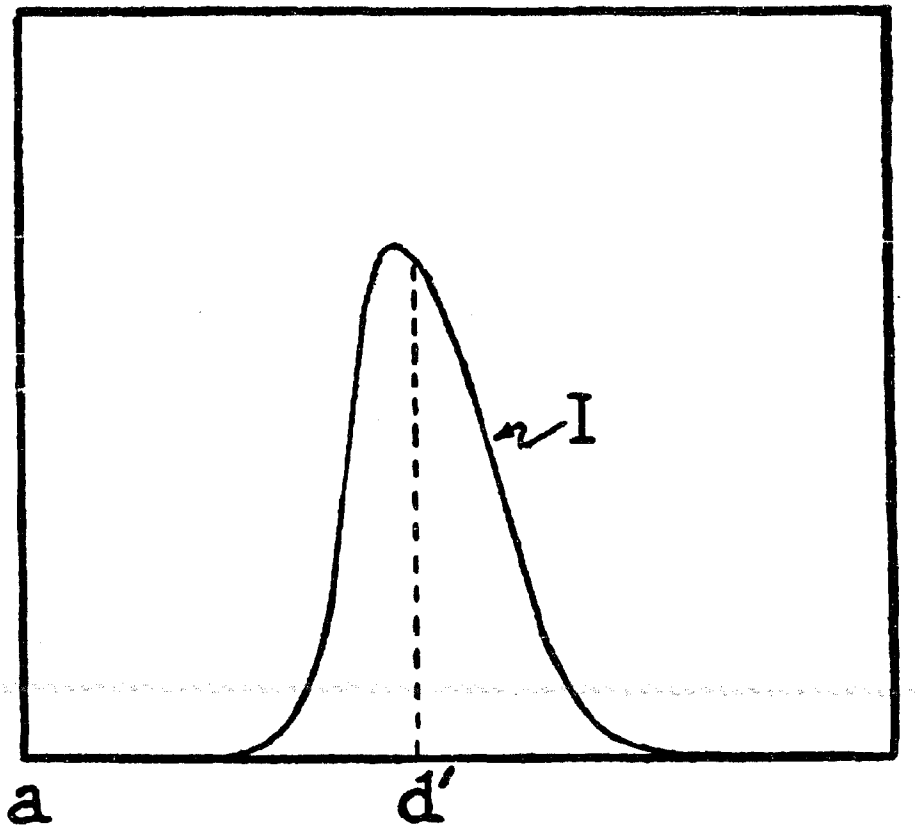


Figure 68. Diagram showing the variation of the refractive index gradient, assumed proportional to the concentration gradient, through a typical boundary. (Longsworth, 66)

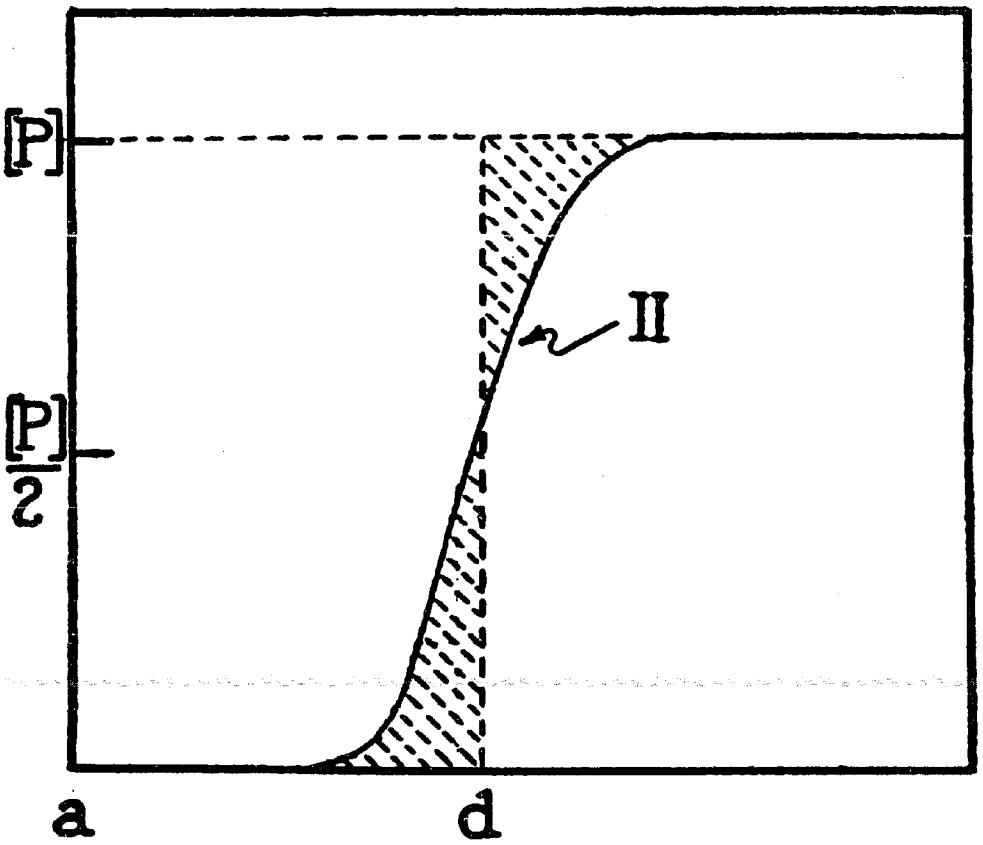


Figure 69. Concentration distance curve obtained by integrating the gradient curve (Figure 68). The ordinate, d, has been located so that it bisects the level in the boundary at which the protein concentration is one half of its origional value. (Longsworth 66)

Average cross sectional area of cell	.7555 cm^2
Average amps. passed during run	.011488
Time of run	10,000 sec.
Specific conductances of protein solution	.00299 $mohs^{-1}$

As an example, the mobility of the albumin component would be,

$$\frac{.7555 \times 11.08 \times .00299}{.011488 \times 10{,}000} \cdot \frac{2.5}{t} = 6.91 \times 10^{-5} \text{ cm}^2/\text{sec/volt}$$

The computation of mobilities from data on the ascending boundary is, however, complicated by the change of protein concentration at the $\underline{s}$ boundary (see discussion on boundary anomalies). Longsworth (95) has shown that if correctly interpreted, the data on rising and descending boundaries will yield the same value $\underline{u}$ of mobility. Equation 33 has been derived to correct ascending mobilities to descending.

$$u_r = \frac{VrKp}{it} \times \frac{Ar + A\frac{Vd}{Vr}}{Ad + A} \qquad (33)$$

in which

Ar = area in arbitrary units of ascending pattern

Ad = area in arbitrary units of descending pattern

A = area in arbitrary units of boundary

In the above treatment of data no correction has been made for volume changes which occur at the electrodes on the passage of a current. Since the observed boundary displacements refer to a plane fixed with respect to the apparatus they should be corrected for solvent displacements if the absolute mobilities are to be determined (95) (96) (97). However, this correction does not affect the order of mobilities.

In determining the relative concentrations of the various components from schlieren scanning photographs, the area of each peak must be determined in some arbitrary unit. In order to determine the size of the areas from the photograph it is necessary to make a more or less arbitrary separation of the peaks since the gradients of the different boundaries overlap. Two methods have been suggested for carrying out this operation. Tiselius and Kabat (98) draw an ordinate from the lowest point between two poles with the exception of the β globulin peak. Svedberg (99) resolves the pattern into a series of symmetrical curves. The peaks in Figure 67 have been separated by both methods. Longsworth (35) finds an average deviation of 15 percent in the areas as determined by these two methods. Because of the simplicity he recommends the method of Tiselius and Kabat. The area of each peak can be determined by the integral

$$\int z\,dh = \int \frac{a}{k}\,dh = \frac{ab}{k}\int \frac{du}{dh}\,dh = \frac{ab}{k}(n_2 - n_1) \qquad (34)$$

where n_2 and n_1 are the refractive indices of the two solutions which meet at the boundary and a, b, and k, are constants of the apparatus. The integration indicated in equation 34 may be carried out with a planimeter. The specific refractive increments of the electrophoretically separable plasma proteins are not known and the concentrations are determined as differences in refractive index and not in terms of protein nitrogen or dry weight. "The available evidence indicates that the refractive index is proportional to the protein concentration as determined by other methods, although the proportionality factor doubtless varies with the nature of the protein" (35).

The measured areas, A, are the sum of an area, P, due to gradients in the protein and an area S due to salt gradients. These salt gradient areas are small and certain assumptions are made concerning them for purposes of calculation. Thus in expressing the concentration of a component relative to another, say albumin, on the descending side it is assumed (1) that S is proportional to P and (2) that the proportionality factor is the same for all components. The plasma globulins and fibrinogen concentrations relative to albumin were calculated from the relationship, Pd/Pad = Ad/Aad and the results are indicated in Figure 67. The calculation may be illustrated from the following data: Area of albumin in planimeter units 157, area of α' globulin 26 units.

$$\frac{26}{157} = .165$$

The first assumption is valid as indicated by experimental evidence (35). The second probably induces a small error.

By making another assumption, namely that the specific refractive increments of the electrophoretically separable components are the same as the increment used in calculating the percentage protein from the dipping refractometer readings (.00185) the concentrations of the various components can be expressed as percentages (81). Thus the total area of Figure 67, excluding the ϵ boundary, is 375 planimeter units. Since the area of the albumin peak is 157 units

$$\frac{157}{375} \times 100 = 41.86 \text{ per cent albumin.}$$

In making the same calculations on the ascending side a third assumption is necessary, namely, that all of the protein components are held in the same proportion through the δ boundary. Values calculated from the ascending side are in fair agreement with values calculated on the descending side even though the third assumption is not strictly valid (35).

BOUNDARY ANOMALIES

Several of the more common boundary anomalies are shown in Figure 61. The δ boundary is more pronounced than the ϵ boundary. (2) The rising albumin boundary is much sharper than the descending one. (3) The concentration distributions in the boundaries are not symmetrical about the maximum as shown by the shapes of the curves. (4) The total area of the rising and descending sides are equal but the partial area of each of the components on the rising side are less than the corresponding areas on the descending sides. (5) Finally, the separation of the alpha globulins is not complete. Longsworth and coworkers have explained these anomalies in some detail. (67), (95), (35), (100), and (92).

As discussed elsewhere in this paper, a difference due to the Donnan equilibrium of salt concentration exists between the protein solution and the buffer after dialysis. In addition, the protein solution contains conducting constituents, the protein ions that are not present in the buffer solution. Referring back to Figure 66 the passage of a certain amount of current causes the boundary, a, to migrate to, d, and in the intervening volume V a buffer solution of composition B' is formed. "This composition has been adjusted in general, to a value different from B in such a way that its "regulating function" has the same value as that of the protein solution it has replaced. This regulating function defines a property of the solution which, at any given point, retains a constant value independent of changes of concentration caused by electrolytic migration. If, as a result of such migration species of ions different from those initially present appear at a point, their concentrations will be adjusted to values compatible with the constant determined by the initial composition of the solution" (67) (101). The boundary ϵ Figure 66, then

forms between two solutions of the same salt, but at different concentrations B and B'. Simple cases of this type have been studied and are understood (102) (103). At the rising boundary r, in the same figure there is a similiar but more complicated adjustment of the composition of the protein solution which replaces the buffer as the boundary rises. The theory for complicated systems of this type has not been developed (95). The δ is more pronounced than the ϵ boundary since the former involves a gradient of protein concentration while the latter does not.

The specific conductance of the protein solution, Kp has been found experimentally to be less than, Kp', of the adjusted buffer solution (95). Therefore variations in the potential gradient occur at the boundary d, of Figure 66. The protein ions in the dilute uppermost layers of the boundary are therefore in weaker fields then are those in the concentrated layers and thus tend to lag behind causing the descending boundary to become diffuse as shown in Figure 69a. However, in the case of the rising boundary the potential gradient is greater in the solution p' than in B - so that the dilute, slowly moving protein ions in the uppermost layer of the r boundary tend to be overtaken by the faster moving ions in the lower part of the boundary. As a result this boundary remains sharp as shown in r, Figure 69a. The variations in potential gradient at the boundaries, as well as pH gradients which may result from Donnan equilibrium, may cause the boundary to be unsymmetrical about the ordinate passing through the maximum of the refractive index gradient curve.

Diffuse boundaries may result from protein boundaries in which the protein molecules forming the boundary have a range of mobilities instead of a single value. Tiselius and Horsfall (104) have found that

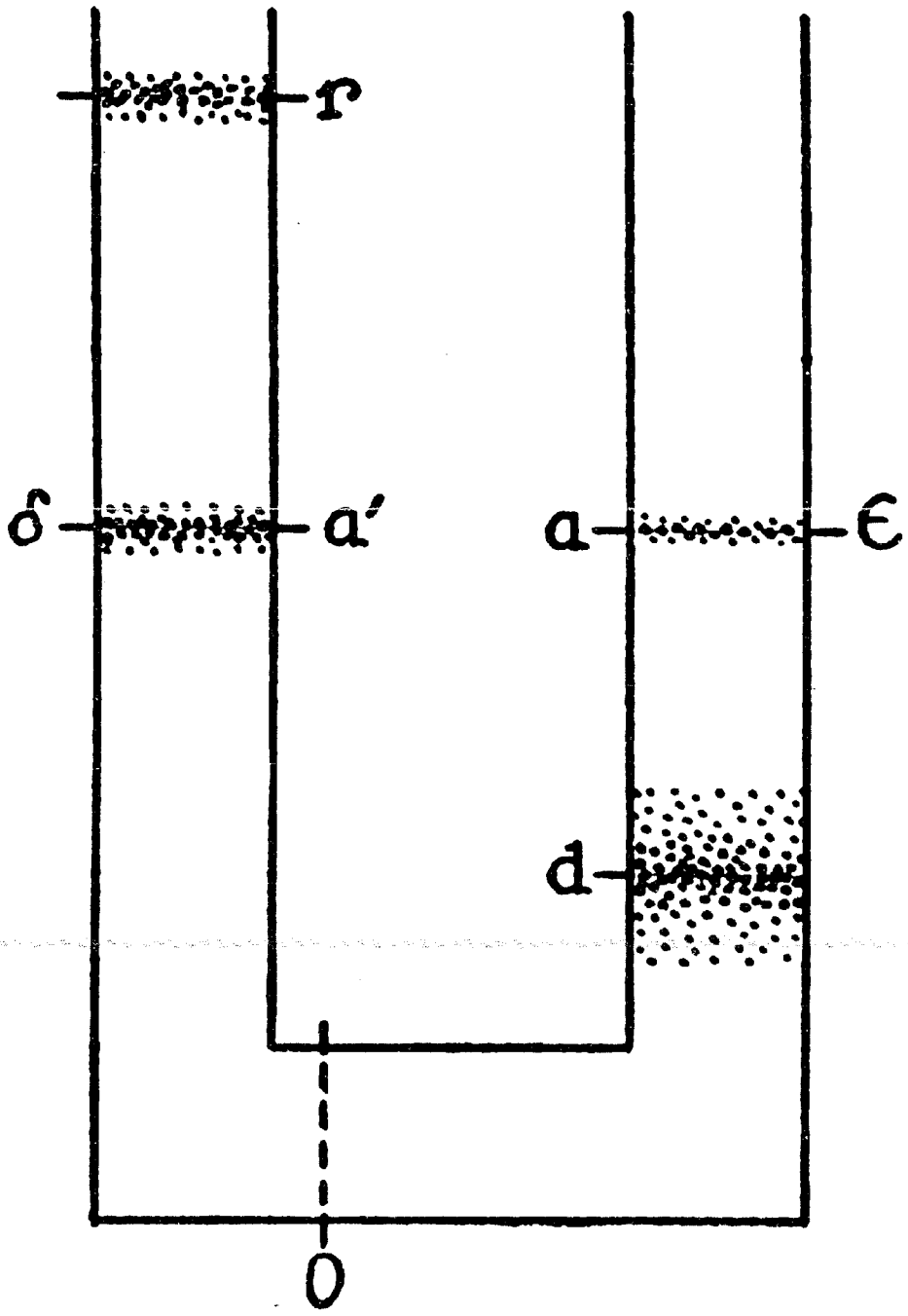


Figure 69a. Diagramatic view of section of electrophoresis cell showing initial boundaries at a-a' and boundaries after electrophoresis at r and d. (Longsworth, 66)

certain proteins show "reversible boundary spreading". This occurs at both the rising and descending boundaries. Longsworth (92) has studied the reversible boundary spreading of ovomuccid. Figure 70 illustrates his results. The initial boundary r, becomes diffuse as current is passed through the solution as evidenced by r^2 and r^3. Reversal of the current caused the boundaries to retain their origional shape. A certain amount of spreading due to diffusion is shown by the fact that the boundaries do not regain their origional sharpness. Spreading due to diffusion occurs independently of the electrophoresis.

The fact that the areas on the two sides of Figure 61 do not exactly compare has been discussed under calculations and is due to the protein gradients in the boundary.

Svensson has stated (105) that a rapid spreading of the descending albumin boundary is not desirable as the adjacent globulin peak is apt to be masked. Figure 61 shows a complete masking of the α' globulin and incomplete separation of the α' and α^2 globulins.

An anomalie of a different type called the "β" globulin disturbance is shown in Figures 71 and 72. The sharp spikes, s, may be due to convection, resulting from reaction in the neighborhood of the boundary following electrophoretic separation of the constituents (82) (107). Moore and Lynn (107) have made a careful study of the anomaly and explain it in the following manner: "The descending pattern of the globulin carries in many cases a spike which extends indefinitely out of the pattern. This corresponds to the presence of turbidity and is not entirely dependent on a refraction gradient. Figure 73 from Moore describes the physics of the production of the anomalous spike. Part A

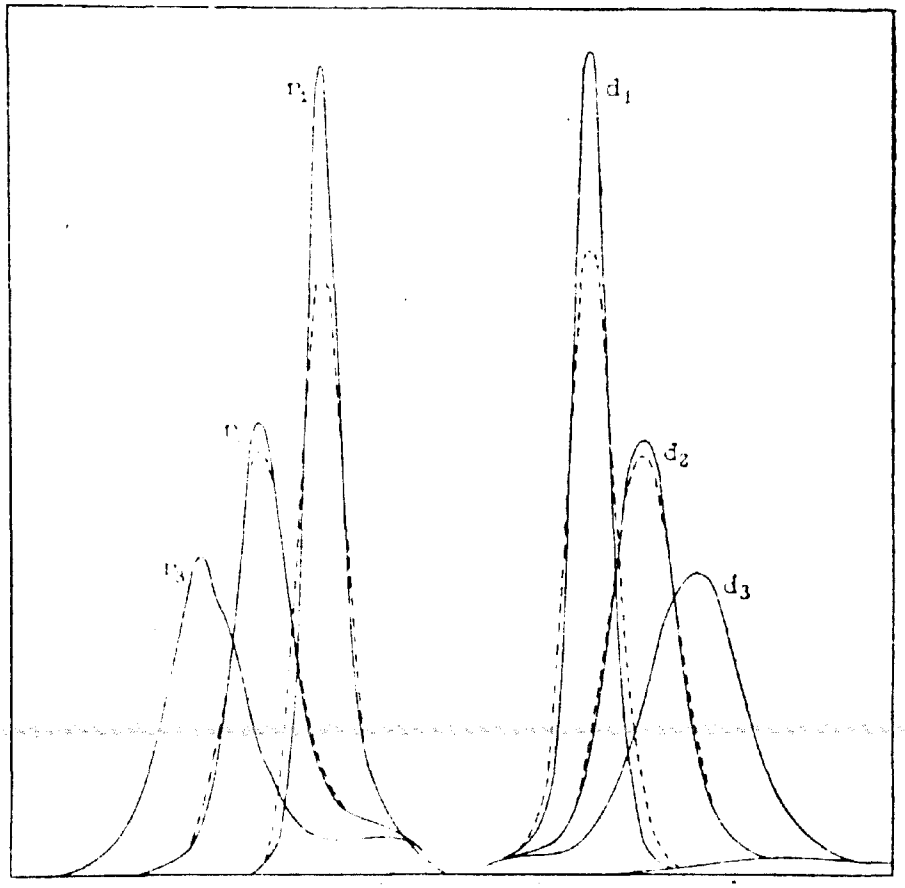


Figure 70. Electrophoretic patterns illustrating the reversible boundary spreading of ovomucoid. (Longsworth, Cannan and MacInnes, 92)

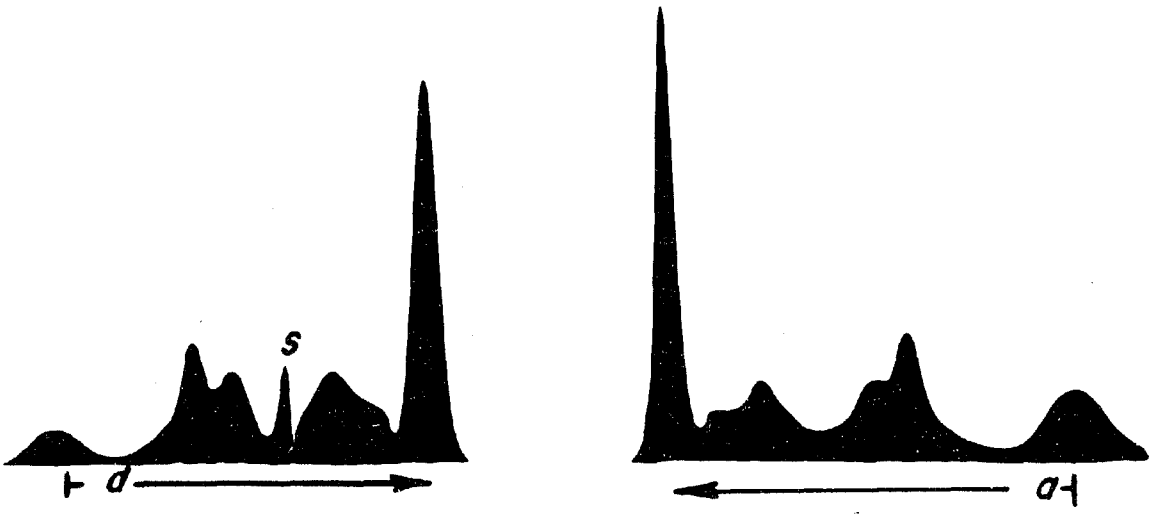


Figure 71. Electrophoretic boundaries of normal bovine plasma diluted to 1.5 per cent in barbiturate buffer of pH 8.6 and ionic strength 0.1. Electrophoresis carried out for 10,000 seconds at a potential gradient of 6.63 volts per centimeter. Boundaries photographed by the schlieren scanning method. The beta boundary disturbance, s, is indicated on the descending side.

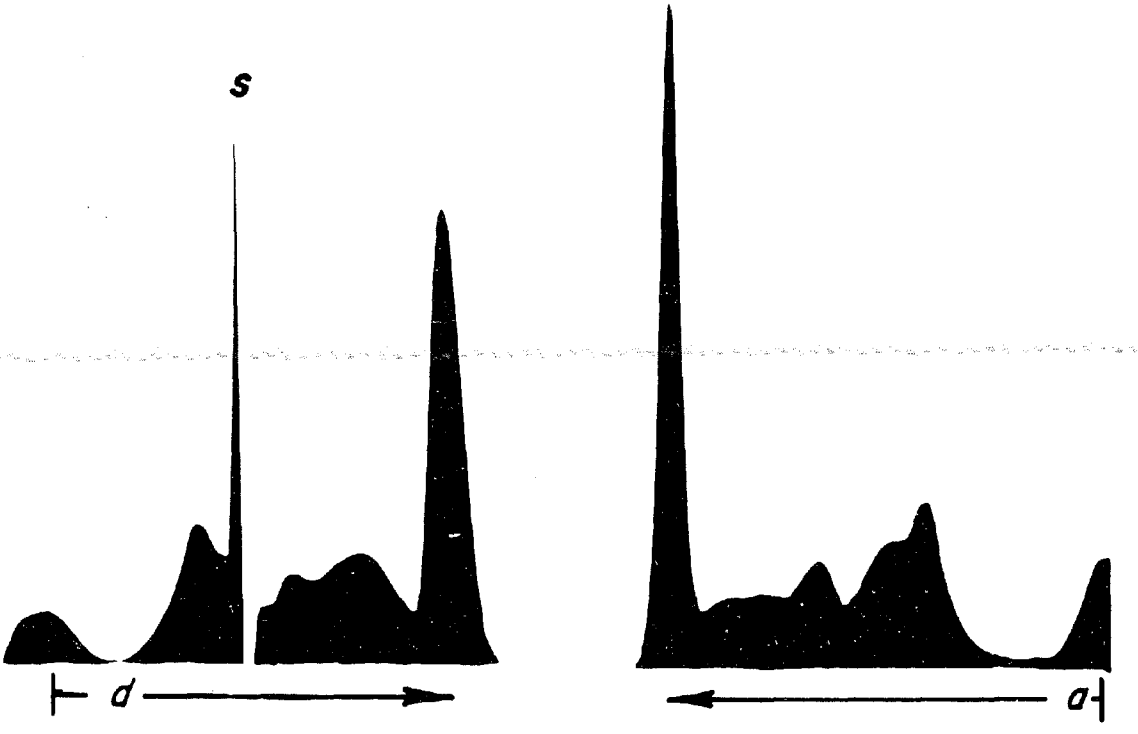


Figure 72. Electrophoretic boundaries of normal fowl plasma diluted to 2 per cent in barbiturate buffer of pH 8.6 and ionic strength 0.1. Electrophoresis carried out for 11,200 seconds at a potential gradient of 6.61 volts per centimeter. Boundaries photographed by the schlieren scanning method. The beta boundary disturbance, s, is indicated on the descending side.

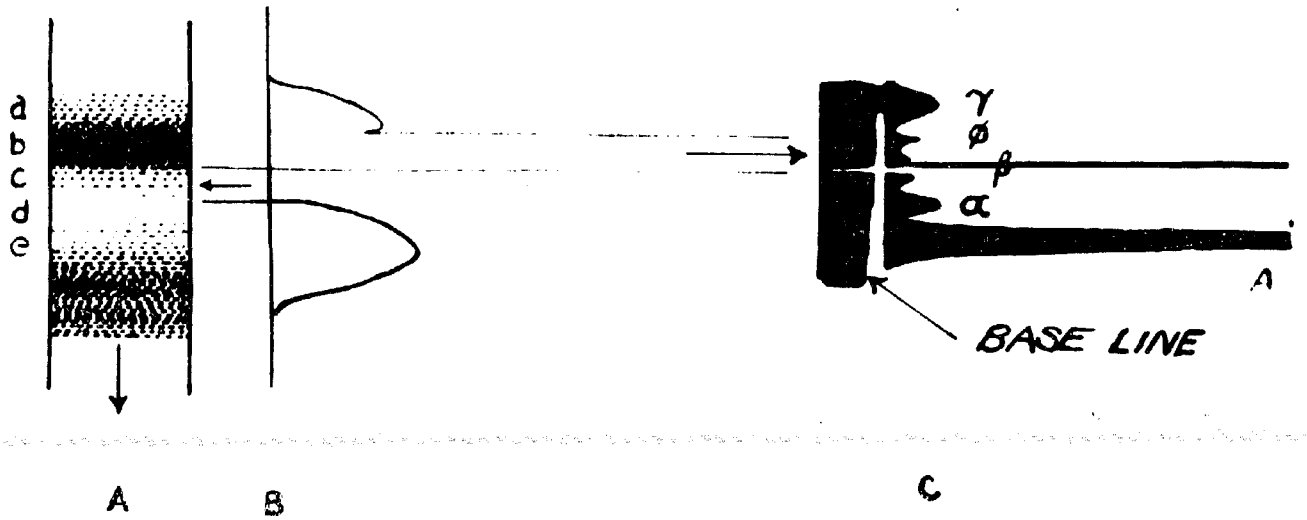


Figure 73. Diagram illustrating a possible explanation of the boundary disturbance. Description in text. (Moore and Lynn, 107)

is a small section of the electrophoresis cell in the region of the descending beta boundary. B represents a greatly magnified β pattern similiar to that in C. Above a, Figure A there is no beta globulin. An increase in the concentration occurs at a and is shown by an elevation in the pattern. At b there is turbid material which transmits no light. The spike is, therefore, of indefinite length. In the region c the concentration is decreasing. This refracts a horizontal light ray upward and causes the projection below the base line. A relatively low concentration is found at d which increases again in the region e leading to the third elevation in the pattern. Below e the concentration of β is uniform. The high concentration in the turbid layer at b accounts for the sparcity at d, so that unless one is able to determine the increment in the dense layer and the decrement below it, it is impossible to calculate the total change in concentration caused by the boundary. In not all cases, however, is the dense layer too opaque to transmit light. In these samples the spike does not have indefinite length but simply shows a steep gradient at that point. The concentration in these cases can be deduced if the area extending below the base line is subtracted from the area above the base line." Some possible explanations for the turbidity may be, (1) an interaction between α and γ globulin (Abramson p 186), (2) a stability change by removal of other components (82), (35). There might be two different globulins of different stabilities, the slower one being more unstable in the absence of the faster moving one (Abramson p 186). Longsworth and coworkers (82), (106), have found the cold ether extraction of certain types of pathological serums causes a marked decrease in the β globulin concentration. However, only unappreciable quantities of the lipids of normal serum can be

extracted with cold ether. Sorensen (108) says "...the perfect clearness of such liquids as serum and plasma, in spite of their contents of lipoids, is explicable only by assuming linkage between lecithin and sterols on one hand and the proteins on the other". Tiselius (109) has found that most of the lipid material of normal serum travels with the β component. Tiselius (24) and Longsworth (106) have noted that the opalescence of normal serum, due presumably to suspended fat globules, migrates with the β globulin.

In view of this evidence it may be possible that the β globulin disturbance is due to the lipid content of the globulin.

In studying mixtures of yeast nucleic acid and ovalbumin electrophoretically, Longsworth and MacInnes (100) found evidence of the formation of a dissociable complex. In buffer solutions of 0.1 ionic strength and at a pH somewhat above the isoelectric point, each of the components migrated independently of the other. However, at a pH near the isoelectric point a new component appeared at the expense of the origional components. High concentrations of the proteins and low ionic strength favored the complex formation. Future work of this type may help in explaining certain boundary anomalies.

An additional type of boundary disturbance was noted by McFarlane (110). He found that suspensions of elementary bodies of vaccinia showed distortions at the moving boundaries in the form of a stream of the particles moving ahead from the center of the boundaries in the direction of the electric current. A stream of suspended material arose in the center of one side of the center of one side of the cell and a corresponding stream of the solvent descended in the center of the other cell. These effects were reversible if the current was reversed. McFarlane (110) attributed

the effect to endosmooses. Shedlovsky and Smadel (111) found that this type of boundary could be obtained also with particles of a similiar size but of an entirely different nature. The lack of density gradients of sufficient magnitude at the boundary were shown to cause these effects. Figure 74a, was obtained with a dilute suspension of washed virus and shows marked streaming 74b, the same virus in a concentrated form showing little streaming 74c, the dilute virus suspension in the presence of added soluble protein showing no streaming.

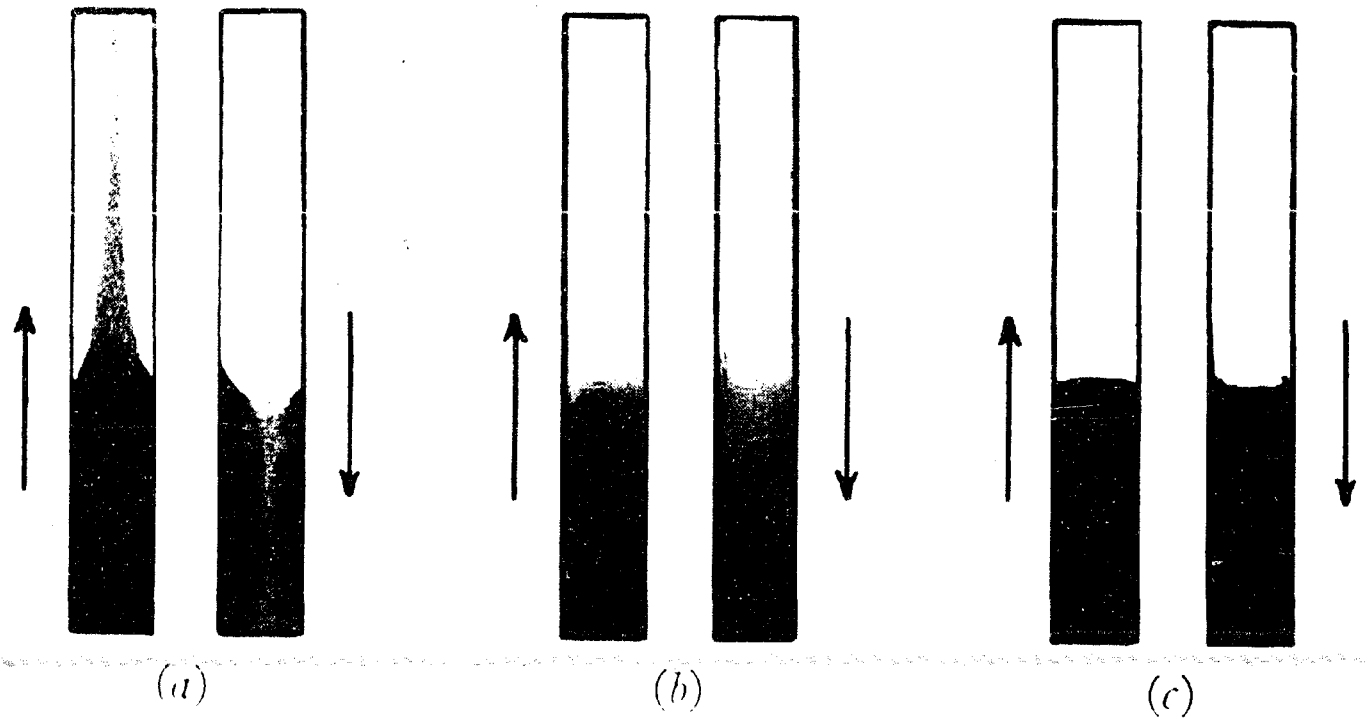


Figure 74. Photographs of electrophoretic boundaries of elementary bodies of vaccinia. (Shedlovsky and Smadel, 111)
(a) Boundaries obtained with dilute suspension of washed virus (.2%) showing marked streaming.
(b) Boundaries obtained with a concentrated suspension of washed virus (.5%) showing little steaming.
(c) Boundaries obtained with a dilute suspension of washed virus in the presence of added soluble protein showing no streaming.

STEPS TO FOLLOW IN A COMPLETE ELECTROPHORETIC ANALYSIS

(1) Determine total protein concentration of sample.

(2) Adjust protein concentration of sample with buffer.

(3) Dialyze sample (Visking casing) against three changes (24 hours for each) of buffer to equilbrate conductance.

(4) Assemble electrophoresis cell.

(5) Place electrophoresis cell in supporting frame.

(6) Place electrode vessels in the supporting frame.

(7) Connect electrode vessels to cell with rubber sleeves.

(8) Fill bottom section with sample and isolate by sliding to right.

(9) Wash out left middle section with buffer and fill with same.

(10) Fill right middle section of cell with sample.

(11) Partially fill electrode vessels with buffer used in 3rd change in step 3.

(12) Place in water bath to equilibrate temperature.

(13) Isolate middle sections of cell by sliding to the left.

(14) Remove supporting frame from water bath and slide bottom section of cell to original position.

(15) Rinse out right side of top cell with buffer.

(16) Fill top of cells and electrode vessels with buffer.

(17) Rinse electrodes with buffer.

(18) Insert electrodes in electrode holders and shake to remove air bubbles.

(19) Place three way stopcock in place in right electrode vessel, upright arm.

(20) Place hollow plug in place in left electrode vessel, upright arm.

(21) Place supporting frame in water bath.

(22) Remove air bubbles from in front and back of center section of cell.

(23) Push supporting frame as near schlieren lens as possible.

(24) Introduce N KCL through silver tube into chamber containing silver electrodes. Admit solution slowly.

(25) Place rubber cap on top of opening of silver tube on closed vessel.

(26) Push up plunger in syringe to remove air bubbles in tubing.

(27) Connect overflow tube to top arm of stopcock.

(28) Check lenses for moisture and dry if necessary.

(29) Turn on light source.

(30) Adjust mask in front of schlieren lens.

(31) Close stopcock on closed side after temperature equilibrium has been reached.

(32) Shove center section of cell to right until aligned with mask.

(33) Check light to see that it is centered on schlieren lens and focused on schlieren diaphram in front of camera lens.

(34) Check base line of Tiselius cells on ground glass plate at end of camera barrel.

(35) Insert proper filter in holder in front of light source. (Wratten #22)

(36) Scann the base line.

(37) Push out boundaries with compensating syringe attached to motor.

(38) Shut off motor when boundaries are out sufficiently.

(39) Photograph both initial boundaries.

(40) Connect leads to electrodes.

(41) Adjust potentiometer to zero volts against standard cell.

(42) Check cells and electrode vessel assembly for current leaks by connecting one pole of current source to frame and one to silver electrode tube.

(43) Determine specific conductance of buffer (0°C).

(44) Determine specific conductance of sample (0°C).

(45) Turn on current and timer.

(46) Check amperage at regular intervals.

(47) Follow migration of boundaries using Philpot-Svensson lens.

(48) Turn off current at completion of run.

(49) Calculate distance plate has to travel to photograph patterns of boundaries.

(50) Place narrow slit mask in position.

(51) Insert plate holder containing photographic plate and connect apparatus for scanning photograph.

(52) Make separate scanning photograph of descending boundaries and ascending boundaries.

(53) Develop plates.

(54) Enlarge photograph 2.5 time in enlarger and trace on graph paper.

(55) Make measurements and calculations.

(56) Retouch negative if necessary for printing.

(57) Make prints from negative on semi-matte bromide paper.
If the length of time of the first experiment has not required more than 12,000 seconds at a potential gradient of not more than 6 volts, one may conduct a second experiment on a new sample without changing the buffer in the electrode vessels by observing the following steps:

(58) Isolate center section of cell.

(59) Remove assembly from water-bath.

(60) Remove glass cocks from top of arms of electrode vessels.

(61) Drain out buffer to point where arms connect with electrode vessels.

(62) Remove rubber sleeves connecting top Tiselius cells.

(63) Remove Tiselius cell assembly.

(64) Replace with clean cells and connect to arms of electrode vessels by means of rubber sleeves.

(65) Repeat steps from 8 to 57 omitting steps, 11, 17, 18, 24, and 25.

APPLICATION

The improved electrophoresis apparatus of Tiselius has found its widest application in the characterization and study of proteins. A brief review of some of the applications of studies of this type will be presented. No attempt will be made to review all of the numerous experiments that have been reported, but it is intended to present enough material so that the wide application of this technique can be appreciated.

Human Serum and Plasma:

The four components of normal human serum were first identified by Stenhagen (93) as albumin, A, and α , β , and γ globulins. Human serum albumin on isolation migrated as a single boundary and was isoelectric at pH 4.74 (u = 0.10). Fibrinogen the fifth component of normal plasma was reported to have an isoelectric point between pH 5.2 and 5.6. Jameson (112) confirmed the presence of four components in normal human serum. From data of Longsworth (106), Scudder (113), and themselves, Moore and Lynn (107) report the average ratios of the components of normal human plasma as follows.

A/G	α/A	β/A	ϕ/A	γ/A
1.99	0.12	0.21	0.08	0.19

Figure 75 from Moore and Lynn (107) shows the range of variations of twelve normal human plasma patterns (shaded areas). These same authors find the average mobilities of human plasma proteins on the descending side to be.

(values x 10^{-5} cm^2 / sec / volt)

Component	A	α	β	ϕ	γ
Mobility	6.6	5.0	3.3	2.1	.4

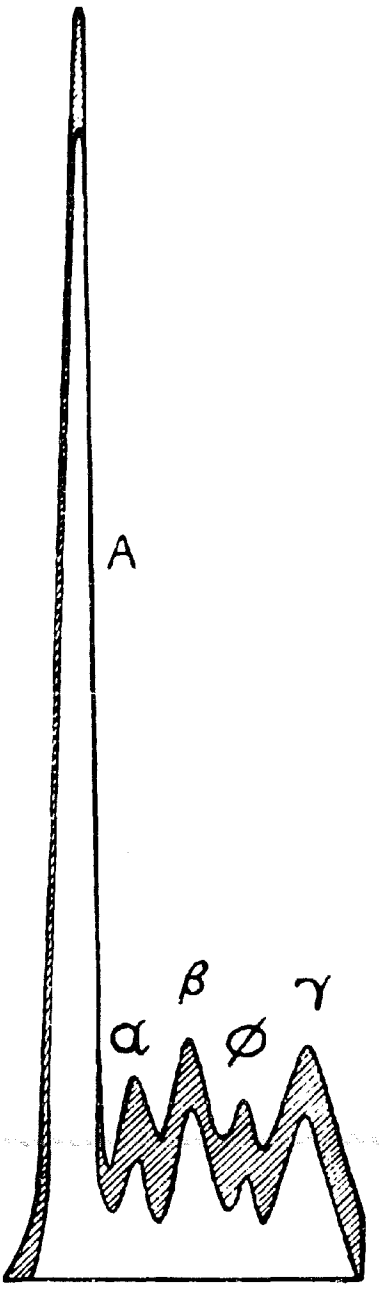
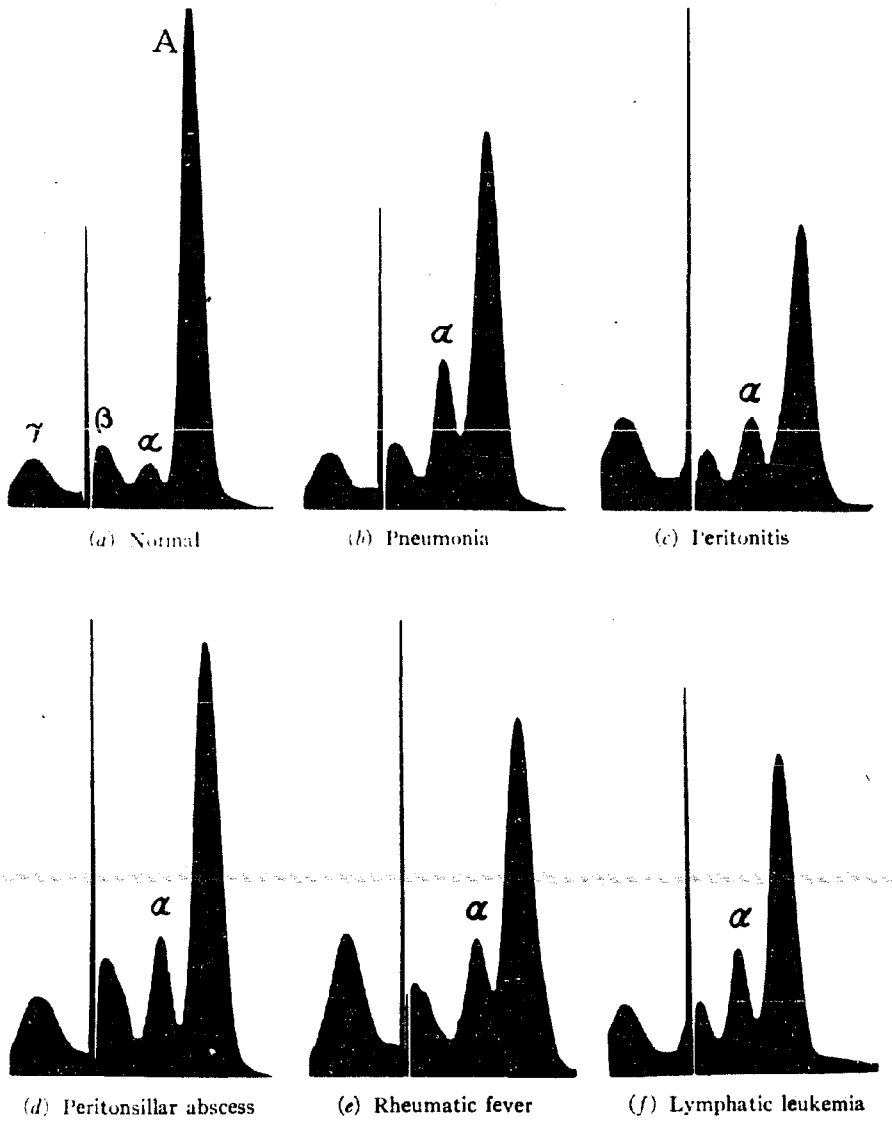


Figure 75. Diagram illustrating the variations in the electrophoretic patterns obtained from twelve normal plasma samples (shaded area). (Moore and Lynn, 107)

Other data on normal human serum and plasma have been reported by Svensson (34), Luetscher (114), Kekwick (115), Longsworth (82), Blix (116), Cohn (117), and Tiselius (24).

Longsworth and associates (82) have made an electrophoretic study of a number of normal and pathological human blood serums and plasmas. The patterns of several of these samples are shown in Figures 76 and 77. It will be noted from Figure 76 that in all of the pathological conditions there is an increase in α globulin. It is pointed out by the authors that a common characteristic of these conditions is that they are accompanied by fever. Figure 77 shows a considerable increase in β globulin, or, more exactly, of material moving with the mobility ascribed to β globulin. The fibrinogen peak of this same pattern is also abnormally high. Figure 77c shows a low albumin peak and an increase in the α and β globulin peaks. Figures 77e and 77f upon analysis showed an abnormal increase in cholesterol. Kekwick (118) found that the albumin globulin ratio of myelomatosis serum is decididly different from the normal. One group of serums of this type contained an additional component, which appeared to be a globulin. Blix (119) has found that pneumonia patients show an increase in α globulin while the β and γ globulins appear normal. Luetscher (114) has found that certain pathological serums reveal a component similiar to the P_2 globulin reported by Green (120).

Shedlovsky and Scudder (121) in a study of normal and pathological human serum and plasma found that an increase in the α globulin level as well as an increase in cell sedimentation rates occur when there is present in the body any considerable inflammation or tissue destruction, irrespective of its cause.



(a) Normal (b) Pneumonia (c) Peritonitis

(d) Peritonsillar abscess (e) Rheumatic fever (f) Lymphatic leukemia

Figure 76. A comparison of several pathological human serums with a normal human serum sample. (Longsworth, Shedlovsky and MacInnes, 82)

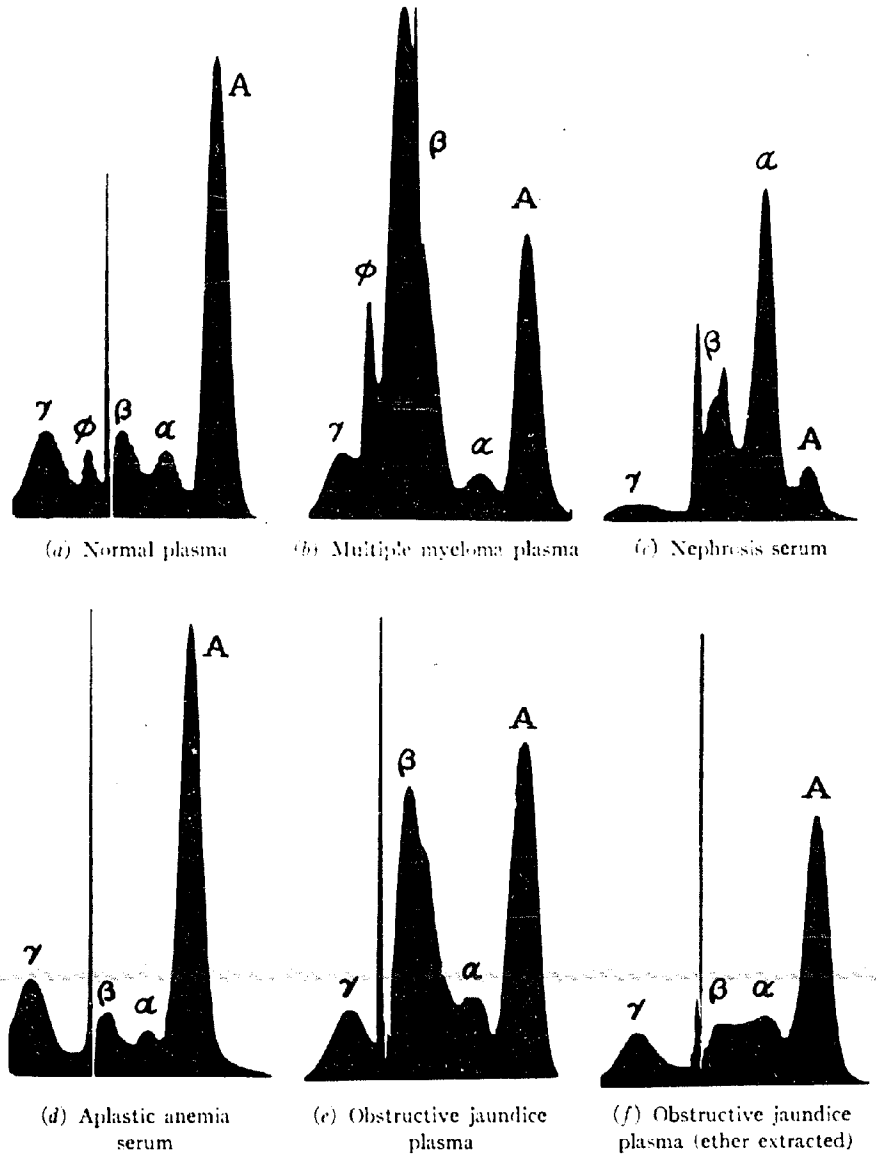


Figure 77. A comparison of several pathological human plasma samples with a normal human plasma sample. (Longsworth, Shedlovsky and MacInnes, 82)

The presence of a large amount of viscous protein which separated on prolonged dialysis, against distilled water from the blood plasma from a patient with multiple myeloma has been reported (122). The protein contained 13.9 per cent nitrogen and following hydrolysis yielded 13.4 per cent reducing substance (as glucose). It coagulated at 64°C. Electrophoretic examination of the plasma revealed a very large peak with a mobility of 1.9×10^{-5} (barbiturate buffer, pH = 7.8, ionic strength = 0.05). This component was shown to be neither fibrinogen nor Bence-Jones protein. The authors summarize their results as follows. "There is some evidence to suggest that it (the large peak) is a normal serum globulin which reacts with another serum component to yield the viscous protein which settles out on freeing the blood plasma of salts by dialysis."

An electrophoretic study of the serum proteins from patients with diseases of the liver revealed the following facts (123): "Electrophoretic analyses of the serum proteins yield lower albumin and higher globulin determinations, and consequently lower albumin globulin ratios, than are obtained by fractional precipitation.

"The distribution of the serum globulin fractions may be definitely abnormal electrophoretically in spite of a normal albumin-globulin ratio on chemical analysis."

Horse serum and plasma:

In describing his new technique of electrophoretic analysis, Tiselius (23) (24) reported on the electrophoretic analysis of normal horse serum. Using a phosphate buffer of pH 8.03 and ionic strength 0.1 he identified four separate components. The faster component with a mobility of 7.61×10^{-5} $cm^2/sec/volt$, was identified with serum albumin.

The three slower moving components were labeled α, β and γ globulins and showed mobilities of 5.79, 4.57 and 1.90 x 10^{-5} cm^2/sec/volt, respectively. The relative concentrations of the various components were reported as A 35.6 percent, α globulin = 12.3 percent, β globulin = 18.7 percent, γ globulin + δ = 33.4 percent. Tiselius was able to separate the four components of horse serum electrophoretically. Each of the components was then studied separately and the following mobilities and isoelectric points were observed.

	Mobility	Isoelectric point
Albumin	7.15	4.64
α globulin	6.16	5.06
β globulin	4.20	5.12
γ globulin	1.51	6.00

The mobilities of the separated constituents agree well with the mobilities observed with whole serum.

In comparative electrophoretic analyses of horse, swine and monkey serum Tiselius (124) found the mobilities of the normal components to be approximately the same. Kekwick (125), Moore (126) and Fell (127) have confirmed the findings with respect to the normal components of horse serum. Svensson (105) identified six components in normal horse serum. In the order of descending mobilities these were albumin, α^1, α^2, β^1, β^2, and γ globulin.

Other Sera and plasma:

San Clemente (57) has made a thorough electrophoretic study of serums from normal and brucellosis infected cattle. The mobilities of the electrophoretically distinct proteins fell into four well defined groups corresponding to albumin, α, β, and γ globulins. The average mobilities of these components were 6.2; 4.4, 3.0, and 1.6 respectively

(barbiturate-sodium chloride buffer of pH 7.9 and ionic strength 0.1). The average relative concentration in per cent of each component with respect to total protein was found to be 42.4 for albumin, 18.3 for α ($\alpha^1 + \alpha^2$) 8.3 for β and 31.1 for γ globulin. The isoelectric points of the various components were determined electrophoretically as shown in Figure 40. In this study it was found that serum from a new born calf is extremely high in α globulin and low in β globulin. Following the ingestion of colostrum there is a marked increase in γ globulin. At the end of two weeks all of the proteins components were in the relative concentration usually found in young heifers.

Jameson (112) found that rat serum shows three electrophoretically separable components, one albumin and two globulins corresponding to the β and γ. By increasing the applied potential a partial disintegration of the globulins occurred and two new components appeared. Later studies indicated that the α component was present but in such low concentrations that the band disappears on dilution (128).

The electrophoretic patterns obtained in this laboratory covering a wide variety of supposedly normal serum and plasma samples are shown in Figures 78 through 85. The terms normal serum and plasma are used to denote that the samples were taken from animals that were not afflicted with any known disease or disorder. Corresponding serum and plasma samples were obtained from the same animal at the same time with the exception of the guinea pig samples which were taken from two different animals.

Figure 78 shows a typical electrophoretic pattern of normal human serum. The various components have been identified as indicated on the patterns. The β boundary disturbance indicated as S, has nearly

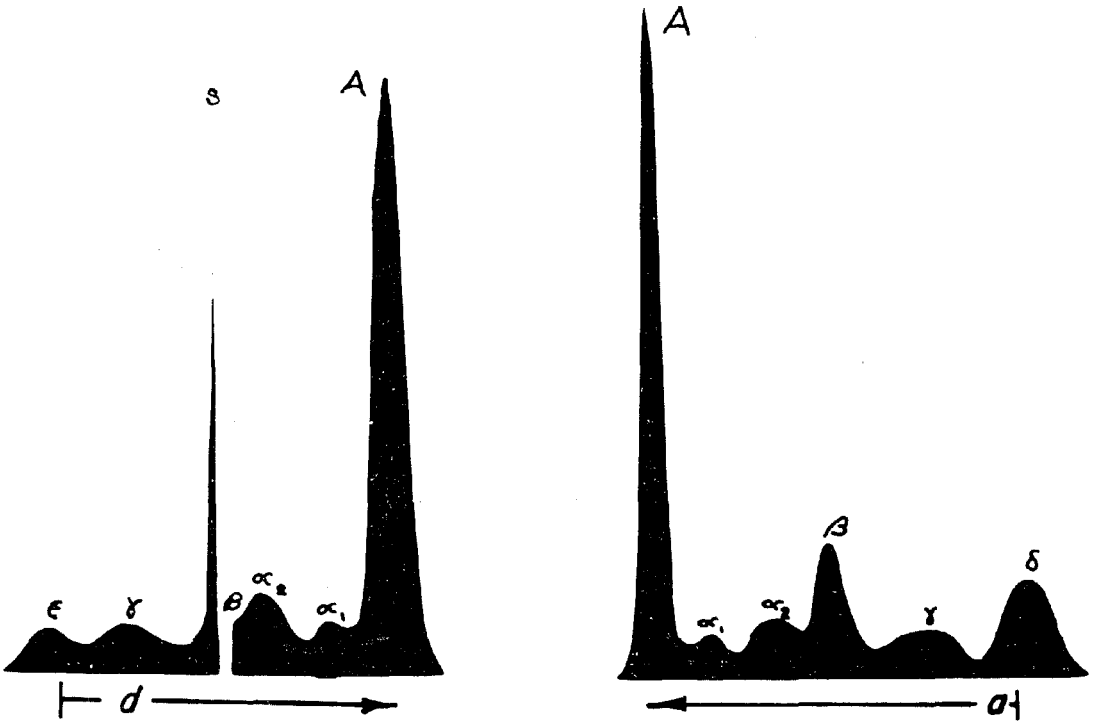


Figure 78. Electrophoretic boundaries of normal human serum diluted to 2.0 per cent in barbital buffer, pH 8.6. Electrophoresis carried out for 10,000 seconds at a potential gradient of 6.90 volts per centimeter. Boundaries photographed by the schlieren scanning method.

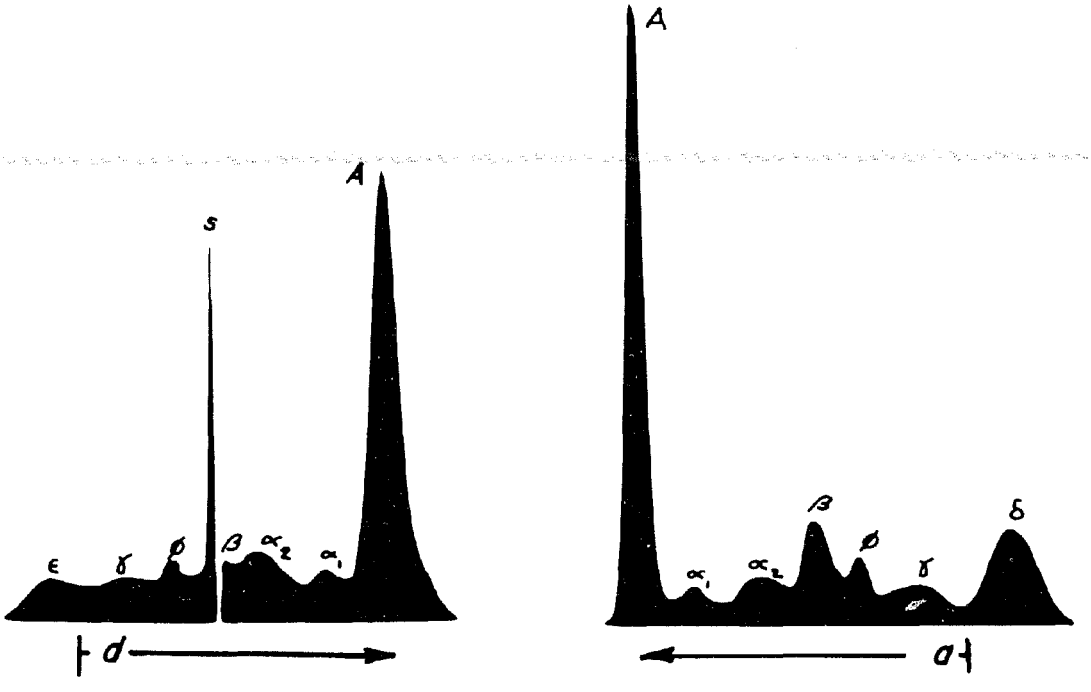


Figure 78a. Electrophoretic boundaries of normal human plasma diluted to 2.0 per cent in barbital buffer, pH 8.6. Electrophoresis carried out for 10,000 seconds at a potential gradient of 6.71 volts per centimeter. Boundaries photographed by the schlieren scanning method.

obscured the β globulin peak on the descending side. Figure 78a shows the corresponding plasma pattern. One additional component, fibrinogen, is present.

The normal components of horse serum and plasma are shown in Figures 79 and 79a. The descending serum pattern shows poor separation of the globulin components. The ascending pattern reveals a better separation and two distinct peaks are shown at the β globulin position. The descending plasma sample (Figure 79a) shows a poor separation of the globulins and fibrinogen. Two components are again evident at the β boundary position. The β boundary disturbance is not evident in either the serum or plasma pattern.

Typical patterns of bovine serum and plasma are illustrated in Figures 80 and 80a. The patterns bear a close resemblance to human serum and plasma patterns except for the β boundary disturbance. This disturbance is not evident in the serum patterns and is not marked in the descending plasma pattern. It is of interest to note that this disturbance migrates well ahead of the β globulin component in bovine serum and plasma while in the case of human serum and plasma it is superimposed on the β globulin peak.

The patterns resulting from the electrophoretic analysis of fowl serum and plasma are described in Figures 81 and 81a. The descending serum and plasma patterns show an incomplete separation of the α' and α^2 globulins while the ascending plasma pattern shows no evidence of the alpha globulins. The β boundary disturbance so prominent in the descending patterns is also discernable in the ascending patterns. In contrast with the human and bovine serum and plasma patterns the β boundary disturbance migrates slower than the β globulin component.

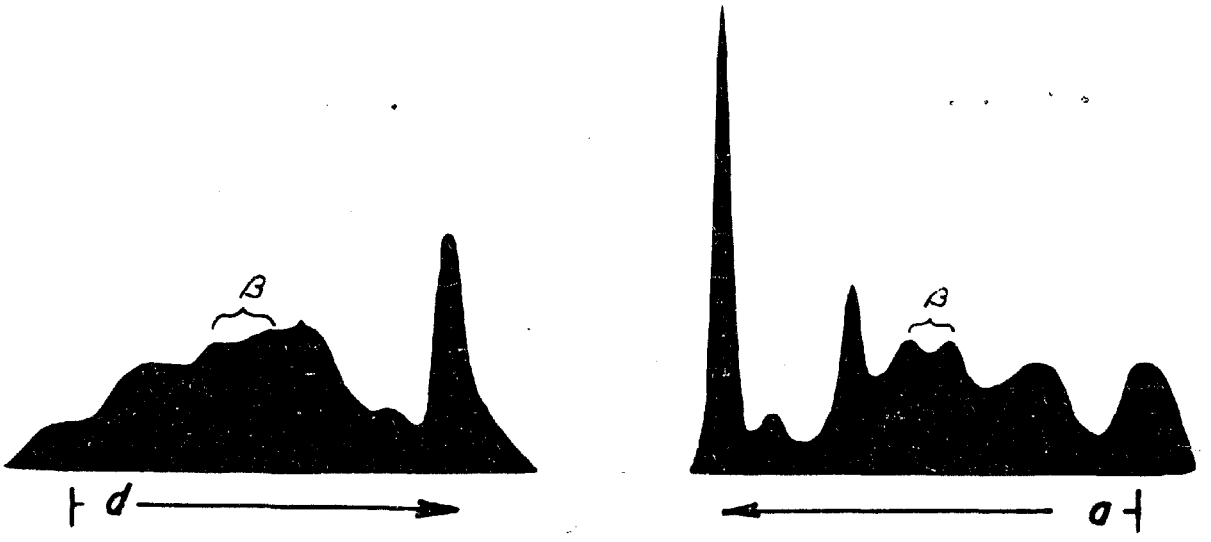


Figure 79. Electrophoretic boundaries of normal horse serum diluted to 2.9 per cent in barbital buffer, pH 8.6. Electrophoresis carried out for 10,000 seconds at a potential gradient of 6.50 volts per centimeter. Boundaries photographed by the schlieren scanning method.

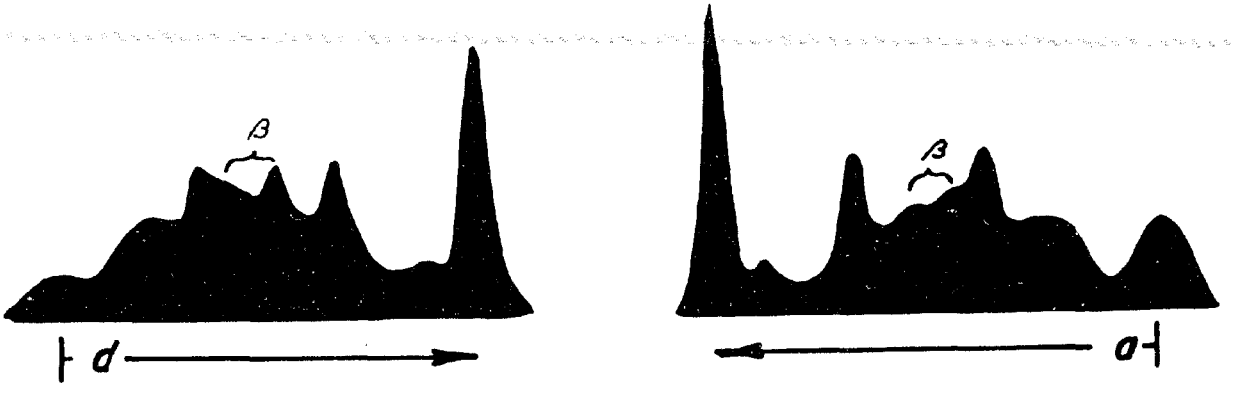


Figure 79a. Electrophoretic boundaries of normal horse plasma diluted to 2.0 per cent in barbital buffer, pH 8.6. Electrophoresis carried out for 10,000 seconds at a potential gradient of 6.61 volts per centimeter. Boundaries photographed by the schlieren scanning method.

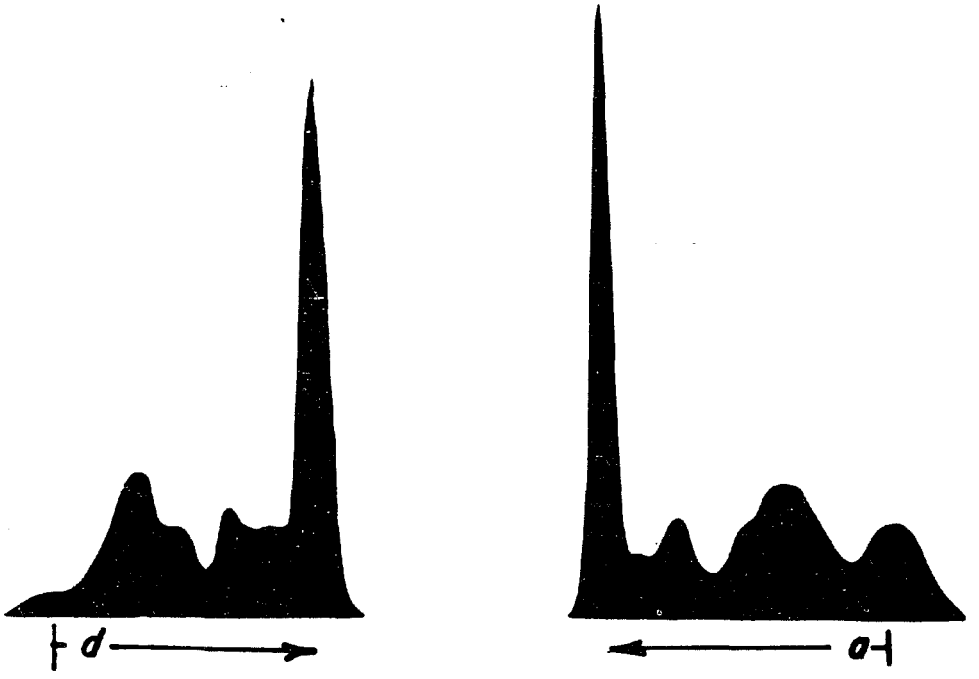


Figure 80. Electrophoretic boundaries of normal bovine serum diluted to 2.0 per cent in barbital buffer, pH 8.6. Electrophoresis carried out for 10,000 seconds at a potential gradient of 5.18 volts per centimeter. Boundaries photographed by the schlieren scanning method.

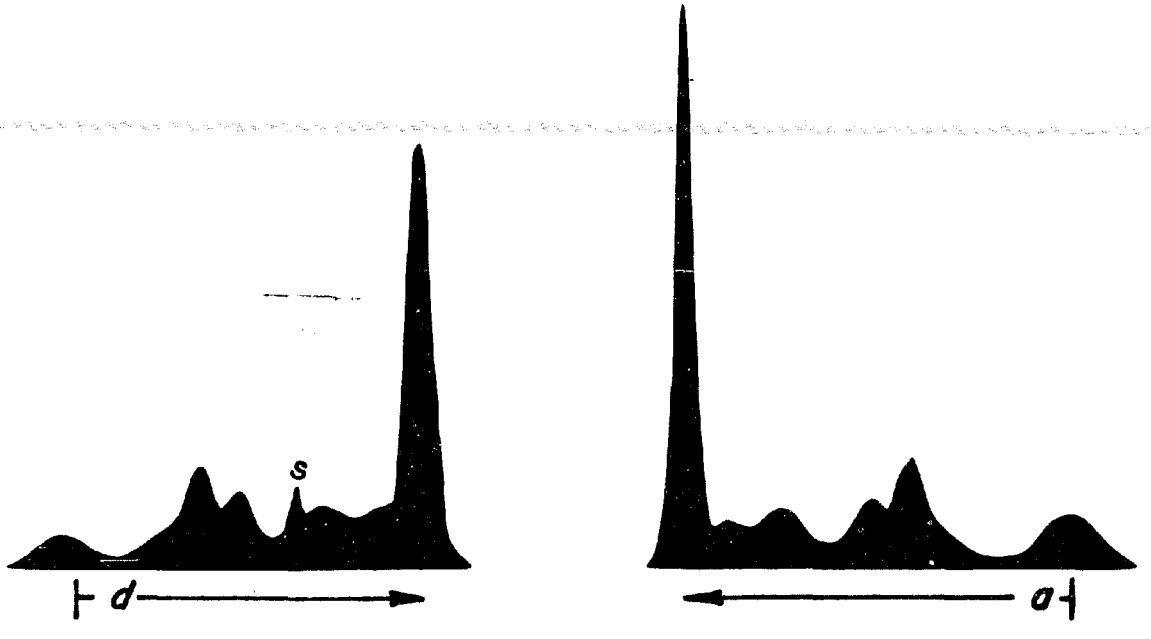


Figure 80a. Electrophoretic boundaries of normal bovine plasma diluted to 2.0 per cent in barbital buffer, pH 8.6. Electrophoresis carried out for 10,000 seconds at a potential gradient of 6.44 volts per centimeter. Boundaries photographed by the schlieren scanning method.

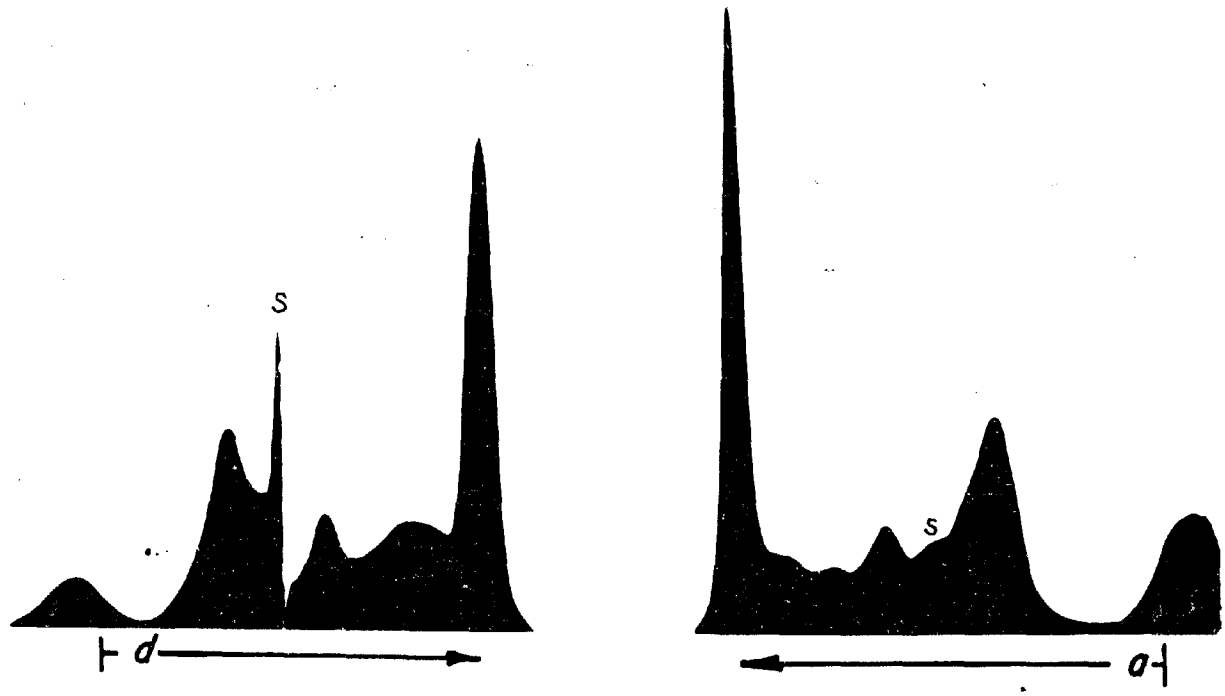


Figure 81. Electrophoretic boundaries of normal fowl serum diluted to 2.0 per cent in barbital buffer, pH 8.6. Electrophoresis carried out for 12,000 seconds at a potential gradient of 6.33 volts per centimeter. Boundaries photographed by the schlieren scanning method.

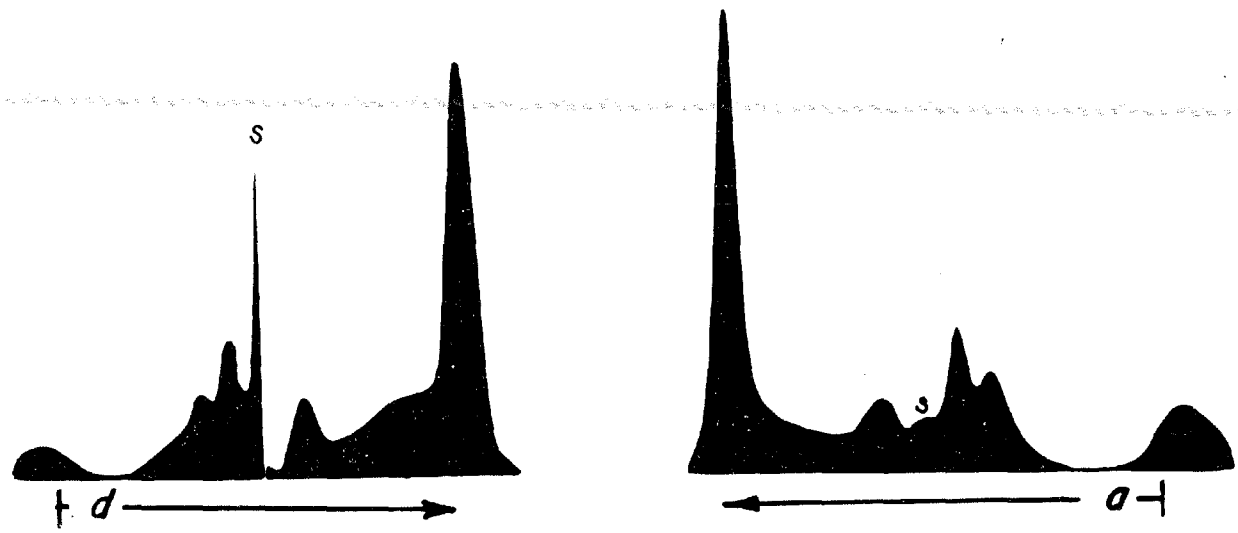


Figure 81a. Electrophoretic boundaries of normal fowl plasma diluted to 2.0 per cent in barbital buffer, pH 8.6. Electrophoresis carried out for 12,000 seconds at a potential gradient of 6.68 volts per centimeter. Boundaries photographed by the schlieren scanning method.

The electrophoretic examination of guinea pig serum and plasma has resulted in the patterns shown in Figures 82 and 82a. The descending serum pattern shows no evidence of the α' globulin component and the α^2 globulin is composed of two peaks. The concentration of β globulin in the pattern is relatively low. The ascending pattern reveals the α' component and both α^2 globulins. In addition the β globulin peak is more pronounced. The guinea pig plasma patterns (plasma from a different animal) show an incomplete separation of fibrinogen and γ globulin. There is no evidence in either the serum or plasma patterns of the β boundary disturbance.

Electrophoretic patterns of dog serum and plasma are shown in Figures 83 and 83a. The descending serum pattern reveals four components in the α' and α^2 globulin regions. The ascending side apparently shows three alpha globulins. Two components can be seen at the β globulin position on the ascending side. The β globulin is not completely separated from the γ globulin. The descending plasma pattern shows two alpha globulins while in the ascending pattern there are four. In both ascending and descending patterns the β globulin has failed to completely separate from the fibrinogen. The β boundary disturbance resembles that of the bovine in that it migrates ahead of the β component.

The sheep serum and plasma patterns, Figures 84 and 84a, are quite different from those of human serum and plasma. The descending serum pattern reveals two alpha globulins. The β globulin peak is nearly obscured by the β boundary disturbance. The ascending pattern shows no α globulins but there appears to be two β globulins. Both patterns reveal an additional component migrating just ahead of the γ globulin. The descending plasma pattern shows one alpha globulin, while in the ascending pattern there are two. The β peak is obscured by the β boundary

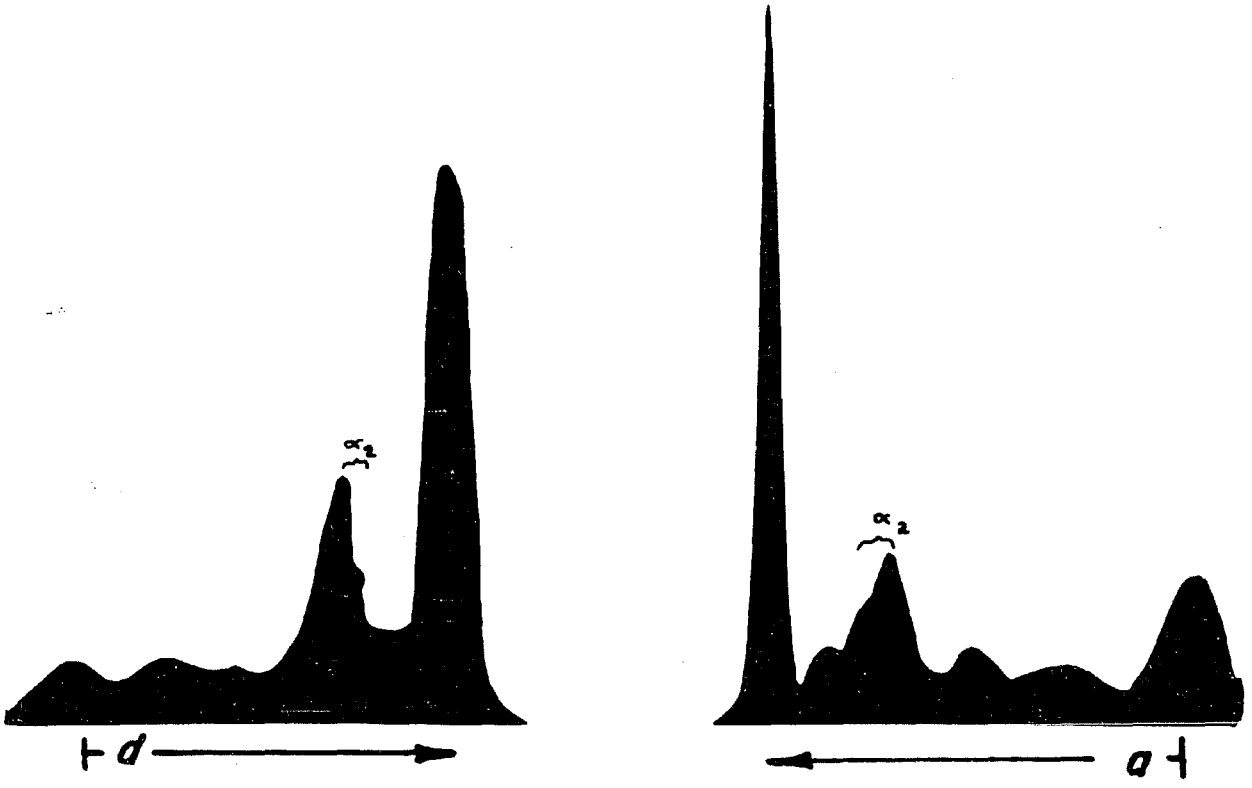


Figure 82. Electrophoretic boundaries of normal guinea pig serum diluted to 2.0 per cent in barbital buffer, Ph 8.6. Electrophoresis carried out for 10,000 seconds at a potential gradient of 6.76 volts per centimeter. Boundaries photographed by the schlieren scanning method.

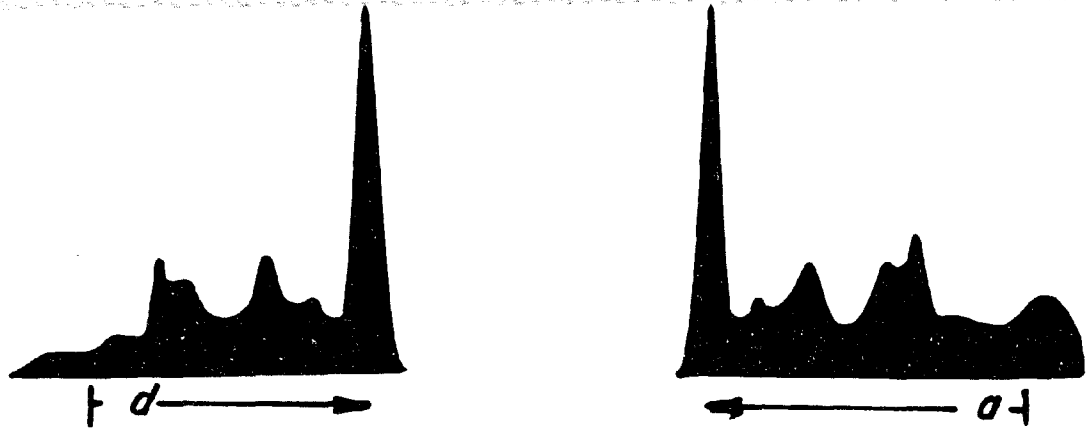


Figure 82a. Electrophoretic boundaries of normal guinea pig plasma diluted to 1.5 per cent in barbital buffer, pH 8.6. Electrophoresis carried out for 10,000 seconds at a potential gradient of 5.79 volts per centimeter. Boundaries photographed by the schlieren scanning method.

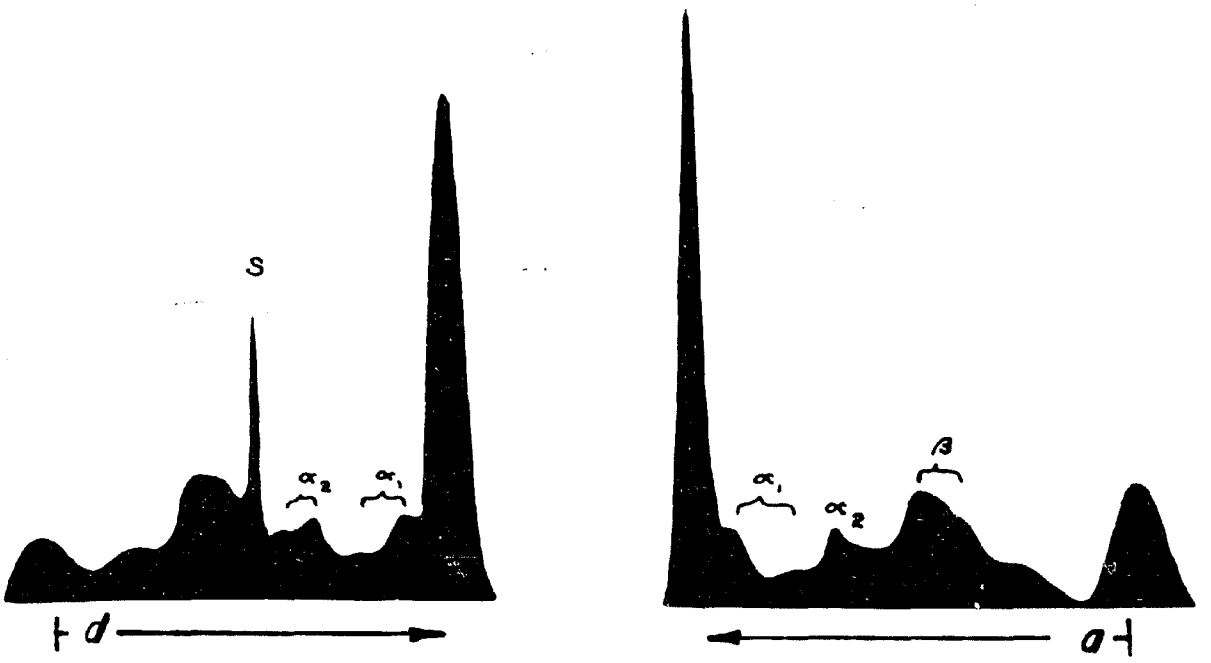


Figure 83. Electrophoretic boundaries of normal dog serum diluted to 2.0 per cent in barbital buffer, pH 8.6. Electrophoresis carried out for 10,000 seconds at a potential gradient of 7.06 volts per centimeter. Boundaries photographed by the schlieren scanning method.

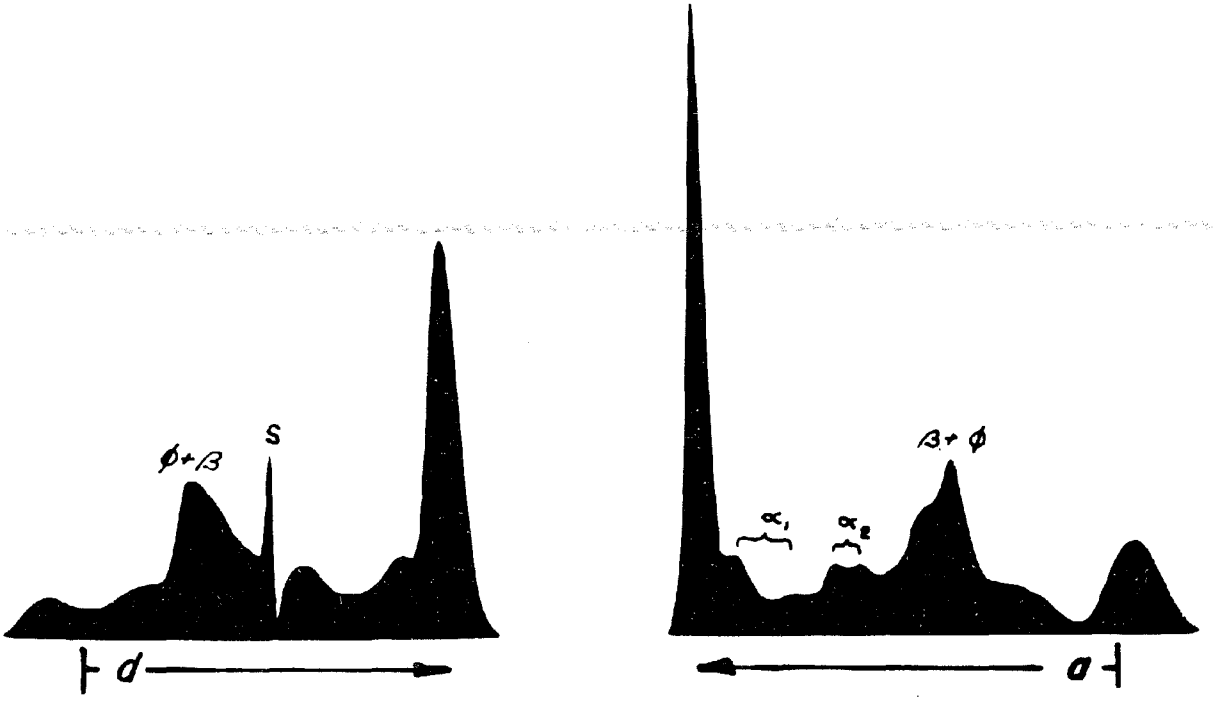


Figure 83a. Electrophoretic boundaries of normal dog plasma diluted to 2.0 per cent in barbital buffer, pH 8.6. Electrophoresis carried out for 10,000 seconds at a potential gradient of 6.73 volts per centimeter. Boundaries photographed by the schlieren scanning method.

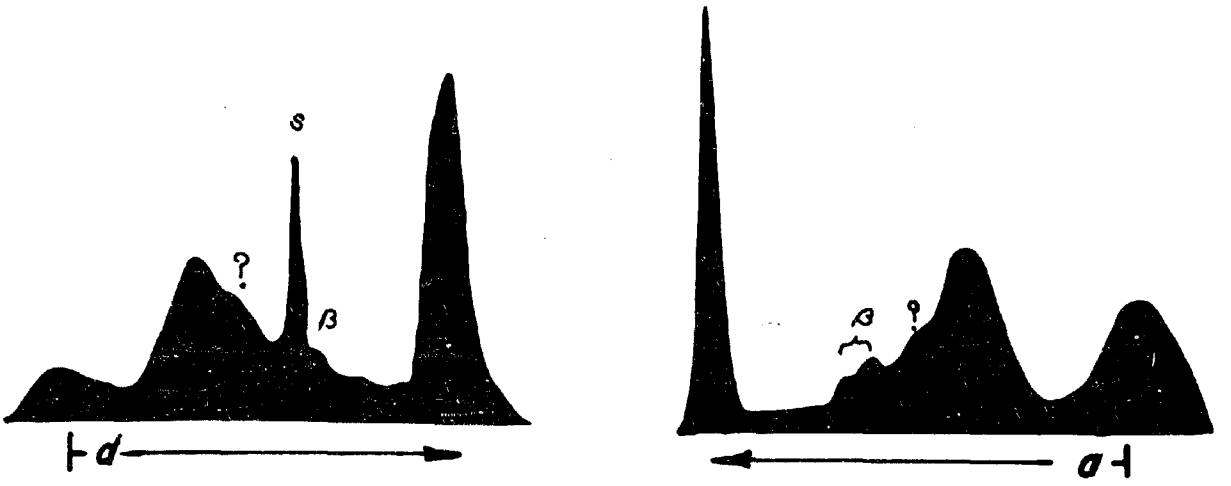


Figure 84. Electrophoretic boundaries of normal sheep serum diluted to 2.0 per cent in barbital buffer, pH 8.6. Electrophoresis carried out for 10,000 seconds at a potential gradient of 6.41 volts per centimeter. Boundaries photographed by the schlieren scanning method.

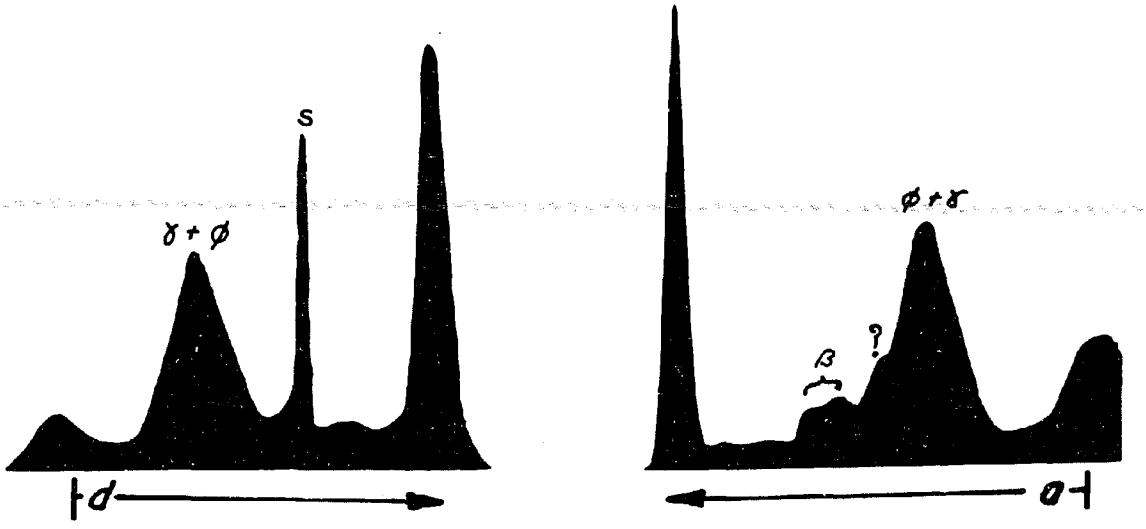


Figure 84a. Electrophoretic boundaries of normal sheep plasma diluted to 2.0 per cent in barbital buffer, pH 8.6. Electrophoresis carried out for 10,000 seconds at a potential gradient of 6.71 volts per centimeter. Boundaries photographed by the schlieren scanning method.

disturbance on the descending side and a double peak is revealed in the ascending pattern. Apparently the fibrinogen has not separated from the γ globulin on either side. The additional component is seen on the ascending side.

Figures 85 and 85a show the electrophoretic patterns of swine serum and plasma. The descending serum pattern is similiar to the human serum pattern. However, the β boundary disturbance migrates well ahead of the β component. The concentration of the γ globulin appears to be rather high. The ascending pattern shows only one alpha globulin and the γ globulin appears to be divided into three closely combined peaks. The β and γ globulins and fibrinogen components in the plasma patterns failed to separate into well divided areas.

Serum and Plasma Fractionation:

In addition to offering a method for fractionating serum and plasma proteins the Tiselius apparatus is a valuable tool for following fractionations by other methods. An excellent review on this subject has been presented by Cohn (117). In this paper Cohn states that "... each of the fractions revealed represents not a single protein but a population of proteins varying in size, shape, solubility, physiological and immunological function, and in many other respects." In fact some believe that blood is a continuous mixture of proteins and that all the apparent fractions are artifacts. This subject has been carefully discussed by Hill (129).

Svensson (105) fractionated serum of several species by adding various percentages of ammonium sulfate. After precipitation was complete the serum was filtered and analyzed electrophoretically. Up to concentrations as high as 60 per cent saturation of ammonium sulfate, only the γ globulin

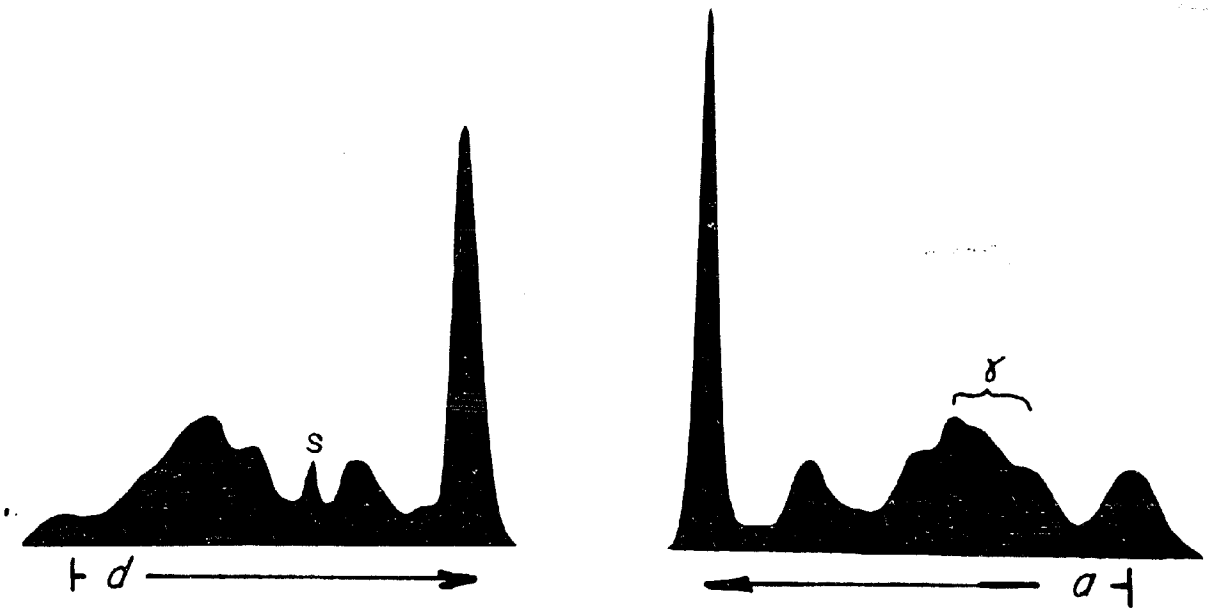


Figure 85. Electrophoretic boundaries of normal swine serum diluted to 2.0 per cent in barbital buffer, pH 8.6. Electrophoresis carried out for 10,000 seconds at a potential gradient of 6.45 volts per centimeter. Boundaries photographed by the schlieren scanning method.

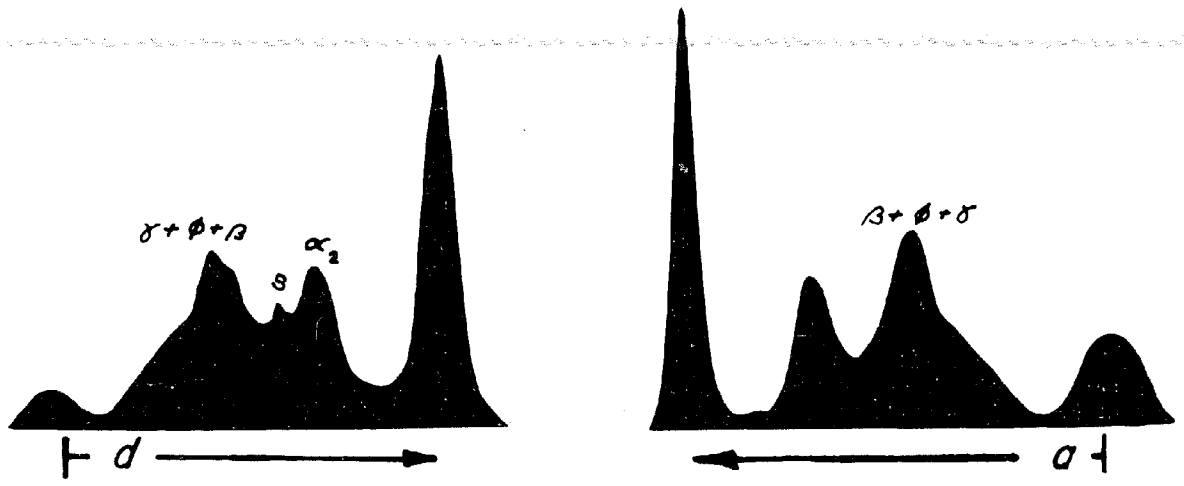


Figure 85a. Electrophoretic boundaries of normal swine plasma diluted to 2.0 per cent in barbital buffer, pH 8.6. Electrophoresis carried out for 10,000 seconds at a potential gradient of 6.58 volts per centimeter. Boundaries photographed by the schlieren scanning method.

was completely precipitated. The α and β globulins were found to have solubilities parallel to their electrophoretic mobilities. Svensson found that euglobulin (water insoluble globulin fraction) is a mixture of α, β and γ globulins. Cohn (130) fractionated normal horse serum by equilibration across cellophane membranes with ammonium sulfate solutions of known pH, concentration, volume and temperature. The course of the fractionation was followed electrophoretically. He too found that the components showing higher mobilities were more soluble than the components with lower mobilities. After separating the globulin fractions and showing that they were electrophoretically homogenous, they were further separated by water dialysis into euglobulins and pseudo-globulins (water soluble globulins). This study demonstrated the presence of many more components than are revealed by analyses of unfractionated serum. In a separate study McMeekin (131) was able to separate normal horse serum albumin into two fractions, one high in carbohydrate and one free from carbohydrate. Both of these preparations were found to be homogenous by ultracentrifuge and electrophoretic analysis. The mobilities of the two albumins in buffer of pH 7.7 and ionic strength 0.2 were 5.3 and 4.5×10^{-5} $cm^2/sec/volt$ respectively for the carbohydrate free and carbohydrate containing albumins.

Cohn and collaborators (132) have developed the method of ethanol-water fractionation of serum and plasma proteins to a high degree. Electrophoretically they have shown that bovine plasma is separated as follows. Fifteen per cent alcohol at 0°C precipitates largely fibrinogen and fibrin. Fraction 2 is precipitated by 20 to 25 per cent ethanol at -5°C and is largely γ globulin. 30 to 40 per cent ethanol precipitates a mixture of the α and β globulins. Fraction 4

remaining in solution at 40 per cent ethanol concentration is largely albumin. Figures 86, 87 and 88 show results obtained in this laboratory in the ethanol-water fractionation of normal bovine serum. All samples were dialyzed against the ethanol-water concentrations indicated at from 0° to -5°C until specific gravity measurements indicated the desired concentration had been obtained. Figure 86 shows the precipitate obtained with 10 per cent ethanol. It is largely fibrinogen but traces of β, and γ globulins and albumin are present. The ascending pattern in particular shows a component traveling behind the γ globulin that has not been identified. The presence of this component in normal unfractionated bovine plasma has been indicated in unpublished work from this laboratory. Figure 87 shows the precipitate obtained from 20 per cent ethanol. The chief component has been identified as γ globulin; β globulin and a trace of albumin are also present. The precipitate formed with 30 to 40 per cent alcohol is shown in Figure 88. It is largely γ globulin with a fairly high concentration of β globulin and traces of α^2 globulin and albumin. The material soluble in 40 per cent alcohol was found to be largely albumin but traces of the alpha and beta globulins were present. As yet we have been unable to prepare pure fractions of any of the normal bovine plasma or serum components by the ethanol-water method.

An interesting and timely application of electrophoretic analysis is in the field of blood plasma and serum processing. Scudder (133) in studying the effects of refrigeration on human blood plasma obtained the patterns shown in Figure 89. Up to 53 days (the upper limit reported) the plasma appears normal. The only change indicated is in the spike due to the β boundary disturbance. It is possible that

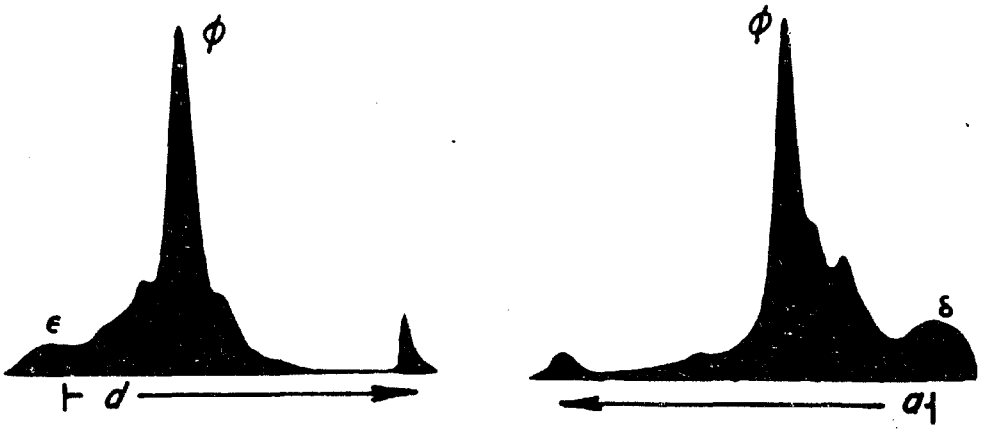


Figure 86. Electrophoretic boundaries of material insoluble when bovine plasma is dialyzed against cold 10 per cent ethanol.

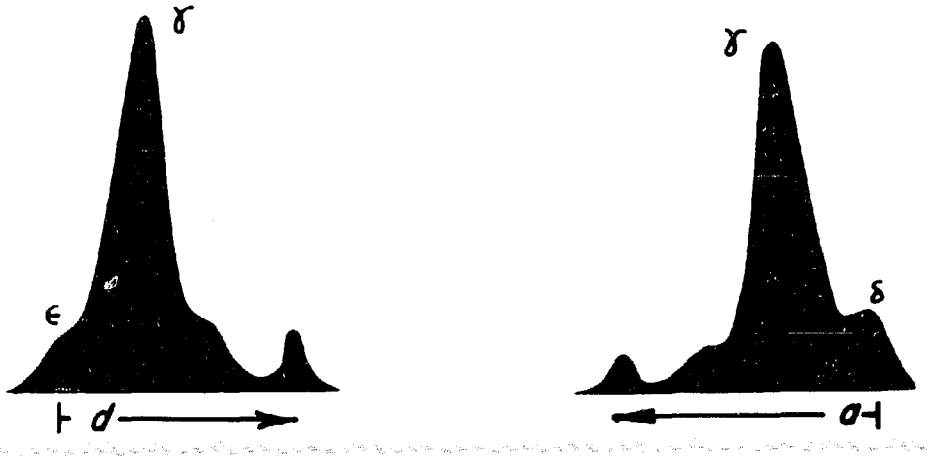


Figure 87. Electrophoretic boundaries of material insoluble when bovine plasma is dialyzed against cold 20 per cent ethanol.

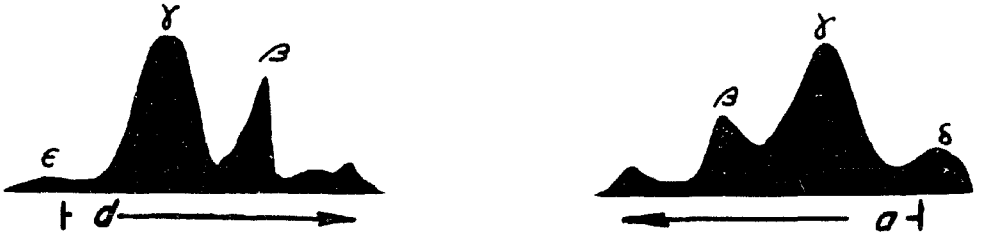


Figure 88. Electrophoretic boundaries of material insoluble when bovine plasma is dialyzed against cold 30-40 per cent ethanol.

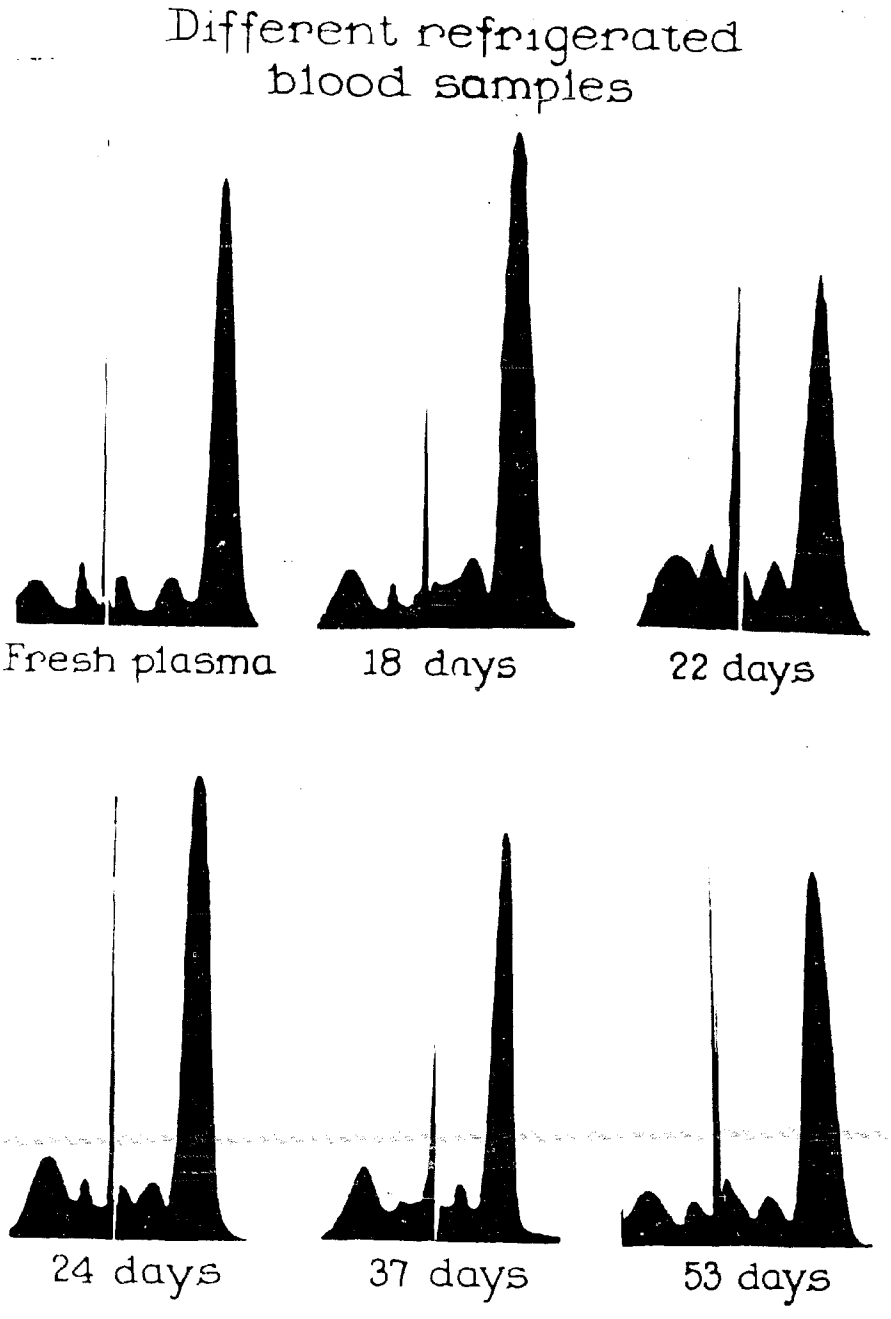


Figure 89. Patterns obtained from refrigerated human blood plasma taken at various intervals after freezing. (Scudder, 133)

aging causes a change in the lipid protein complex to which some have ascribed the basis of this anomaly. Figure 90 shows a normal human plasma sample and the same sample after drying. It appears that no noticeable changes occur in the pattern when plasma is dried either by the lyophile process or under partial vacuum.

Studies On Other Body Fluids:

In a study of nephrotic urine Longsworth (106) found that the anomaly is not apparent, which may indicate that one of the components of the constituents of the complex is missing or that conditions which favor its formation are lacking. Nephrotic urine patterns resembled those of normal serum.

Kabat (134) found that the proteins of cerebrospinal fluid are similiar to those of the plasma proteins. However the normal fluid shows neither α globulin or fibrinogen.

Other investigations have been made on urine, pleural effusions, cerebrospinal fluid, hydrocele testes, ascites fluid, cow aqueus humor and pericardial fluid. (135). In several cases the faster moving component was isolated and identified as albumin. Fractions corresponding to α, β, and γ globulins were found in some or all of the samples. Blix (136) studied hyalmucoid from the vitreous body of the eye, synovial mucin and submaxillary mucin. Hesselvek (137) studied human synovial fluid.

Immunological studies:

In an early work on antipneumococcus serums from the horse, swine, and monkey, Tiselius (124) (138) demonstrated the presence of a new component, all of which appeared to be antibody, since it was absent

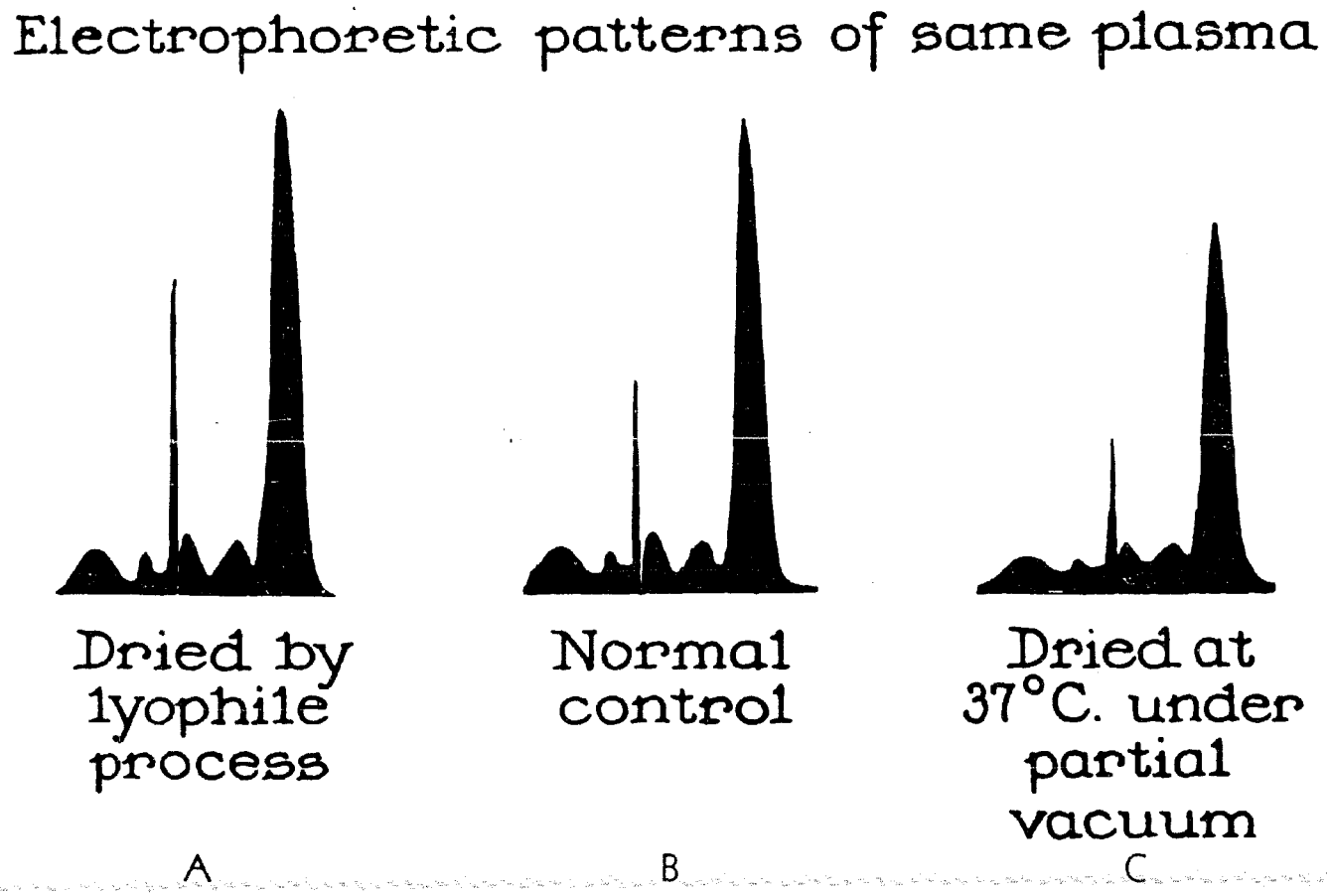


Figure 90. A comparison of the electrophoretic patterns obtained from normal human plasma, dried by two different methods. (Scudder, 133)

on electrophoresis of the same serum after absorption of the antibody with antigen. The new component migrated between the β and γ globulins. The new component was found in swine serum and in this case its mobility very closely approached that of the γ globulin. In the same study, rabbit anti-egg albumin serum was investigated. The antibody was found to be identical with the γ component of the serum. Another investigation of anti-pneumococcal horse serums was made by Vander Scheer and coworkers (126) (139). They found that the antibodys always showed the same mobility as γ globulin and no new component was demonstrated. These authors explain the descrepency between their results and those of Tiselius (124) (138) as follows. "It has been thought that the different mobilities found for pneumococcal antibodies arise from the use of different pneumoccal antigens. It has also been proposed that the electrical mobilities of antibody molecules as well as their molecular weights change with the length of time over which hyperimmunization has been proceeding." In one case the new component as described by Tiselius was demonstrated. Fell (127) has found both types of antibodies in antipneumococcus horse serum.

In electrophoretic studies with horse tetanus antitoxic serums the presence of a new component not present in normal horse serum was demonstrated (140) (141). The new component was labeled "T" and was found to have an average mobility of 1.98×10^{-5} $cm^2/sec/volt$ in a phosphate-sodium chloride buffer of pH 7.6. The same component was demonstrated in certain other antiserums (142) (see plate I Figures 3,4,5,6,7,8,9,10,11,12,14, and 15.) In a summary of these results the authors state. "In some of these sera antibody is expressed by an increase in amount of the normally present γ globulin . In others its

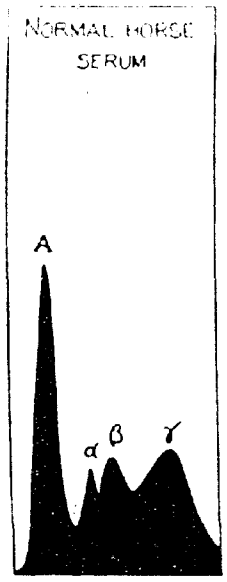


FIGURE 1

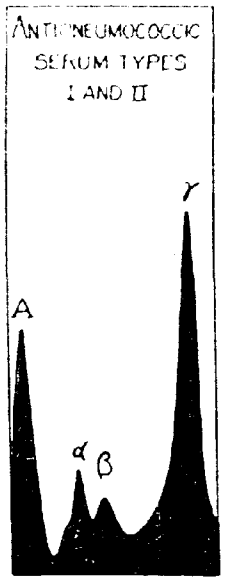


FIGURE 2

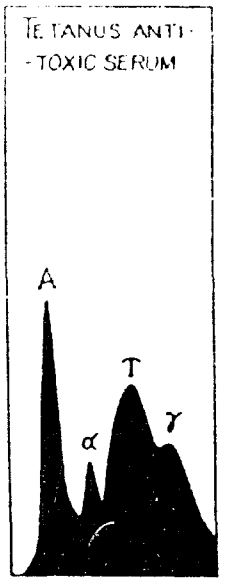


FIGURE 3

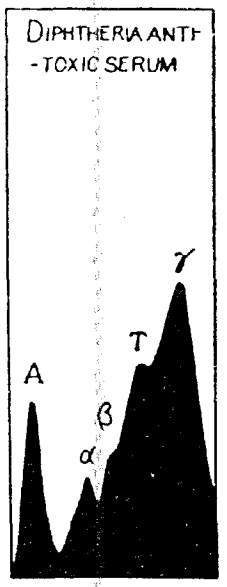


FIGURE 4

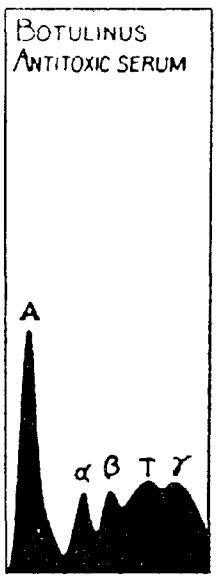


FIGURE 9

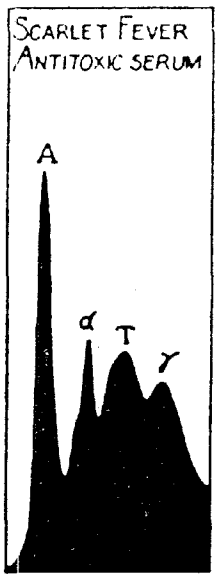


FIGURE 10

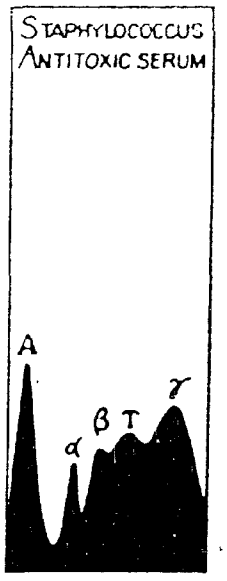


FIGURE 11

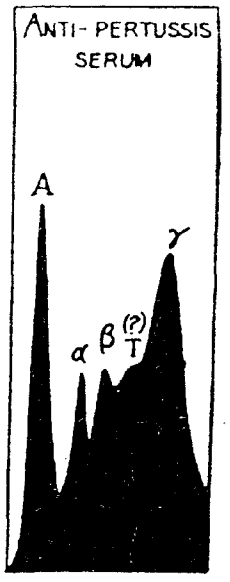


FIGURE 12

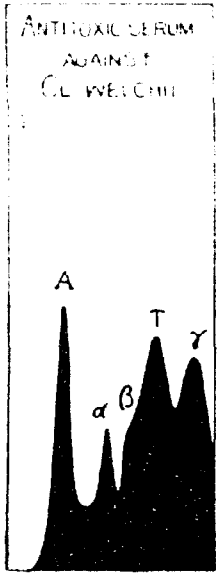


FIGURE 5

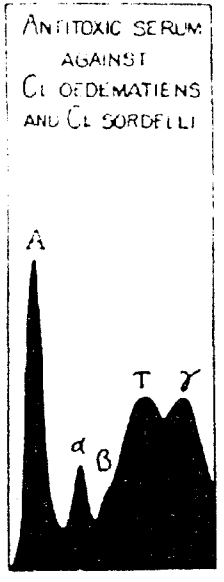


FIGURE 6

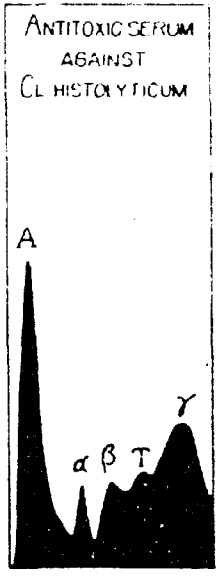


FIGURE 7

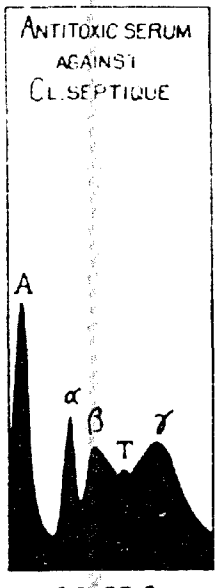


FIGURE 8

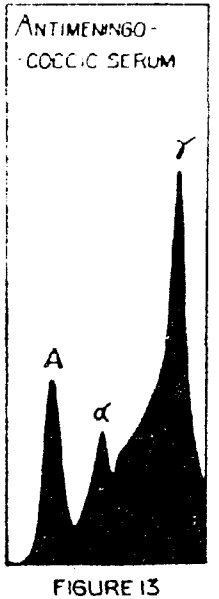


FIGURE 13

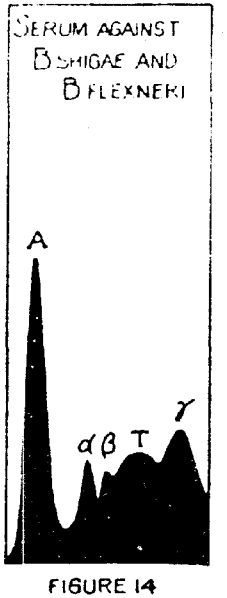


FIGURE 14

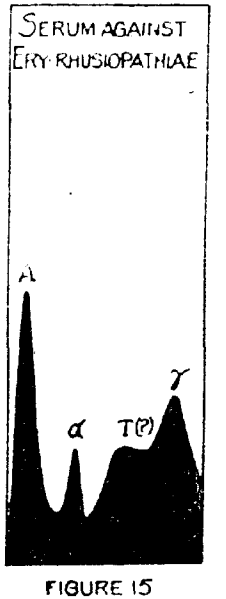


FIGURE 15

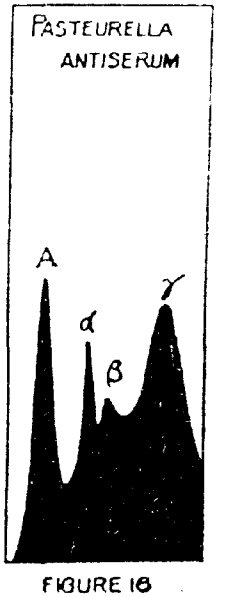


FIGURE 16

Plate I (Van der Scheer, Wyckoff and Clarke, 1942)

appearance is accompanied by the development of a new "T" component." It was stated further, that in spite of this obvious association of antitoxic activity with "T", the areas under the "T" peaks cannot be taken as proportional to the measured antitoxic activity.

Kekwick (125) followed the changes in horse serum during the course of producing diptheria antitoxin. The normal constituents were found to remain but their relative concentrations changed. All antitoxic serums showed a β' and β^2 globulin component. The β^2 component may be the same as the "T" component but the mobility was so close to that of β globulin that it did not completely separate. The antitoxic serums were fractionated and the β and γ globulins were the only fractions that showed antitoxic activity. Rothen (143) found that a large increase in γ globulin occured during immunization of horses against diphtheria toxin.

It is known that certain antitoxins can be partially digested with pepsin without losing their abilities to neutralize toxin (144). Diphtheria antitoxic horse plasma when digested with pepsin under certain conditions shows a 69 per cent loss of coagulable protein which is accompanied by only a 20 per cent loss of antitoxic activity. By this procedure the antigenicity of the antiserum is greatly reduced. Figure 91 from Van der Scheer (145) shows the electrophoretic changes accompaning pepsin digestion of defibrinated diphtheria-antitoxic horse plasma. The digestion was carried out with acid pepsin (pH 4.0) at 37°C. Figure 91-1 shows the electrophoretic pattern of the antiserum before digestion. In Figure 91-2 the "T" component is indicated only after 1/2 hours digestion. Figure 91-3 shows the results after 48 hours digestion. That the heat by itself does not cause this effect is shown in Figure 91-4.

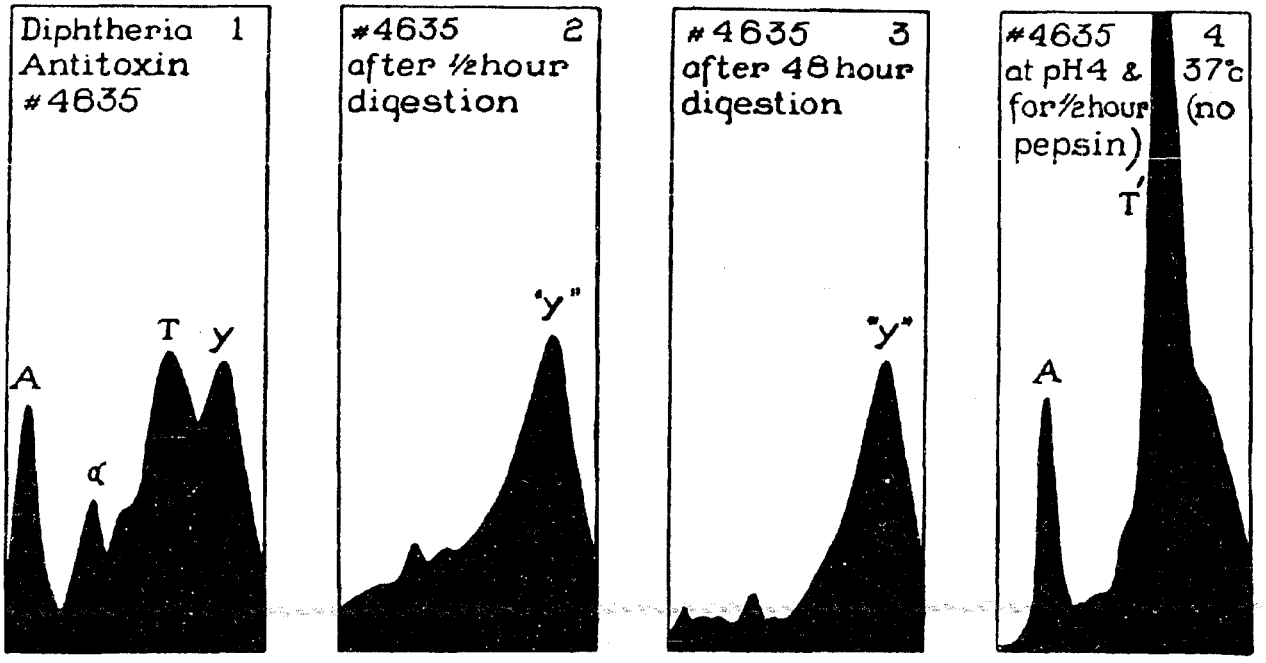


Figure 91. Electrophoretic patterns showing the effect of pepsin digestion of diphtheria-antitoxic horse serum. Discussion in text. (Van der Scheer, Wyckoff and Clarke, 145)

It has been observed previously that many diseases cause a marked change in the blood protein albumin-globulin ratios. Frequently the globulin concentrations, gamma in particular, show a marked increase. A comparison of Figure 91a, a normal bovine plasma pattern with Figure 91b, obtained from a brucellosis infected cow shows a marked increase in the gamma globulin concentration. By combining agglutinin absorption techniques with electrophoretic analyses, San Clemente (57) was able to show that Brucella agglutinins migrate with the gamma globulin component. It was not possible to remove all of the gamma globulin by the absorption method indicating that the gamma globulin fraction was a mixture.

Figures 91c and 91d show the electrophoretic patterns obtained from serum and plasma of a brucellosis infected horse. The globulin concentrations are abnormally high as can be seen by comparing these patterns with normal horse serum and plasma (see Figures 79a and 79b). It is also of interest to note that the alpha two globulin concentration approaches that of the albumin.

In a study of the proteins of tuberculin (146) and the blood serum response in tuberculosis (147) Siebert has made the following observations. Two distinct proteins are present in tuberculin. In phosphate buffers of pH 7.6-7.6 (ionic strength 0.1) the mobilities of these two proteins are of the order of 3 to 4 and 6 to 7 x 10^{-5} cm^2/sec/volt the mobility varying slightly with various preparations. It is shown that there is always a progressive decrease in the amount and percentage of serum albumin with progression of tuberculosis. In early tuberculosis the α globulins increase in percentage. At the same time a new component, X, with a mobility greater than albumin occurs.

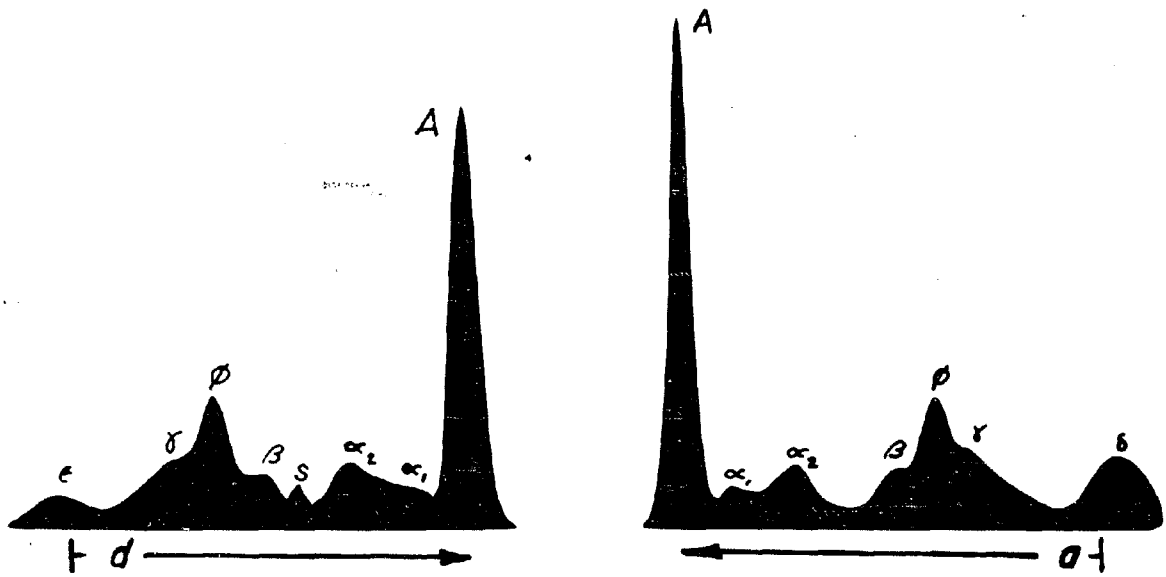


Figure 91a. Electrophoretic boundaries of normal bovine plasma diluted to 2.0 per cent in barbital buffer, pH 8.6. Electrophoresis carried out for 10,000 seconds at a potential gradient of 6.32 volts per centimeter. Boundaries photographed by the schlieren scanning method.

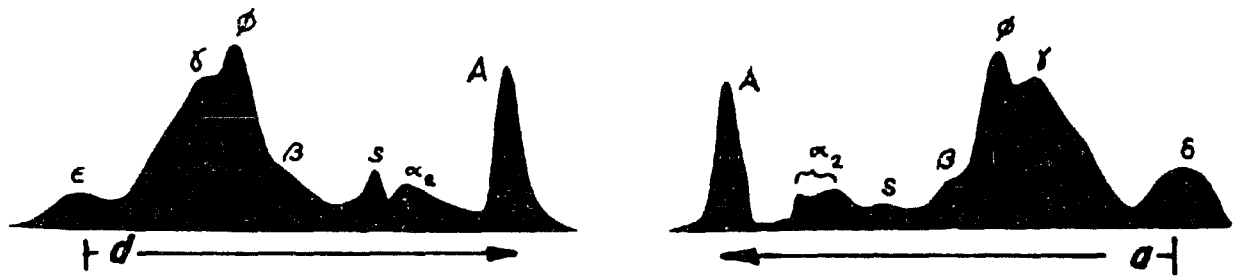


Figure 91b. Electrophoretic boundaries of brucellosis infected bovine plasma diluted to 2.0 per cent in barbital buffer, pH 8.6. Electrophoresis carried out for 10,000 seconds at a potential gradient of 6.43 volts per centimeter. Boundaries photographed by the schlieren scanning method.

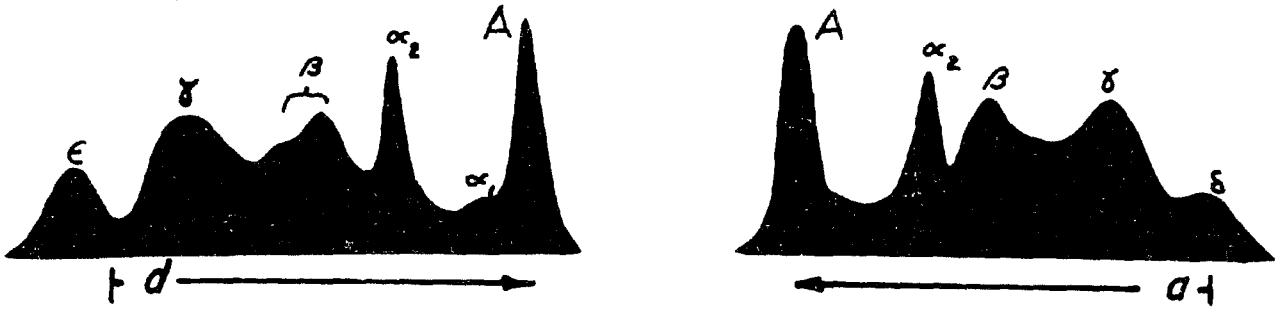


Figure 91c. Electrophoretic boundaries of brucellosis infected horse serum diluted to 2.0 per cent in barbital buffer, pH 8.6. Electrophoresis carried out for 10,000 seconds at a potential gradient of 6.80 volts per centimeter. Boundaries photographed by the schlieren scanning method.

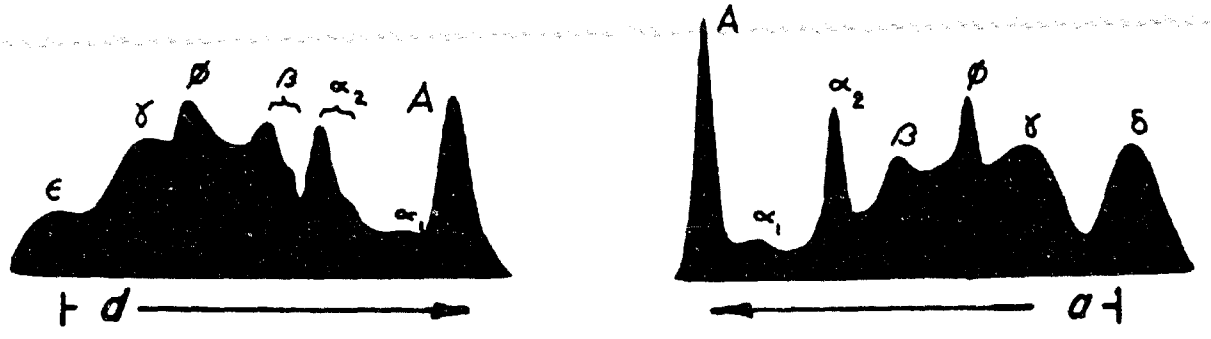


Figure 91d. Electrophoretic boundaries of brucellosis infected horse plasma diluted to 2.0 per cent in barbital buffer, pH 8.6. Electrophoresis carried out for 10,000 seconds at a potential gradient of 6.94 volts per centimeter. Boundaries photographed by the schlieren scanning method.

It is believed that these changes represent sensitization to the tuberculin protein. In the terminal stages of tuberculosis the β globulin increases. There was some evidence obtained which indicated that a rise in γ globulin accompanied resistance to the disease. In another study Siebert (148) separated the protein, nucleic acid, and polysaccharide fractions of tuberculin electrophoretically. The polysaccharide fractions were of two types electrophoretically. One fraction showed no migration in the electric field and the other a slow migration.

Electrophoretic studies on guinea pigs compliment fractions have shown the following (149). The mid-piece has been separated as a euglobulin, with an electrophoretic mobility of 2.9×10^{-5} in phosphate buffer of ionic strength 0.2 at pH 7.7. The end piece and fourth component were found together in a euglobulin fraction of serum which contained 10.3 per cent carbohydrate and had an electrophoretic mobility of 4.2×10^{-5} in phosphate buffer of ionic strength 0.2 at pH 7.7.

After certain of the viruses had been isolated in supposedly pure form, electrophoretic studies were carried out in order to obtain physical property measurements and to determine the homogeniety of the preparations.

In a study on tobacco mosaic virus (150) solutions of the pure virus protein were found to be isoelectric at pH 3.49. Electrophoretically the preparations were uniform but they were not monodisperse in the ultracentrifuge. It is of interest to note that Pfankuck (151) has reported that it is possible to split the particle of tobacco mosaic virus protein into 50 to 100 pieces having identical electrophoretic properties.

McFarlane (152) noted that the tomato bushy stunt protein was monodisperse electrophoretically over a pH range of 2.4 to 8.6 and isoelectric at pH 4.11.

An attempt to electrophoretically isolate and crystallize the vaccinia virus was made by Douglas (153). The virus was obtained mixed with rabbit testes proteins. The testes proteins were isoelectric at pH 6.8 and the virus proteins carried a negative charge from pH 5.5 to 8.4. By carrying out electrophoresis studies below pH 6.8 and above pH 5.5 the testes proteins migrated as cations and the virus proteins as anions. Separations were reported in this manner but a pure crystalline product was not obtained.

An electrophoretic separation of the fowl leucosis virus was claimed by Lee (154), using a U tube apparatus. The progress of the separation was followed by injecting material from the anode, cathode and center sections of the cell into susceptible animals. The isoelectric point of the virus protein was found to lie somewhere between pH 6.01 and 7.01.

Using electrophoretic techniques Smadel and Shedlovsky (155) have shown the virus of vaccinia to have a complex structure. At least five antibodies developed in animals following injection or hyperimmunization with active elementary bodies, viz., a neutralizing antibody, an agglutinin designated X, antibody against a nucleoprotein constituent (NP) of the virus, and finally, antibodies against a heat-labile (L) and heat stable (S) soluble antigen. L and S antigens, although immunologically distinct, are not separate substances; they are components of a single substance, LS.

Comphrensive studies have been carried out by Abramson (156) on pollen extracts causing hay fever. A wide variety of pollens have been analyzed electrophoretically. The patterns are all similar and contain a pronounced peak which may be a protein, and several pigment peaks. Because of the low molecular weights of the proteins (about 5,000) it is believed that they may be intermediate between polypeptides and proteins. Some of the pigments have been separated electrophoretically and like the protein component, they are biologically active.

Other Studies of Biological Interest:

Ringer (157) was possibly the first to attempt to purify enzymes by electrophoresis. He found that purified pepsin had no apparent isoelectric point. Tiselius (158) found on electrophoretic analysis that unless special precautions are observed, most supposedly pure pepsin preparations contain one main component and several contaminants. Upon further purification (electrophoretic) the contaminants were removed and pepsin acted like an acid remaining negative at all values of pH investigated. Herriot (159) has confirmed these observations and reports the isoelectric point of pepsin to be below pH 1.5.

Theorell (160) succeeded in isolating and purifying the yellow respiratory enzyme of Warburg and Christian by electrophoretic technique. He was able to identify the purified enzyme as a protein. Cytochrome- c was isolated electrophoretically by this same worker (161).

Other electrophoretic studies on enzymes have been reported. Choline esterase (162), ribonuclease (163), zymohexase (164), yeast carboxylase (165), and chymotrypsin (166). In the case of zymohexase it is of interest to note that the isoelectric point of the purified native

enzyme and of heat coagulated particles was pH 6.3 in all cases. Figure 93 shows the electrophoretic pattern obtained in this laboratory on a beef liver catalase preparation in barbital buffer, pH 8.6, u 0.1. The preparation appears to be quite homogenous and has a mobility of 3.12 cm^2/sec/volt calculated from the descending pattern.

Studies on the pitiutary-lactogenic hormone have been reported by Shipley (167) and Li (168). Pure preparations of the hormone appear to be homogenous. The crude gland extracts are largely composed of physiologically inert proteins. The isoelectric point of the pure hormone appears to be somewhere in the neighborhood of pH 5.6 to 5.7.

Shedlovsky and coworkers (169) (170) have succeeded in the electrophoretic isolation of the interstitial cell-stimulating (luteinizing) hormone from swine pituitary glands. The initial extract after chemical purification showed three electrophoretically distinct components. The main component was electrophoretically separated and found to contain all of the biological activity. The main component after separation was homogenous in both electrophoretic and ultracentrifuge studies with an isoelectric point of pH 7.45.

Other electrophoretic studies on hormones have been reported as follows: gonadotrophic hormones (171) (172), posterior pituitary (173), (174), (175) and thryoglobulin (176).

A comparison of the mobilities of various hemoglobins from different species has been made by Landsteiner (177). The mobilities of some of the hemoglobins from unrelated species were very similiar.

Muscle juice obtained by pressing muscle has been shown to contain the following proteins: myohemoglobin, myogens A and B, globulin X, and myoalbumin. Herbert (178) claims that at least 50 enzymes are present in varying amounts in muscle juice.

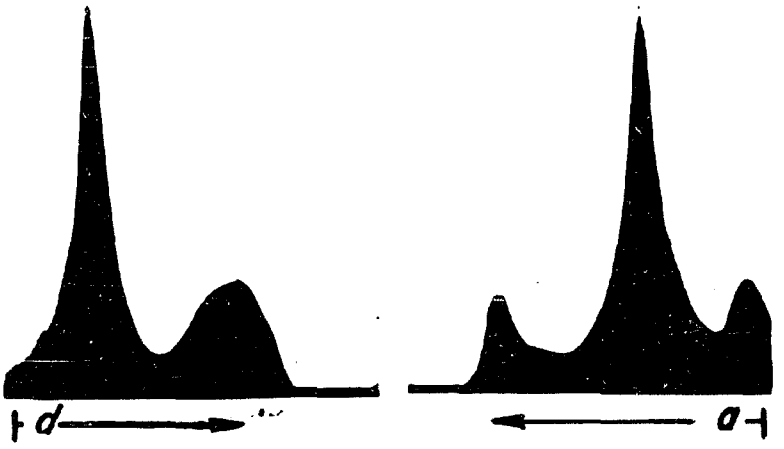


Figure 92. Electrophoretic boundaries of Brucella protein nucleate. Electrophoresis carried out for 3,400 seconds in phosphate buffer of pH 7.6 and ionic strength 0.1 at a potential gradient of 5.38 volts per centimeter.

Figure 93. Electrophoretic patterns of beef liver catalase. Description in text.

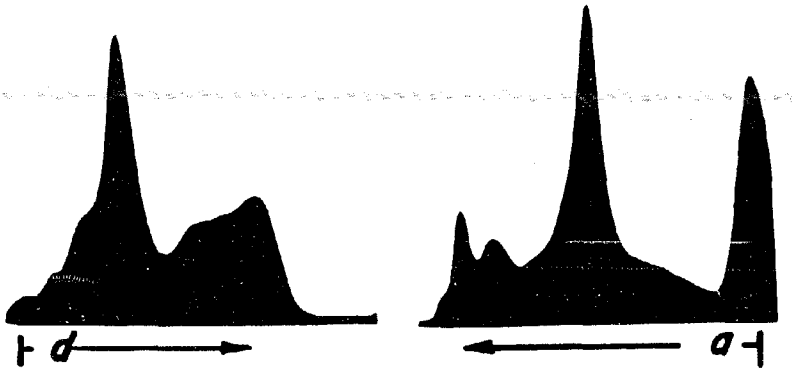


Figure 94. Electrophoretic boundaries of Brucella protein nucleate. Electrophoresis carried out for 2,900 seconds in phosphate buffer of pH 7.6 and ionic strength 0.02 at a potential gradient of 5.30 volts per centimeter.

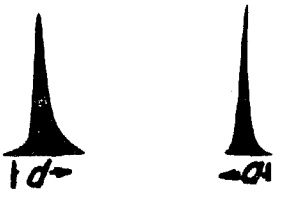


Figure 95. Electrophoretic patterns of Brucella gluco-lipid. Description in text.

A chlorophyll-protein complex from leaves has been reported by various workers. One electrophoretic study has shown that suspensions of these particles are isoelectric in acetate buffers (M/50 at a pH of 4.7). (179). These particles are believed to have uniform surfaces.

Fibrous proteins have been investigated as follows: Wool (180), (181), silk (60), hair, skin and fingernails (182), (183), and steer collagen (184).

Martin (185) and Kemp (186) have studied extracts of wheat proteins. The isoelectric point of gliaden was found by Kemp to be near pH 6.6 Glutenin was isoelectric at about pH 4.9. The isoelectric points of some other prolamines have been reported as follows: zein fractions, pH 6.29, 7.17 and 6.95, (187), and secalin pH 6.67 (in 63 per cent alcohol by weight) (188).

Cow casein (189) has been shown to consist of three electrophoretically separable components called α, β and γ casein in order of their decreasing mobilities. A boundary corresponding to lactalbumin was also observed.

The electrophoretic behaviour of certain nucleoproteins has been investigated (190). The nucleohistone of the calf thymus, two additional nucleoproteins of the calf thymus, and the nucleoprotein of the hog thyroid were characterized as uniform substances from their electrophoretic behavior. Figures 92 and 94 show patterns obtained in this laboratory on the protein nucleate from Brucella cells (191). The major component is the bacterial protein. The faster moving component is nucleic acid. The effect of ionic strength on electrophoretic separations is readily seen by comparing Figures 92 and 94.

Figure 95 shows the electrophoretic pattern obtained with the endo-antigen from Brucella cells (192). The electrophoretic homogeniety

of this substance is apparent. This is interesting since the endo-antigen has been found to contain a nitrogenous substance, glucose on hydrolysis and lipid.

It has been shown that when blood serum is heated to about 65°C it becomes opalescent and prolonged heating causes the entire sample to gel. Electrophoretic examination of serum heated at 65°C. showed the presence of a new component (193) called "C". On further study on this component it became evident that it was a colloidal aggregation product resulting from denaturation of components of the serum. Experiments in this laboratory have shown conclusively that glucose has an inhibitive action on "C" component formation when samples of serum or plasma are heated at 65°C in the presence of glucose (194). Figure 96 shows the pattern of a sample of normal bovine plasma. Figure 97 shows the pattern that results from heating the same sample at 65°C. The "C" component is very much in evidence. As discussed elsewhere (194) the peaks labeled X and Y are believed to be lipid disturbances. Figure 98 shows the pattern of the sample saturated with glucose and heated at 65°C for one hour. The "C" component is not formed under this condition of heating.

The use of electrophoretic techniques in following chemical separations has been clearly demonstrated by Longsworth (92) in an electrophoretic study of the proteins of egg white. Figure 99a shows the pattern of untreated egg white at pH 3.92. G_1, G_2, and G_3, are globulin fractions, C the conalbumin fraction, A the ovalbumin and O, the ovomucoid fraction. Part (b) shows the soluble fraction resulting from half saturation with ammonium sulfate. This fraction contains all of the components shown in (a) with the possible exception of globulin G_2.

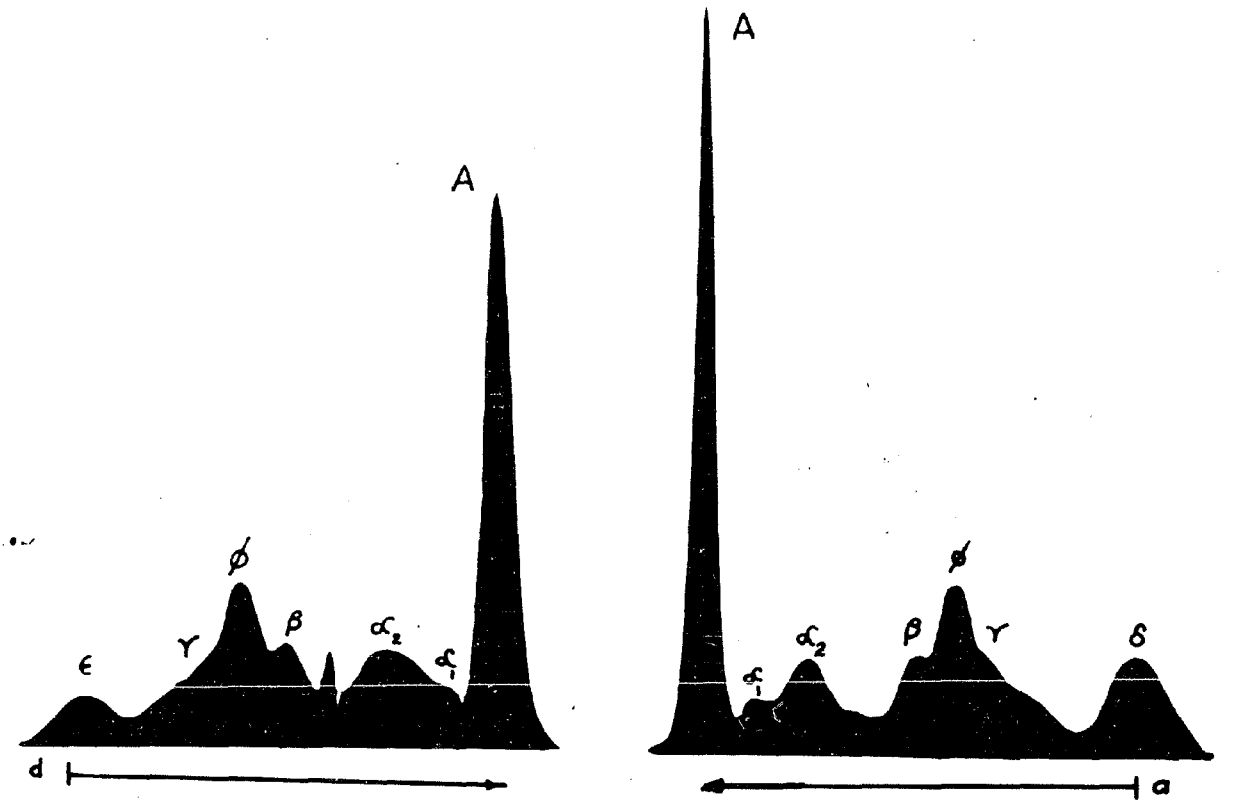


Figure 96

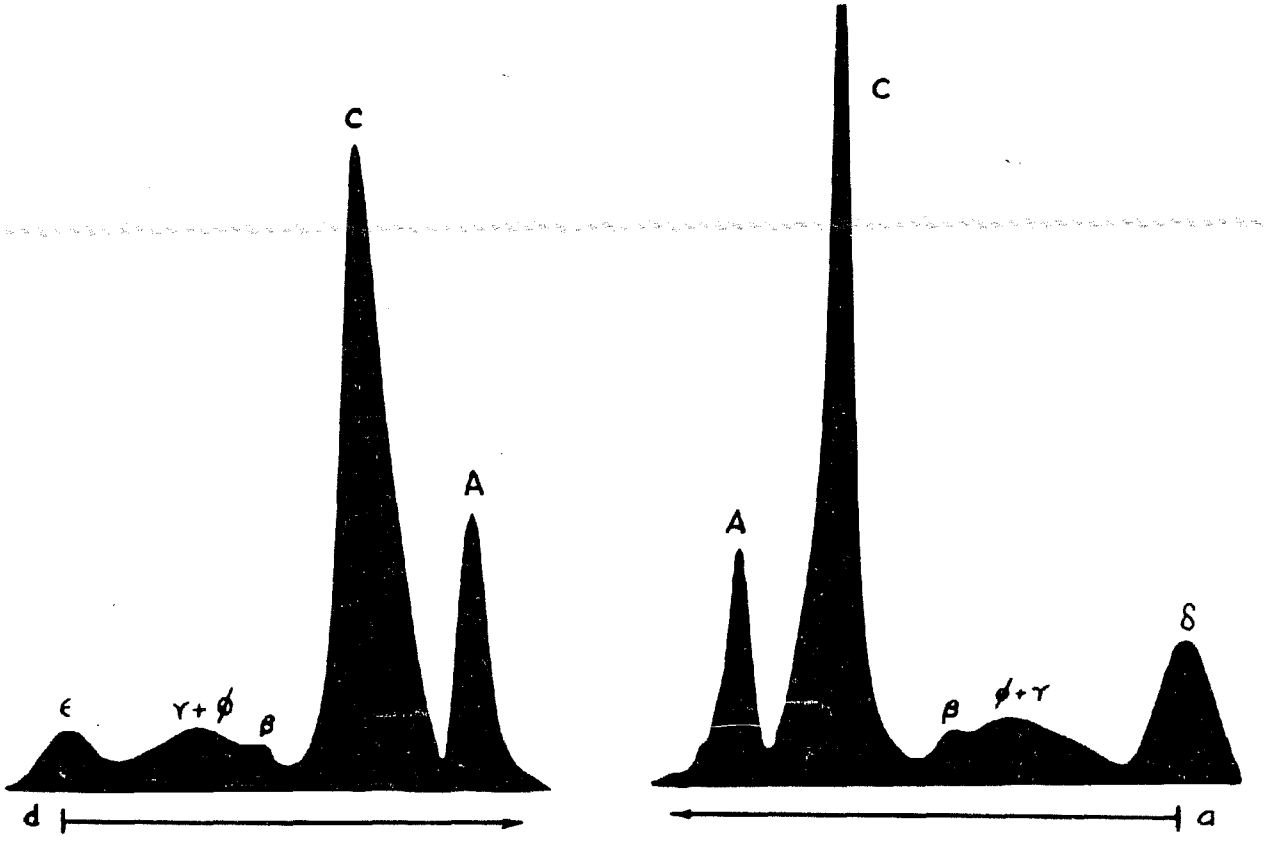


Figure 97

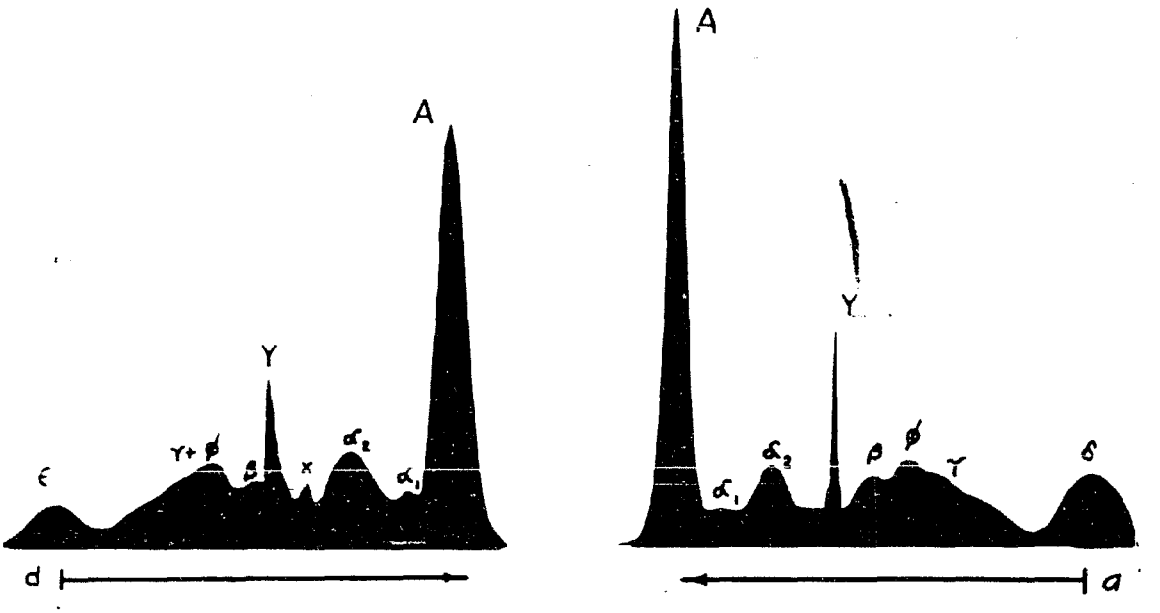


Figure 98

Figures 96, 97, and 98. Electrophoretic patterns of bovine plasma in barbiturate buffer of pH 8.6 and ionic strength 0.1. Electrophoresis of all samples carried out for 10,000 seconds at a potential gradient of 6.00 volts. Boundaries photographed by schlieren scanning method. Description in text.

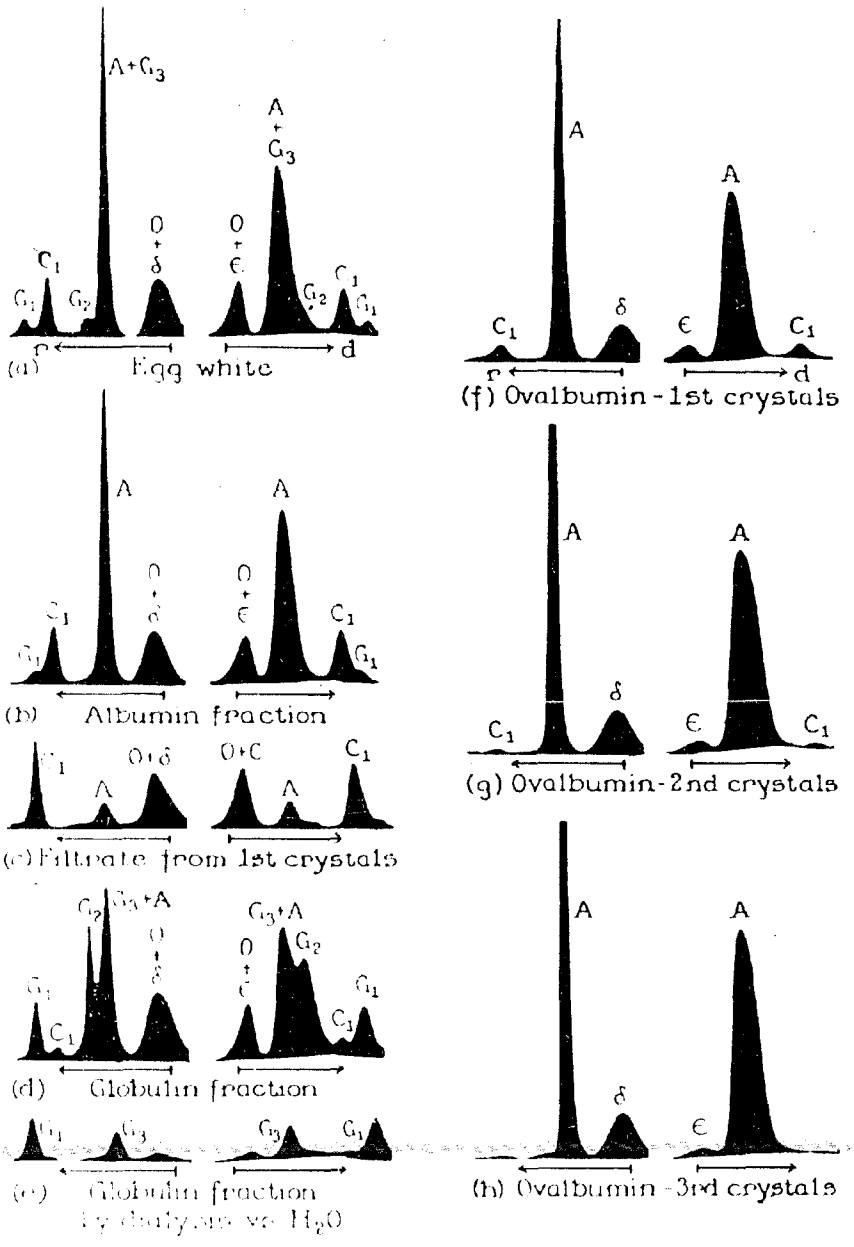


Figure 99. Electrophoretic patterns of egg white proteins illustrating how chemical fractionations can be followed electrophoretically. (Longsworth, Cannon and MacInnes, 92)

The insoluble fraction is shown in (d) and it too is a mixture of all of the original components. Figure 99 (f) shows the effect of crystallization of the albumin fraction. The G, globulin has dissappeared and the ovomucoid component is reduced. Figures 99 (g) and (h) illustrate the effect of further recrystallization of the ovalbumin fraction.

The chemical fractionation of the water soluble, crushed cell fraction obtained from Brucella cells as described by Huddleson (195) has been followed electrophoretically. Figure 100 shows a typical pattern of a crushed cell fraction. Electrophoresis of this sample was carried out for 3,800 seconds at a potential gradient of 4.12 volts per centimeter in a barbiturate-sodium chloride buffer of pH 7.8 and ionic strength of 0.1. The mobilities computed from the descending boundaries were as follows: (a) 4.40×10^{-5}, (b) 9.43×10^{-5}, (c) 11.84×10^{-5}, (d) 14.76×10^{-5} cm^2/sec/volt. Figure 100b shows the insoluble fraction obtained by dialyzing the crushed cell fraction against 45 per cent ammonium sulfate. Electrophoresis of this fraction and the subsequent fractions was carried out at a potential gradient of 6 volts for 3,000 seconds in a barbiturate buffer of pH 8.6 and ionic strength 0.1. The electrophoretic pattern shows that the d component has been removed. In addition the concentration of the a component has been reduced and at the same time the relative concentrations of the b and c components have been increased. Figure 100 c was obtained by electrophoresis of the material remaining in solution after the crushed cell fraction was made 30 per cent (by weight) with respect to p-dioxane (10°C). Three components are now evident. The steepest peak does not migrate under the conditions of this experiment and is believed to be an electrophoretically inert polysaccharide. The other two components are believed to be the b and c fractions. Figure 100 d was obtained upon

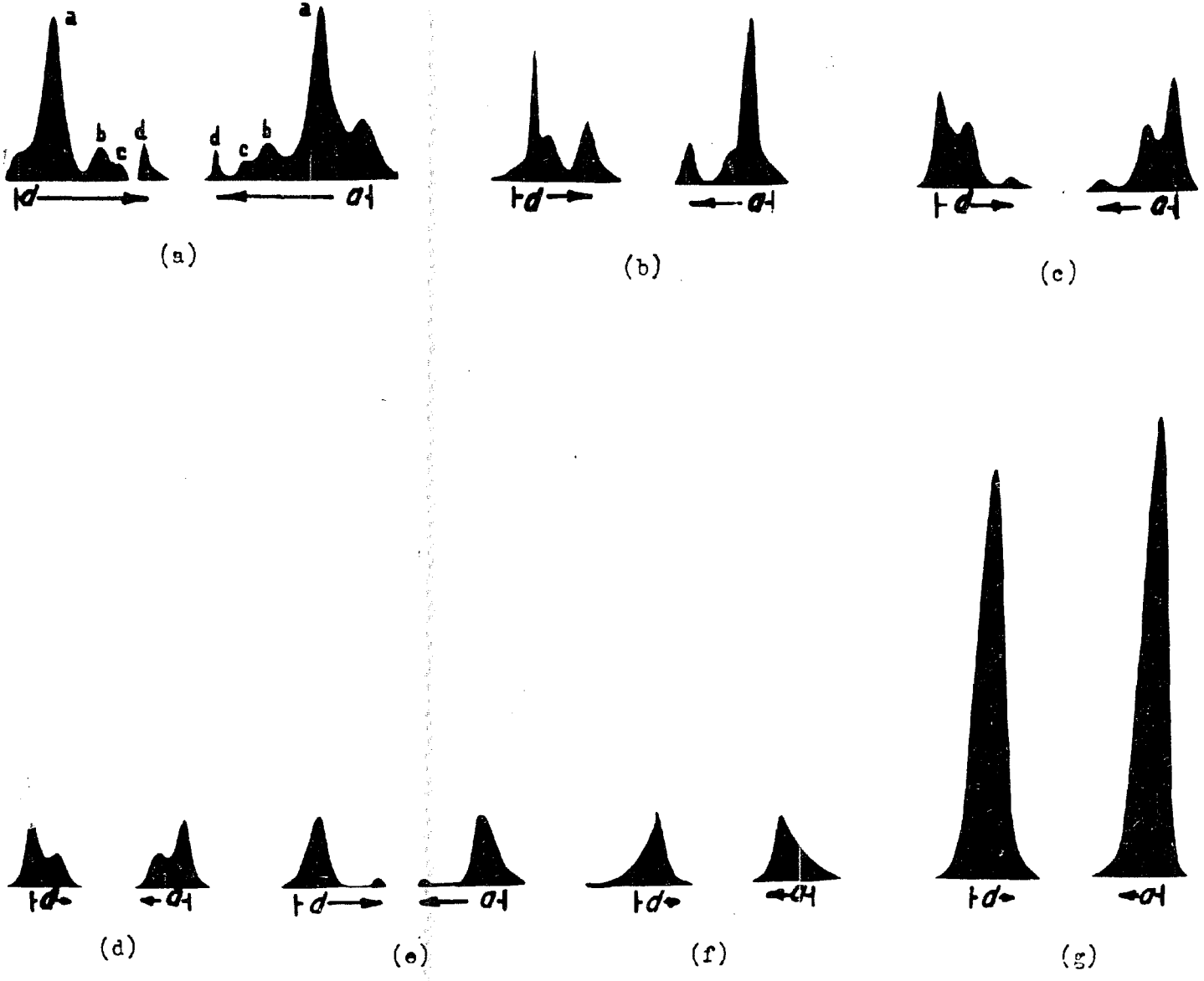


Figure 100. Electrophoretic patterns of water soluble Brucella crushed cell fractions in barbiturate buffer. Description in text.

electrophoretic analysis of the material soluble in 40 percent dioxane. The inert material is still evident and the other component has a mobility indicating that it is the b fraction. After dialysis of the crushed cell fraction against 30 percent ethanol (0°C.) electrophoretic analyses showed the b and d components to be present as shown in Figure 100 e. Increasing the ethanol concentration to 40 per cent removed the d component as shown in Figure 100 f. The electrophoretic pattern shown in Figure 100 g was obtained from the supernatent after the crushed cell fraction had been dialyzed against an acetate buffer of pH 3.91 and ionic strength of 0.1 (concentrated four times). This material is electrophoretically inert and does not give a positive biuret test. The molisch test is positive indicating that it may be a polysaccharide. Immunization studies have been carried out using these various fractions and the results will be published at a later date.

The standard Tiselius electrophoresis cell is applicable to and satisfactory for diffusion studies (66). Boundaries are formed as in electrophoresis experiments and then shifted into view. The center sections of the cell are then isolated by displacement to one side and diffusion patterns (schlieren scanning photographs of the refractive index gradients) are obtained at intervals until the diffusion process has approached the closed ends of the sections. At zero degrees this may take several days. Monopolization of the equipment can be avoided, by supporting the diffusion cells in the water bath in such a manner that they can be moved into view without distrubing the diffusion. In the case of ideal diffusion the maximum value of the refractive index gradient $\left(\frac{dn}{dx}\right)_m$ varies inversely as the square root of the time, t, according to the relation $\left(\frac{dn}{dx}\right)_m = \Delta m / \sqrt{4\pi D t}$

in which D is the diffusion coefficient and Δn is the refractive index increment. In practice the following procedure may be followed (66). "A series of ordinates are drawn on a sheet of coordinate paper at values of the abscissae equal to the reciprocals of the square roots of the times at which patterns were obtained. An enlarged image of the pattern is then traced on the paper after the position of the latter has been shifted to bring the base line of the pattern into coincidence with the axis of abscissae and the proper ordinate passes through the maximum value of the gradient. If diffusion has been normal these maxima fall as illustrated in Figure 101, on a straight line passing through the origin. The diffusion coefficient is then computed from the slope m, of this line and the areas under the curves, the latter being proportional to the refractive index Δn according to the formula $D = \frac{1}{4\pi}\left(\frac{\Delta n}{m}\right)^2$ The areas A, under each of the curves are indicated in Figure 101 and it will be noted that they are essentially constant as required by the simple theory."

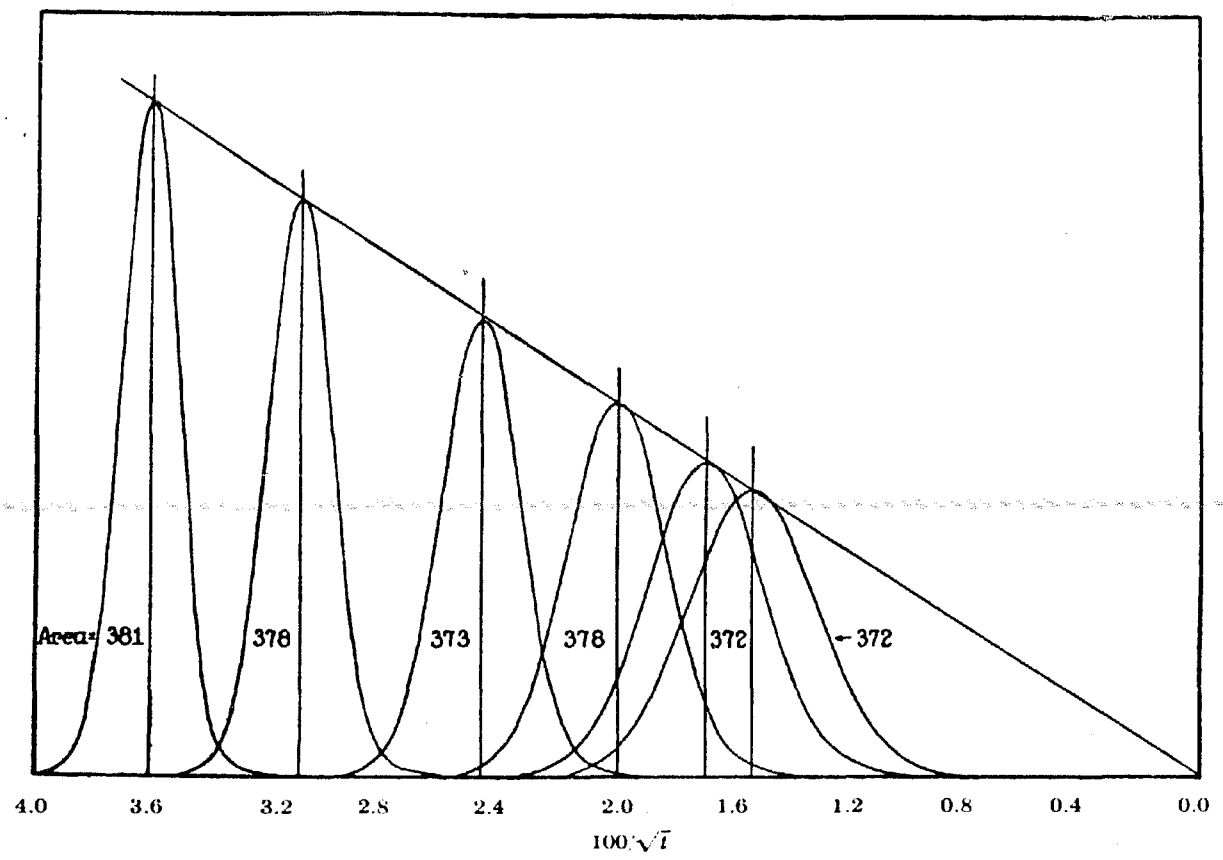


Figure 101. Diagram illustrating the adaptability of the Tiselius apparatus to diffusion studies. (Longsworth, 66)

LITERATURE CITED

1. Abramson, H. A., L. S. Moyer, and M. H. Gorin., Electrophoresis of proteins, Reinhold Publishing Co. New York. (1942), p. 13.

2. Abramson, H. A., The microscopic method of electrophoresis. J. Phys. Chem. 36 1454 (1932) See also reference (1) pages 43-44.

3. Tiselius, A., Thesis. Nova. Acta. Soc. Sci. Upsala IV, 7 4 (1930).

4. Reuss, F. F., Notice sur un nouvel effet de l'ectricite galvanique. Mem. Soc. Imp. des Naturalistes de Moscou. 2 327-337 (1809).

5. Porret, R., Curious galvanic experiments. Annals of Philosophy p. 74-76 July (1816).

6. Kuhne, W., Uber das Porret'sche phänomen am muskel. Arch. Anat. Physiol. 2 542 (1860).

7. Stern, K. G., The moving boundary method for studying electrophoresis. Ann. N. Y. Acad. Sci. XXXIX. 147-186 (1939).

8. Quincke, G., Uber die fortfuhrung materieller theilchen durch strömende elektricität. Pogg. Ann. 107, 1 (1859); 113. 513-598 (1861).

9. Jürgensen, T., Uber die bewegung fester in flüssigkeiten suspendirter körper unter dem einfluss des elektrischen stroms. Arch. Anat. Physiol. 2 673-687 (1860).

10. Lodge, O., Report of the British Association for the Advancement of Science. 389 (1886).

11. Picton, H., and Linder, S., Solution and pseudosolution. J. Chem. Soc. 61, 148-172 (1892).

12. Hardy, W. B., On the coagulation of proteid by electricity. J. Physiol. 24 288-304 (1899).

13. Hardy, W. B., Colloidal solution. The globulins. J. Physiol. 33 251-337 (1905).

14. Pauli, W., Untersuchung über physikalische zustandsänderungen der kolloide. Fünfte mitteilung. Die elektrische ladung von eiweiss. Beitr. Chem. Physiol. Path. 7 531-547 (1906).

15. Michaelis, L., Die Wasserstoffionenkonzentration. Berlin (1914), (1922).

16. Pauli, W., and Valko, L., Electrochemie der Kolloide. Dresden and Leipzig (1933).

17. Prausnitz, P. H., and Reitstotter, J., Electrophorese, Electroosmose, Electrodialyse in Flussigkeiten. Dresden and Leipzig (1931).

18. Svedberg, T., and Jette, E. R., The cataphoresis of proteins. J. Am. Chem. Soc. 45, 954-957 (1923).

19. Scott, V. D., and Svedberg, T., Measurements of the mobility of egg albumin at different acidities. J. Am. Chem. Soc. 46, 2700-2707 (1924).

20. Svedberg, T., and Tiselius, A., A new method for determination of the mobility of proteins. J. Am. Chem. Soc. 48, 2272-2278 (1926).

21. Theorell, H., Ein neuer kataphoreseapparat für untersuchungswecke. Biochem. Zeit. 275 1-10 (1934).

22. Theorell, H., Ein kataphoreseapparat für präparative zwecke. Ibid 278 291-297 (1935).

23. Tiselius, A., A new apparatus for electrophoretic analysis of colloidal mixtures. Trans. Faraday Soc. 33 524-531 (1937).

24. Tiselius, A., Electrophoretic analysis of normal and immune sera. Biochem.J. 31, 1464-1477 (1937).

25. Topler, A., Optische studien nach der methode der schlierenbeobachtung. Pogg. Ann. 131 33-55 (1867).

26. Tiselius, A., and H. Svensson., The influence of electrolyte concentration on the electrophoretic mobility of egg albumin. Trans. Farsday Soc. 36 16-22 (1940).

27. Longsworth, L. G., The observation of electrophoretic boundaries. Ann. N. Y. Acad. Sci. 34 187-202 (1939).

28. Schardin, H., Das toeplersche schlierenverfahren. Grundlagen für seine anwendung and quantitative anwertung VDI - Verlag. G. m.b.H. Berlin (1934).

29. Abramson, H. A., and D. H. Moore., A discussion of electrophoresis with special reference to serum and allergens. J. of Lab. and Clin. Med. 26, 176-184 (1940).

30. Lamm, O., Nova Acta Regiae Soc. Scient. Upsaliensis. IV, 10, 6 (1937).

31. Kekwick, R. A., Observations at the electrophoretic moving boundary with Lamm's scale method. Trans. Faraday Soc. 36 47-52 (1940).

32. Longsworth, L. G., A Modification of the schlieren method for use in electrophoretic analysis. J. Am. Chem. Soc. 61, 529-530 (1939).

33. Philpot, J. St. L., Direct photography of ultracentrifuge sedimentation curves. Nature 141, 282-283 (1938).

34. Svensson, H. V., Direkte photographische aufnahme von elektrophorese-diagrammen. Koll. Zeit. 87 181-186 (1939); 90 141-149 (1940).

35. Longsworth, L. G., Recent advances in the study of proteins by electrophoresis. Chem. Rev. 30 323-339 (1942).

36. Stern, K. G., and D. DuBois., Aids for the optical analysis of electrophoretic diagrams. Yale J. Biol. Med. 13 509-512 (1941).

37. Siebenmann, C., Refractometric methods for determining total protein. Biochem. J., 31 205-211 (1937).

38. Reiss, P., Eine neue methode der quantitativen eiweissbestimmug Arch. Exp. Path. Pharmack., 51, 18-29 (1904).

39. Quoted as a personal communication by A. S. McFarlane. An ultracentrifugal investigation of the serum proteins. Biochem. J. 29, 407-429 (1935).

40. Bierry, H., and R. Vivario., Determination of albumins in blood plasma. Compt. Rend. Soc. Biol. 89, 13-15 (1923).

41. Guillaumin, C., R. Wahl, and M. Laurencin., Determination of serum proteins. Comparison of results obtained by the gravimetric method and determination of nitrogen and refractive indices. Bull. Soc. Chem. Biol. 11, 387-399 (1929).

42. Microkjeldahl nitrogen method. J. Assoc. Official Agr. Chem. 24, 97-100 (1941).

43. Niederl, J. B., and V. Niederl, Micromethods of quantitative organic analysis., John Wiley and Sons, Inc., New York, (1942) p. 69-78.

44. Wagner, E. C., Titration of ammonia in presence of boric acid. Ind. Eng. Chem. Anal. Ed. 12 771-772 (1940).

45. Sobel, A. E., H. Yuska, and J. Cohen., A convenient method of determining small amounts of ammonia and other bases by the use of boric acid. J. Biol. Chem. 118, 443-446 (1937).

46. Ball, C. D., Associate Professor of Chemistry. Mich. State College., East Lansing, Michigan. Personal Communication.

47. Kingsley, G. R., The determination of serum total protein, albumin, and globulin by the biuret reaction. J. Biol. Chem. 131, 197-200 (1939).

48. Riegler, E., A colorimetric method for the determination of albumin. Z. Anal. Chem. 53, 242-247 (1914).

49. Adams, E. C., Relations between the constants of dibasic acids and of amphoteric electrolytes. J. Am. Chem. Soc. 38, 1503-1510 (1916).

50. Bjerrum, N., Die konstitution der ampholyte, besonders der aminosäuren und ihre dissoziationkonstanten. Z. Physik. Chem. 104, 147-173 (1923).

51. Gortner, R. A., Outlines of biochemistry. John Wiley and Sons Inc., New York (1938) p. 359.

52. Davis, B. D., and E. J. Cohn., The influence of ionic strength and pH on electrophoretic mobility. Ann. N. Y. Acad. Sci. 39, 209-212 (1939).

53. Sörensen, S. P. L., Studies on proteins. Compt. rend. Lab. Carlsberg. 12, (1917).

54. Schmidt, C. L. A., The chemistry of the amino acids and proteins. Charles C. Thomas, Baltimore, (1938) p. 597.

55. Brönsted, J. N., The acid-base function of molecules and its dependency on the electric charge type. J. Phys. Chem. 30, 777-790 (1926).

56. Hill, T. H., Theory of the isoelectric point. J. Phys. Chem. 46 417-428 (1942), Ibid 46 597-605 (1942), Ibid 46 621-624 (1942).

57. San Clemente, C. L., and I. F. Huddleson., Electrophoretic studies of the proteins of bovine serums with respect to Brucella. Mich. Agr. Exp. Sta. Bul. 182 3-44 (1943).

58. Slater, J. C., Introduction to chemical physics. McGraw Hill Book Co.. New York (1939) p 363.

59. Fouss, R. M., and C. A. Kraus., Properties of electrolytic solutions. J. Am. Chem. Soc. 55, 476, 1019, 2387, (1933).

60. Sookne, A. M., and J. M. Harris., Electrophoresis studies of silk. J. Research Nat. Bur. Standards 23, 299-308 (1939).

61. See reference number 1, page 161.

62. Lewis, G. N., and M. Randall., The activity coefficient of strong electrolytes. J. Am. Chem. Soc. 43, 1112-1154 (1921).

63. Gorin, M. A., and H. A. Abramson and L. S. Moyer., The effect of phosphate buffers on the electric mobility of hemoglobin. J. Am. Chem. Soc. 62, 643-644 (1940).

64. Cannan, R. K., The effect of neutral salts on the hydrogen ion dissociation curves of proteins. Cold Spring Harbor Symposia on Quantitative Biology. 6, 1-8 (1938).

65. Smith, E. R. B., The influence of method of preparation and of cations on the isoelectric point of ovalbumin. J. Biol. Chem, 113, 473-478 (1936).

66. Longsworth, L. G., The influence of pH on the mobility and diffusion of ovalbumin. Ann. N. Y. Acad. Sci. 41, 267-287 (1941).

67. Longsworth, L. G., and D. A. MacInnes., Electrophoresis of proteins by the Tiselius method. Chem. Rev. 24, 271-287 (1939).

68. Davies, C. W., The conductivity of Solutions. John Wiley and Sons Inc., New York (1933) p 6.

69. Shedlovsky, T., Personal Communication.

70. Henderson, L. J., A diagramatic representation of equilibria between acids and bases in solution. J. Am. Chem. Soc. 30, 954-960 (1908).

71. Washburn, E. W., The theory and practice of the iodometric determination of arsenious acid. J. Am. Chem. Soc. 30, 31-46 (1908).

72. Michaelis, L., and R. Kruger., Weitere ausarbeitung der indikatoren-methode ohne puffer. Bio. Zeit. 119 307-327 (1921).

73. Cohn, E. J., The activity coefficients of the ions in certain phosphate solutions. A contribution to the theory of buffer action. J. Am. Chem. Soc. 49, 173-193 (1927).

74. Clark, W. M., The determination of hydrogen ions. Williams and Wilkins. Baltimore (1928) p. 217, 678.

75. Robinson, H. W., The influence of neutral Salts on the pH of phosphate buffer mixtures. J. Biol. Chem. 82, 775-802 (1929).

76. Cohn, E. J., F. F. Heyroth and M. F. Menken., The dissociation constant of acetic acid and the activity coefficients of the ions in certain acetate solutions. J. Am. Chem. Soc. 50, 696-714 (1928).

77. Green, A. A., The preparation of acetate and phosphate buffer solutions of known pH and ionic strength. J. Am. Chem. Soc. 55, 2331-2336 (1933).

78. Perlman, E., J. G. Bullowa, and R. Goodkind., An immunological and electrophoretic comparison of the antibody to C Polysaccharide and the C reactive protein of acute phase serum. J. Exp. Med. 77 97-110 (1943).

79. MacInnes, D. A., The principles of electrochemistry. Reinhold Publishing Corp. New York. (1939) p. 40.

80. Getman, F. H., and F. Daniels., Outlines of theoretical chemistry. John Wiley and Sons Inc. New York. (1937) pp. 365-367.

81. Shedlovsky, T., The electrolytic conductivity of some uni-univalent electrolytes in water at 25°. J. Am. Chem. Soc 54, 1411 (1932).

82. Longsworth, L. G., T. Shedlovsky, and D. A. MacInnes, Electrophoretic patterns of normal and pathological human blood plasma and serum. J. Exp. Med. 70, 399-413 (1939).

83. Jones, G., and B. C. Bradshaw., A redetermination of the conductance of standard potassium chloride solutions in absolute units. J. Am. Chem. Soc. 55, 1780-1800 (1933).

84. Daniels, F., H. J. Mathews., and J. W. Williams., Experimental physical chemistry. McGraw Hill Book Co., New York (1941) Third edition. p 369.

85. Pierce, W. C., and E. L. Haenisch., Quantitative analysis. John Wiley and Sons Inc. New York (1940), Second edition. P. 36.

86. Handbook of Chemistry and Physics. Chemical Rubber Publishing Co., Cleveland Ohio. (1941) Twenty fifth edition P. 1287.

87. Broazeale, W. M., A vacuum tube alternating current bridge detector. Review of Scientific Instruments. 8, 250-251 (1936).

88. Garman, R. L., A bridge balance indicator. Review of Scientific Instruments 8, 327-329 (1937).

89. Clark, J. C., Associate Professor of Physics. Mich.State College, East Lansing, Michigan. Personal Communication.

90. Donnan, F. G., Theorie der membrang-leichgewichte und membranpotentiale bei vorhandensein von nicht dialysierenden elecktrolyten. Ein beitrag zur physikalisch-chemischen physiologie. Z. Elektrochem. 17, 572-581 (1911).

91. Longsworth, L. G., Personal communication.

92. Longsworth L. G., R. K. Cannan, and D. A. MacInnes., An electrophoretic study of the proteins of egg white., J. Am. Chem. Soc. 62 2580-2590 (1940).

93. Stenhagen, E., Electrophoresis of human blood plasma. Biochem. J. 32 714-718 (1938).

94. Smith, E. R., A new design of apparatus for the moving boundary method of determining transference numbers. J. Am. Chem. Soc. 50 1904-1906 (1928).

95. Longsworth, L. G., and D. A. MacInnes., The interpretation of simple electrophoretic patterns. J. Am. Chem. Soc. 62 705-711 (1940).

96. Longsworth, L. G., and D. A. MacInnes., Transference numbers. Chem. Rev. 11, 171-230 (1932).

97. Miller, W. L., The theory of the direct method of determining transport numbers. Z. physik Chem. 69 436-441 (1909).

98. Tiselius, A., and E. A. Kabat, An electrophoretic study of immune sera and purified antibody preparations. J. Exp. Med. 69 119-131 (1939).

99. Svedberg, T., and K. O. Pedersen., The ultra-centrifuge. Oxford University Press. London (1940). p. 296.

100. Longsworth, L. G., and D. A. MacInnes., An electrophoretic study of mixtures of ovalbumin and yeast nucleic acid. J. Gen. Phys. 25 507-516 (1942).

101. Denison, R. B., and B. D. Steele. Uber eine methode zur genauen messung von uberführungszahlen. Z. physik. Chem. 57 110-127 (1907).

102. Longsworth, L. G., An application of moving boundaries to a study of aqueus mixtures of hydrogen chloride and potassium chloride. J. Am. Chem. Soc. 52 1897-1910 (1930).

103. Henry, D. C., and J. Brittain., A comparison of the results of measurements by the transport and moving boundary methods, and a theory of the latter method Trans. Faraday Soc. 29 798-814 (1939).

104. Tiselius, A., and Horsfall., Ark. Kemi. Minera Geo. 13a No. 18 (1939).

105. Svensson, H., Fractionation of serum with ammonium sulfate and water dialysis, studied by electrophoresis. J. Biol. Chem. 139 805-825 (1941).

106. Longsworth, L. G., and D. A. MacInnes., An electrophoretic study of nephrotic sera and urine. J. Exp. Med. 71 77-82 (1940).

107. Moore, D. H., and J. Lynn. Electrophoretic measurements on normal human plasma. J. Biol. Chem. 141 819-825 (1941).

108. Sörensen, S. P. L. quoted by Longsworth, L. G. and D. A. MacInnes (106).

109. Tiselius, A., Electrophoretische messungen am eiweiss, Kolloid Z. 85 129-137 (1938).

110. McFarlane, A. S., The electric double layer and virus stability. Trans. Faraday Soc. 36 257-264 (1940).

111. Shedlovsky, T., and J. E. Smadel., Electrophoretic Studies on elementary bodies of vaccinia. J. Exp. Med. 72 511-521 (1940).

112. Jameson, E., and Alvarez-Tostado., Qualitative separation of proteins of rat serum by electrophoresis. Proc. Soc. Exp. Biol. Med., 40 476-478 (1939).

113. Scudder, J., Symposium on fluid and electrolyte needs of surgical patients; studies in blood preservation; stability of plasma proteins. J. Ann. Surg. 112 502-519 (1940).

114. Luetscher, J. A. Jr., Electrophoretic analysis of plasma and urinary proteins. J. Clin. Inv. 19 313-320 (1940)., 20 99 (1941).

115. Kekwick, R. A., The electrophoretic analysis of normal human serum. Biochem. J. 33 1122-1129 (1939).

116. Blix, G., Quantitative determination of α-, β- and γ-globulin in normal serum and pneumonia serum. Hygiea 100 77-84 (1938).

117. Cohn, E. J., The properties and functions of the plasma proteins with a consideration of the methods for their separation and purification. Chem. Rev. 28 395-417 (1941).

118. Kekwick, R. A., The serum proteins in multiple myelomatosis. Biochem. J. 34 1248-1257 (1940).

119. Blix, G., Quantitative bestimmung von elektrophoretisch getrennten serum globulinen. Z. ges. exp. Med. 105 595-598 (1939).

120. Green, A. A., The amphoteric properties of certain globulin fractions of normal horse serum. J. Am. Chem. Soc. 60 1108-1115 (1931).

121. Shedlovsky, T., and J. Scudder., A comparison of erythrocyte sedimentation rates and electrophoretic patterns of normal and pathological human blood. J. Exp. Med. 75 119-126 (1942).

122. Shapiro, S., V. Ross, and D. H. Moore., A viscous protein obtained in large amount from the serum of a patient with multiple myeloma. J. Clin. Inv. 22 137-142 (1943).

123. Gray, S. J., and E. S. Guzman Barron., The electrophoretic analyses of the serum proteins in diseases of the liver. J. Clin. Inv. 22 191-200 (1943).

124. Tiselius, A., and E. Kabat., An electrophoretic study of immune sera and purified antibody preparations. J. Exp. Med. 69 119-131 (1939).

125. Kekwick, R. A., and B. R. Record, Some physical properties of diphtheria antitoxic horse sera. Brit. J. Exp. Path. 22 29-44 (1941).

126. Moore, D. H., J. van der Scheer and R. W. G. Wyckoff., An electrophoretic study of antipneumoccal horse sera. J. Imm. 38 221-229 (1940).

127. Fell, N. K., G. Stern and R. D. Coghill., A physical-Chemical study of normal and immune horse sera. J. Imm. 39 223-245 (1940).

128. Jameson E., and C. Alvarez-Tostado., A study of blood serum proteins by electrophoresis. J. Phys. Chem. 43 1165-1172 (1939).

129. Hill, R. M., and V. Trevorrow., Normal variation in the concentration of fibrinogen, albumin, and globulin in blood plasma. J. Phys. Chem. 46 1117-1129 (1942).

130. Cohn, E. J., T. L. McMeekin, J. L. Oncley, J. M. Newell and W. L. Hughes., Preparation and properties of serum and plasma proteins I. J. Am. Chem. Soc. 62 3386-3393 (1940).

131. McMeekin, T. L., Preparation and properties of serum and plasma proteins II. J. Am. Chem. Soc. 62 3393-3396 (1940).

132. Cohn, E. J., J. A. Luetscher Jr., J. L. Oncley, S. H. Armstrong Jr., and B. D. Davis., Preparation and properties of serum and plasma proteins. III. J. Am. Chem. Soc. 62 3396-3400 (1940).

133. Scudder, J., Electrophoretic patterns of Serum and plasma. Blood substitutes and blood transfusions. Mudd, S., and W. Thalhimer. Charles C. Thomas. Baltimore (1942) p 126-139.

134. Kabat, E. A., H. Landow and D. H. Moore., Electrophoretic patterns of concentrated cerebrospinal fluid. Proc. Soc. Exp. Biol. Med. 49 260-263 (1942).

135. Hesselvik, L., Electrophoretical investigation on proteins of eye lens and vitreous body. Acta Physiol. Scand. 101 151-156 (1939).

136. Blix, G., Glucoproteins. Acta Physicl. Scand. 102 29-42 (1940).

137. Hesselvik, L., Electrophoretical study of normal and pathological body fluids. Acta Med. Scand. 101 461-464 (1939).

138. Tiselius, A., and E. A. Kabat., Electrophoresis on Immune serum. Science 87 416-417 (1938).

139. Van der Scheer, J. J., B. Lagsdin and R. W. G. Wyckoff., Electrophoretic and ultracentrifugal analyses of antipneumococcal horse sera. J. Imm. 41 209-223 (1941).

140. Van der Scheer, J., and R. W. G. Wyckoff., An electrophoretic study of tetanus antitoxin sera. Proc. Soc. Exp. Biol. Med. 43 427-428 (1940).

141. Van der Scheer, J., R. W. G. Wyckoff and F. H. Clarke., The electrophoretic analysis of tetanal antitoxic horse-sera. J. Imm. 40, 173-177 (1941).

142. Van der Scheer J., R. W. G. Wyckoff and F. H. Clarke., The electrophoretic analysis of several hyperimmune horse sera. J. Imm. 39 65-71 (1940).

143. Rothen, A., Purified diphtheria antitoxin in the ultracentrifuge and in the electrophoresis apparatus. J. Gen. Physiol. 25 487-496 (1942).

144. Parfentjev, I. A., U. S. Patents No. 2065196 and No. 2123198.

145. Van der Scheer, J., R. W. G. Wyckoff and F. H. Clarke., An electrophoretic examination of digested sera. J. Imm. 41 349-360 (1941).

146. Siebert, F. B., and J. W. Nelson., Proteins of Tuberculin. J. Am. Chem. Soc. 65 272-278 (1943).

147. Siebert, F. B., and J. W. Nelson., Electrophoretic study of the blood protein response in tuberculosis. J. Biol. Chem. 143 29-38 (1942).

148. Siebert, F. B., and D. W. Watson., Isolation of the polysaccharides and nucleic acid of tuberculin by electrophoresis. J. Biol. Chem. 140 55-69 (1941).

149. Pillmer, I., E. E. Ecker, J. L. Oncley and E. J. Cohn. The preparation and physicochemical characterization of the serum protein components of complement. J. Exp. Med. 74 297-308 (1941).

150. Quensel, I. B. E., and T. Svedberg., Sedimentation and electrophoresis of the tobacco mosaic virus protein. J. Am. Chem. Soc. 58 1863-1867 (1936).

151. Pfankuck, E., Clevage of the virus proteins of the tobacco mosaic variety., Biochem. Z. 306 125-129 (1940).

152. McFarlane, Physical properties of bushy stunt virus protein. Biochem J. 32 1607-1613 (1938).

153. Douglas, S. R., and W. Smith., Cataphoresis experiments with the virus of vaccinia. Brit. J. Exp. Path. 9 213-215 (1928).

154. Lee, C. D., and H. L. Wilcke., The migration of the etiologic agent of fowl leukosis when subjected to electrophoresis. J. Am. Vet. Med. Ass. 94 178-186 (1939).

155. Smadel, J. E., and T. Shedlovsky., Antigens of vaccina. Ann. N. Y. Acad. Sci. XLIII 35-46 (942).

156. Abramson, H. A., D. H. Moore and H. G. Gettner., A general electrophoretic pattern in extracts of pollens causing hay fever. J. Phys. Chem. 46 1129-1139 (1942).

157. Ringer, W. E., Weitere studien am pekelharingschn pepsin. Z. Physiol. Chem. 95 195-258 (1915).

158. Tiselius, A., G. E. Henschen and P. Svenson., Electrophoresis of pepsin. Biochem. J. 32 1814-1818 (1938).

159. Herriot, R. M., V. Desreux and J. H. Northrop., Electrophoresis of pepsin. J. Gen Physiol. 23 439-447 (1940).

160. Theorell, H., Ein neuer kataphoreseapparat fur untersuchungszwecke. Biochem. Z. 275 1-36 (1935)., Ein kataphoreseapparat fur paraparative zwecke. Biochem. Z. 278 291-297 (1935).

161. Theorell, H., Reines cytochrome C. Biochem. Z. 279 463-464 (1935).

162. Vahlquist, B., The esterase activity of human blood plasma. Skand. Arch. Physiol. 72 133-160 (1935).

163. Rothen, A., Moelcular weight and electrophoresis of crystalline ribonuclease. J. Gen. Physiol. 24 203-211 (1940-41).

164. Bate-Smith, E. C., Cataphoretic behaviour of zymohexase. Biochem. J. 34 1122 (1940).

165. Melnick, J. L., and R. G. Stern., Carboxylase, Enzymologia 8 129-151 (1940).

166. Kunitz, J., Formation of new crystalline enzymes from chymotrypsin. J. Gen. Physiol. 22 207-237 (1938).

167. Shipley, R. A., K. G. Stern, and A. White., Electrophoresis of anterior pituitary proteins. J. Exp. Med. 69 785-800 (1939).

168. Li, C. H., W. R. Lyons and H. M. Evans., Studies on pituitary lactogenic hormone. J. Gen. Physiol. 23 433-438 (1940).

169. Shedlovsky, T., A. Rothen, R. O. Greep, H. B. Van Dyke, and B. F. Chow., The isolation in pure form of the interstitial cell-stimulating (luteinizing) hormone of the anterior lobe of the pituitary gland. Science 92 178-180 (1940).

170. Chow, B. F., H. B. Van Dyke, R. O. Greep, A. Rothen, and T. Shedlovsky., Chemical aspects of metakentrin (Interstial cell-stimulating hormone). J. Biol. Chem. 140 XXVI-XXVII (1941).

171. Li, C. H., H. M. Evans and D. H. Wonder., Electrophoretic homogeneity of Pregnant mare serum. Gonadotrophin. J. Gen. Physiol. 23 733-739 (1939-40).

172. Gurin, S., C. Bachman, and D. W. Wilson., Homogenity of gonadotropic hormone preparations isolated from pregnancy urine. J. Am. Chem. Soc. 61 2251 (1939).

173. Cohn, M., G. W. Irving Jr; and V. du Vigneaud., The amphoteric nature of the pressor principle of the posterior lobe of the pituitary gland. J. Biol. Chem. 137 635-642 (1941).

174. du Vigneaud, V., G. W. Irving Jr., H. M. Dyer, and R. R. Sealock., Electrophoresis of posterior pituitary gland preparations. J. Biol. Chem. 123 45-55 (1938).

175. Irving, G. W. Jr., and V. du Vigneaud., The differential migration of the pressor and oxytocic hormones in electrophoretic studies of the untreated pressjuice of the posterior lobe of the pituitary gland. J. Biol. Chem. 123 485-489 (1938).

176. Heidelberger, M., and K. O. Pedersen., The molecular weight and isoelectric point of thyroglobulin J. Gen. Physiol. 19 95-108 (1935-36).

177. Landsteiner, K., L. G. Longsworth, and J. Van der Scheer., Electrophoresis experiments with egg albumins and hemoglobins. Science 88 83-85 (1938).

178. Herbert, D., H. Gordon, V. Subrahmanyan and D. E. Green., Zymohexase. Biochem. J. 34 1108-1123 (1940).

179. Fishman, M., and L. S. Moyer., Electrophoresis of the chlorphyll-protein complex. Science 95 128-129 (1942).

180. Harris, M., The isoelectric point of wool. J. Research Nat. Bur. Standard 8 779-782 (1931).

181. Sookne, A. M., and M. Harris. Electrophoretic studies of wool. J. Research Nat. Bur. Standards 23 471-477 (1939).

182. Wilkerson, V. A., The chemistry of human epidermis II. The isoelectric points of the stratum corneum, hair, and nails as determined by electrophoresis. J. Biol. Chem. 112 329-335 (1935-36).

183. Wilkerson, V. A., The Chemistry of human skin. IV The electrokinetic effect of various ions upon suspended particles of stratum corneum. J. Gen. Physiol. 23 165-170 (1939-40).

184. Beek, J. Jr., and A. M. Sookne., Electrophoresis of collagen. J. Research Nat. Bur. Standard 23 271-273 (1939).

185. Martin, W. H., Electrokenetic properties of proteins. I Iseelectric point and solubility of wheat proteins in aqueus solutions of alcohol. J. Phys. Chem. 35 2065-2090 (1931).

186. Kemp, I., Trans. Faraday Soc., 32 837 (1936).

187. Williams, J. W., and C. C. Watson., The physical chemistry of the prolamines. Cold Spring Harbor Symposia on Quantitative Biology. 6 208-217 (1938).

188. Andrews, A. C., The physical chemistry of secalin. Electrophoresis and diffusion constant studies of the prolamine of rye. J. Am. Chem. Soc. 62 942-948 (1940).

189. Mellander, O., Elektrophoretische untersuchung von casein. Biochem. Z. 300 240-245 (1939).

190. Hall, J. M., The physical chemical investigation of certain nucleoproteins. II. General electrophoretic behavior. J. Am. Chem. Soc. 63 794-798 (1941).

191. Stahl, W. H., R. B. Pennell and I. F. Huddleson., A study of the protein nucleates of the species of the genus Brucella. Tech. Bull. 168 Mich. Agr. Exp. Sta. (1937).

192. Pennell, R. B., and I. F. Huddleson., Chemical constitution and biological properties of the endo-antigen of the Brucella group of microorganisma. Tech. Bul. 156. Mich. Agr. Exp. Sta. (1937).

193. Van der Scheer J., R. W. G. Wyckoff, and F. L. Clarke., An electrophoretic study of heated horse serum. J. Imm. 40 39-45 (1941).

194. Hardt., C. R., I. F. Huddleson and C. D. Ball., The protective action of glucose of bovine plasma against heat coagulation. To appear in Science.

195. Huddleson, I. F., The immunization of guinea pigs against Brucella infaction. Mich. Agr. Exp. Bul. 182 45-56 (1943).