Part II. Clinical Applications

DISC ELECTROPHORESIS—II

METHOD AND APPLICATION TO HUMAN SERUM PROTEINS*

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INTRODUCTION

The purpose of this paper is to describe a technique of zone electrophoresis in polyacrylamide gels. The salient characteristics of the technique reside in (1) the controlled variation of the gel pore size for the purpose of increasing the resolution of ions based on dimensional differences and (2) an electrophoretic step for concentrating the sample ions into a narrow starting zone prior to electrophoretic separation.

Zone electrophoresis provides a comparatively innocuous method for the separation of ionic mixtures and is based on differences among the electrophoretic mobilities of the constituent ions. In most zone techniques separation is dependent solely on the differences in the free electrophoretic mobilities among the constituent ions, and the supporting matrix, e.g., filter paper, is used primarily to reduce convection. In those instances when the differences among the free mobilities are small, satisfactory separation may not be achieved. With the development of zone electrophoresis in starch gel matrices, the capacity of gels to "sieve" high molecular weight substances such as proteins was recognized to provide a simple but elegant means to resolve ions of similar, and even identical, free mobilities.[1] A gel matrix, unlike other porous media such as filter paper or starch granules, is a latticed structure with pores of molecular dimensions that can impose an appreciable frictional resistance to the passage of ions, provided the size of the pores approaches the dimensions of the migrating ions. If, as is the case with starch gels,[2] the average pore size approaches the range of dimensions of proteins, the various protein fractions will be differentially retarded to degrees proportional to their dimensions. Thus the separation of mixtures is achieved in such a gel matrix through dimensional as well as charge differences.

The substantial increase in resolving power of sieving gel matrices over that of conventional filter paper electrophoresis is effectively demonstrated with a complex protein mixture such as human serum. In a single electrophoretic analysis (pH 8.6) a filter paper technique commonly resolves five to seven protein fractions, while starch gel resolves 20 to 30 fractions.

This discovery by Smithies[1,2] of the additional resolving power provided by the sieving capacity of gels prompted our investigation of polyacrylamide gels. Polyacrylamide gels are synthetic polymers formed from low molecular weight chemicals obtainable in high purity. The pore size of polyacrylamide gels can be varied through a wide range by adjustment of the monomer concentration, and preparation of the gels is a simple and rapid procedure. Furthermore, these gels are transparent to visible radiation through a wide range of monomer

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concentrations. Polyacrylamide gel lattices are carbon-carbon polymers with pendant amide groups, are relatively inert chemically, and have few or no ionic side groups. Anionic or cationic groups can, however, if desired, be incorporated into the polymer. The gels are also mechanically strong over a wide range of pore sizes, and the mechanical properties can be easily adjusted by changing the proportions of the gel ingredients. Since these characteristics offer kinds of flexibility and versatility not easily attainable with starch gels at the present time, it was felt that polyacrylamide gel electrophoresis would provide a valuable complement to starch gel electrophoresis.

The successful utilization of polyacrylamide gels for zone electrophoresis was reported as early as 1959 from this laboratory,[3,4] as well as independently by another group.[5]

Since diffusion during electrophoresis continuously dilutes the separating fractions and blurs their boundaries, it is desirable to reduce the running time to the minimum necessary to achieve a desired separation of the constituent ions. For this reason the thickness of the starting zone critically affects the resolution of a mixture of ions; the thinner the starting zone, the shorter the running time necessary for a given separation of the constituents. Thus an additional gain in electrophoretic resolution can be achieved by means of a step that reduces the thickness of the sample starting zone. In the technique we have named disc electrophoresis, this step is based on simple electrochemical laws, as formulated in the Kohlrausch Regulating Function,[6] and the rationale of the procedure, although only implicit in a report by Kendall *et al.*,[7] is virtually identical to that of their technique for the purification and concentration of mesothorium present in small amounts from a solution of barium chloride.

The concentration step is achieved by introducing the mixture of sample ions into an electrophoretic column near the boundary of two ions whose sign is like that of the sample ions at a given pH. One ion is faster, the other (an ion of a weak acid or base) slower than all of the sample ions at this pH. Electrical polarity is set so that the fast ion is situated ahead, i.e., in the direction of migration of the sample and slow ions. Application of a voltage results in the segregation from one another and stacking of the constituent ions of the sample into contiguous zones in order of their relative mobilities, the entire sample sandwiched between the slow and fast ions. The final concentration of each constituent in the stack is independent of its original concentration in the mixture but is proportional to the concentration of the fast ion. Thus a dilute sample can be concentrated into a zone the thickness of which is fixed by the amount of ionic material present in the sample and by the chosen concentration of the fast ion.

Shortly after the sample ions have reached fixed high concentrations, they are arranged to migrate into a region of the electrophoretic column where a new set of conditions of pH or pore size or both obtains so that the mobility of the ion of the weak acid or base now exceeds that of, for example, the fastest protein. The ions of the weak acid or base now continuously overtake and pass through the sample species, establishing a comparatively uniform voltage gradient in which electrophoretic separation'of the sample occurs.

This paper will be devoted to a description and discussion of the rationale of a technique of preparing and handling polyacrylamide gels and the results obtained from the electrophoretic analysis of human serum proteins.*

• A detailed discussion of the theoretical aspects and potential applications of these phenomena, as well as a useful bibliography, can be found in the companion paper by Ornstein (this *Annal).*[8]

MATERIALS AND METHODS

General

The polyacrylamide gel column, formed in a suitable container, e.g., cylindrical glass tube, is composed of three layers: (l) a large-pore gel (sample gel) containing the sample ions in which electrophoretic concentration of these ions is initiated; (2) a large-pore gel (spacer gel) in which electrophoretic concentration of the sample ions is completed; (3) a small-pore gel in which electrophoretic separation takes place. The large-pore gels are intended to serve primarily as anticonvection media, while the small-pore gel serves as a sieving as well as an anticonvection medium. Electrophoresis is ordinarily performed with the column in a vertical position, the gel container attached, sample gel uppermost, to an upper buffer reservoir and the lower end submerged in the buffer solution of a lower reservoir. Electrodes are placed. in each reservoir, and the polarity is set so that the sample ions migrate toward the small-pore gel. A voltage is applied for a specified time. The gel is then removed from the container and placed for a period of time in a solution of protein fixative and stain. Unbound dye is removed from the gel by electrophoresis, or more slowly by washing, and the gel is then preserved in a suitable solution.

*Apparatus**

Buffer reservoirs are prepared from an inert, nonconductive material such as polystyrene, methacrylate, or polyester, available as sheets or circular or rectangular containers. The material is chosen on the basis of availability, convenient shape, and machinability.

In this laboratory a pair of polystyrene refrigerator dishes, four to six inches in diameter and about two and one-half inches in depth have been found especially convenient. An upper buffer reservoir is made by drilling up to twelve holes, 3/8 inch in diameter, in the bottom of one dish at equal intervals along a circumference about 1/2 inch in from the periphery. Rubber or water-resistant polyvinyl chloride electrical grommets, of 1/4 inch inner diameter (i.d.), which can be fitted snugly around the tubular gel containers, are inserted into the holes and serve as inlets for the gel containers. The upper reservoir is supported above a lower reservoir, an undrilled dish of similar dimensions, by means of a tripod, such as three plastic legs cemented to the sidewall of the upper reservoir or, alternatively, a support ring attached to a ring stand (FIGURE 1).

Cylindrical graphite electrodes, removed from flashlight batteries (size "D") and thoroughly cleaned, are satisfactory and have the advantages of convenient dimensions, ready availability, and economy. Electrode leads of insulated wire are soldered to the metal caps of the electrodes. An electrode is attached to each reservoir and held in a vertical position by insertion into the hollow end of a rubber stopper that has been cemented to the center of the bottom of each reservoir. The hollow rubber stoppers, with a cylindrical well about 7 mm. in depth and an i.d. of about 6 mm. of B-D Vacutainer† test tubes, which have been used and discarded by the clinical taboratories of a hospital, are quite suitable. Once used as cathode and anode the graphite electrodes should not be interchanged, otherwise decomposition products resulting from reaction between the gel catalyst and substances in the anode will be released and migrate through and discolor the gels. Alternatively, platinum

* Apparatus and reagents for disc electrophoresis can be obtained from CANALCO, Bethesda, Md.

† Becton, Dickinson, and Company—Columbus, Nebraska, and Rutherford, N. J.

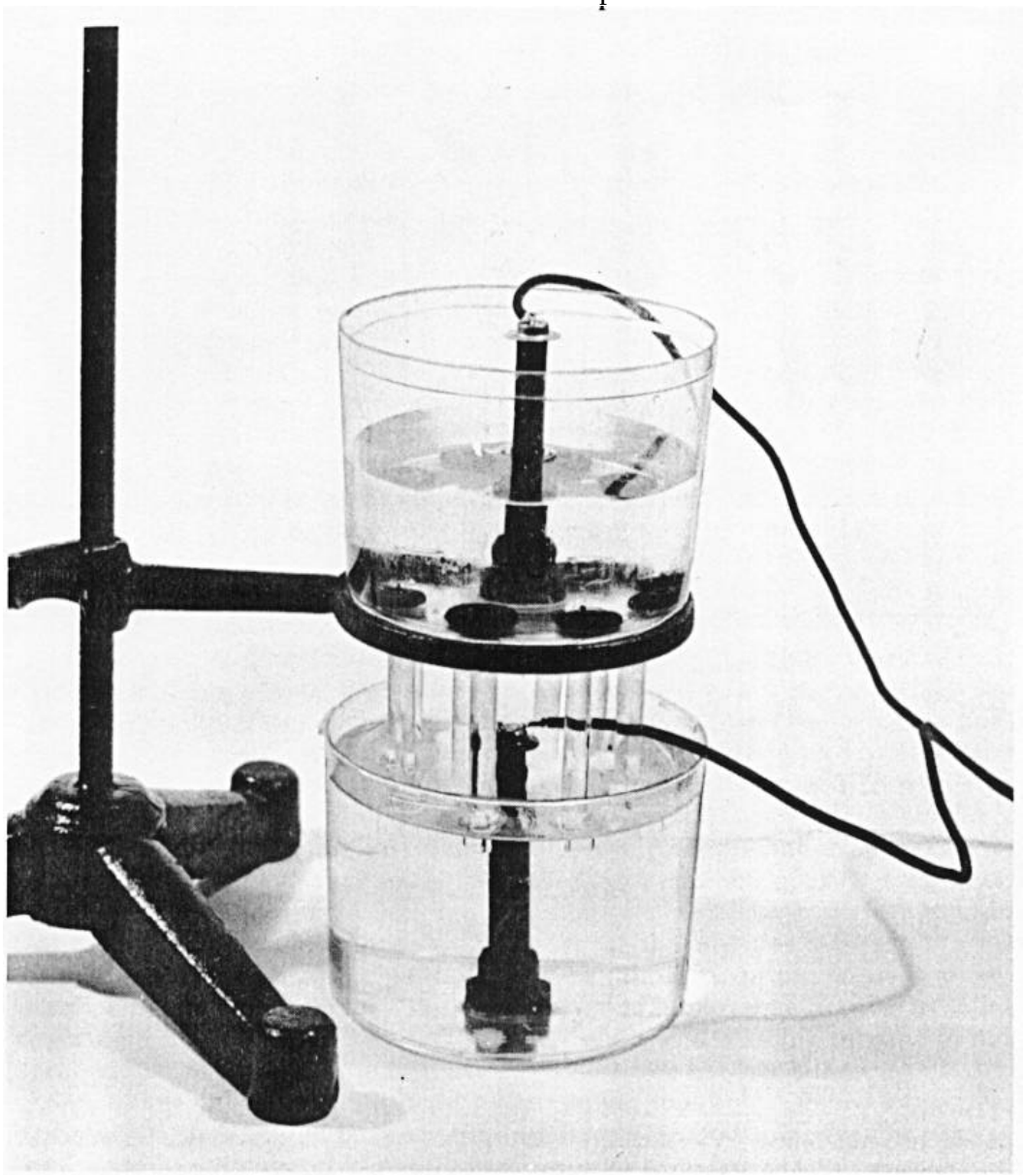

FIGURE 1. Complete electrophoretic apparatus.

electrodes, which are nonreactive, and hence superior to battery electrodes, can be made by wrapping 2 turns of #30 guage wire around the long axis of a cylinder (6.5 to 7.0 mm. in diameter; about 60 mm. Iength) of glass or rigid plastic, the faces of which have been notched to prevent slippage of the wire. The two ends of wire, resting in the notch of one face, are soldered together along with an electrode lead. This face is insulated with a one-inch length of plastic tubing such as polyvinyl chloride tubing. The electrodes are attached to the reservoirs by inserting the lead-free faces of the cylinders into the test tube caps as described for the graphite electrodes.

Regardless of the size or shape of the reservoirs, the electrodes should be equidistant from all of the grommets and the grommets equidistant from one another so that the voltage drop between the electrode and each of the grommets will be the same.

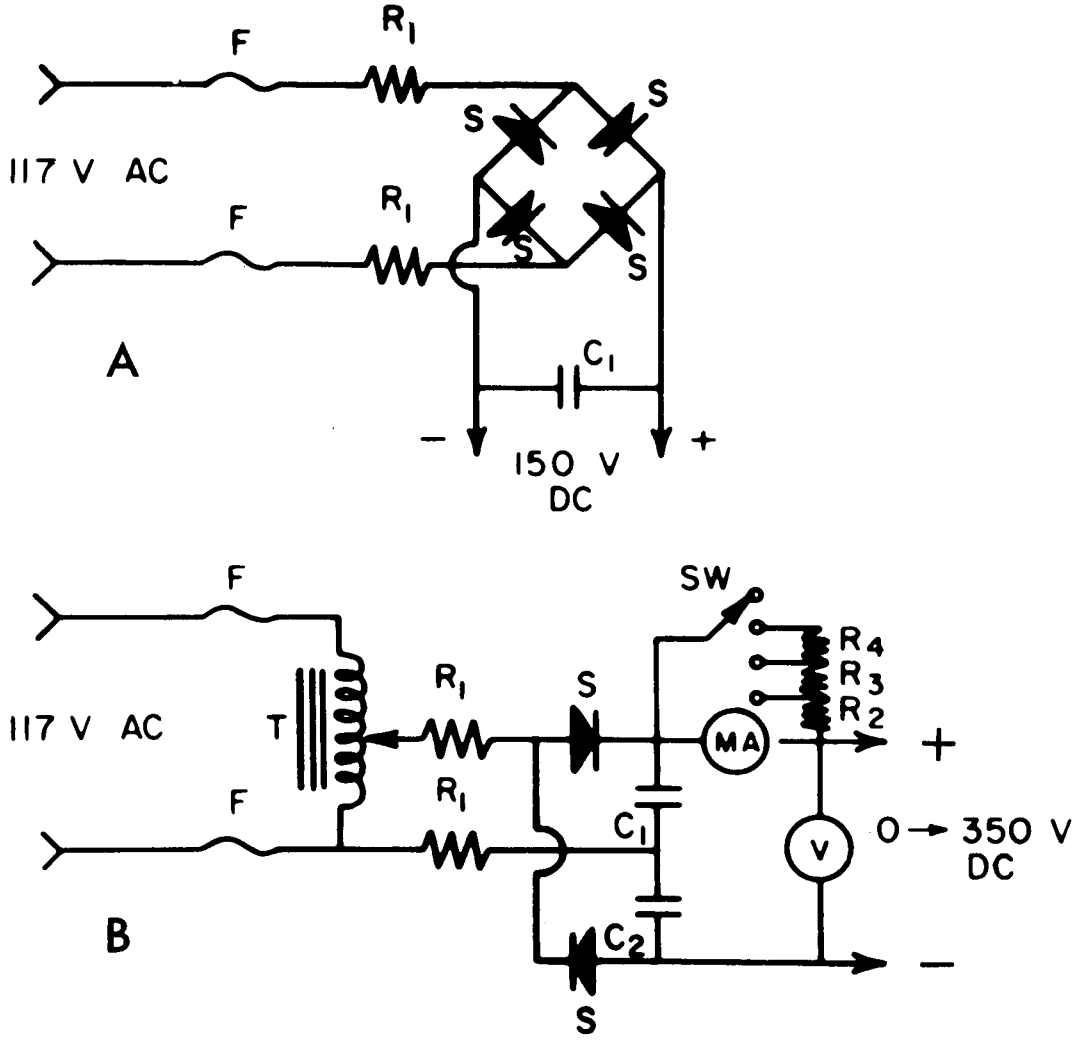

FIGURE 2. (A) Simple 150 volt supply; (B) variable 350 volt supply: components as indicated: F—1 Amp. slow-blow fuses; S—0.5 Amp., 200V silicon rectifiers; C_1—50 mfd., 200 **WVDC** electrolytic capacitors; C_2—300 mfd., 200 **WVDC** electrolytic capacitors- T—160 watt variable auto-transformer; V—voltmeter, 0–400 **V DC;** MA—milliameter, 0.25 mAmp. **DC**; SW—4 position switch to shunt MA for currents larger than 25 mAmps; R_1—2.7 ohms, 2 watt resistors; R_2, R_3, R_4,—milliameter shunt resistors.

A very simple 150 volt, direct current 1 ampere power supply, useful for both electrophoretic separation and destaining, may be constructed as indicated in FIGURE 2A. FIGURE 2B illustrates a more versatile 0 to 350 volt D.C. 350 milliampere power supply.*

Containers for the gel columns are cut from cylindrical glass or rigid plastic (e.g. cellulose acetate-butyrate) tubing The gel-tubes are about 63 mm. in length and have a 7.0 mm. outside diameter (o.d.) and 5.0 mm. i.d. and fit snugly into the grommets of the buffer reservoir. The ends of the gel tubes are square cut. Sharp edges are easily removed with Carborundum cloth (fire polishing the edges may reduce the size of the orifice and is not recommended). Although the diameter and length of the tubes are not critical, all the gel tubes hat are to be

*These power supplies are potentially lethal. The circuit should be housed in an insulated or grounded chassis, and great care should be taken to avoid contact between the user and the electrodes or buffer when the circuit is attached to the alternating current line.

used simultaneously should be cut to the same length and from the same piece of tubing (therefore to more nearly the same i.d.), to reduce differences in the electrical resistance from column to column.

During the filling of the gel tubes and the polymerization of the ingredients, the gel tubes are fixed in a tube stand. Stands are conveniently made by cementing sets of from 4 to 6 hollow rubber stoppers, e.g. the B-D Vacutainer stoppers previously described, in a single row about 10 mm. apart, closed end down, to a flat piece of wood or plastic. The open end of a cap should fit snugly around a gel tube to prevent leakage of the ingredients.

During the preparation of the gel column it is necessary to layer water over gel solution in order both to eliminate a meniscus and to obtain a flat smooth gel surface. The water must be added at a slow, regular, controlled rate, and care must be taken to minimize mixing. A variety of pipettes, e.g. mouth or constriction pipettes, can be made or adapted for this purpose. It has been found convenient to use a disposable one or two milliliter syringe with #25 gauge hypodermic needle.

Following electrophoresis, the gels are removed from the gel tubes by rimming with a piece of wire while introducing water to lubricate the advancing wire and the separated surfaces of tube and gel. Stainless steel wire about 70 mm. in length and not greater than 1 mm. in diameter, the sharp ends of which have been polished smooth, is satisfactory; as is a blunted standard biological "teasing" or "dissecting" needle or the stylet of a needle used for tapping spinal fluid.

Following fixation and staining, the unbound dye is removed from the gels by elution or electrophoresis. Electrophoretic destaining is performed in an apparatus identical to that just described. Glass tubing is cut into lengths one centimeter longer than that of the gel tubes, and one end of each tube is constricted by fire polishing so that the diameter of this orifice is slightly less than that of the gel. Medicine droppers of the proper size are also satisfactory. Since the gels swell to a variable degree, dependent primarily on the concentration of crosslinking monomer, the i.d. of the destaining tubes should be 1 to 2 mm. Iarger than that of the gel tubes. If swelling is slight, destaining tubes with o.d.'s only slightly larger than that of the gel tubes can be used and these can be connected to the previously described upper buffer reservoir. On the other hand, if slightly larger destaining tubes are r.equired, a second upper buffer reservoir can be made with slightly larger holes (same size grommets as previously described) to accommodate these tubes.

Reagents

(l) Acrylamide (Eastman 5521).* Acrylamide is a white crystalline solid that is best stored in a cool, dark, dry place to reduce slow spontaneous polymerization and hydrolysis. The average shelf life of commercial samples is several years. (2) N,N'-Methylenebisacrylamide (Eastman 8383)* (BIS). BIS is a white crystalline solid best stored in a cool, dark, dry place. Slow spontaneous polymerization may occur during long storage. (3) 2-Amino-2-(hydroxymethyl)-l, 3-propanediol: also known as tris(hydroxymethyl) aminomethane (TRIZMA BASE)† (TRIS). (4) N,N,N',N'-Tetramethylethylenediamine (Eastman 8178)*. (TEMED). (5) Riboflavin (Eastman 5181).* (6) Hydrochloric acid (HCl), reagent grade, one Normal (N). (7) Ammonium persulfate, reagent grade. (8) Glycine (Ammonia free). (9) Galcial acetic acid. (10) Amido Schwarz: also known a Naphthol Blue Black, Color Index

* Distillation Products Industries, Division of Eastman Kodak Company, Rochester, N. Y.

† Sigma Chemical Company, St. Louis, Mo.

Number 20470 (first edition:246). (11) Bromphenol Blue. (12) Sucrose.

Stock Solutions

These solutions are prepared using distilled water and are filtered and stored in brown glass bottles in a refrigerator. The shelf life of these solutions is up to several months.

TABLE 1

STOCK SOLUTIONS

(A)			(B)		
1N HCl		48 ml.	1N HCl	approximately 48 ml.	
TRIS		36.6 gm.	TRIS		5.98 gm.
TEMED		0.23 ml.	TEMED		0.46 ml.
water	to	100 ml.	water	to	100 ml.
(pH 8.9)			(pH 6.7)		
(C)			(D)		
Acrylamide		28.0 gm.	Acrylamide		10.0 gm.
BIS		0.735 gm.	BIS		2.5 gm.
water	to	100 ml.	water	to	100 ml.
(E)			(F)		
Riboflavin		4 mg.	Sucrose		40.0 gm.
water	to	100 ml.	water	to	100 ml.

* pH adjusted by titrating with 1N HCl

Working Solutions

These solutions should be used the day they are prepared, with the exception of the persulfate solution which should be used within 7 days of preparation. The persulfate solution should be strored in a refrigerator.

TABLE 2

WORKING SOLUTIONS

Small-pore Solution #1	Small-pore Solution #2	Large-pore Solution	Stock buffer solution for reservoirs*
1 part A	Ammonium persulfate	1 part B	TRIS 6.0 gm.
2 parts C	0.14 gm.	2 patrs D	Glycine 28.8 gm.
1 part water	water to 100 ml.	1 part E	water to 1 liter
pH 8.9 (8.8-9.0)		4 parts F	pH 8.3
		pH 6.7 (6.6-6.8)	

* Up to 12 samples may be run with 500 ml. 0f a 1/10 strength buffer before it is discarded.

WASH-SOLUTION FOR DESTAINING AND STORING GELS			FIXATIVE-STAIN SOLUTION	
Glacial acetic acid		70 ml.	Amido Schwarz	1 gm.
water	to	1 liter	7% Acetic acid	100 ml.

The dye is added to the acetic acid, the mixture stirred and filtered. This solution can be reused a number of times. As the solution is reused, a black insoluble precipitate forms that

may coat the gels. This precipitate should be removed by filtering the solution prior to reuse. It should be noted that the fixative-stain solution, after it has been reused a number of times, will tend to stain the protein black rather than the expected dark blue.

POLYACRYLAMIDE SOLUTION FOR DESTAINING

Acrylamide	6.0 gm.
Riboflavin	0.5 mg.
TEMED	0.05 ml.
water	to 100 ml.

This solution is exposed to a daylight fluorescent light in a 100 ml. beaker at a distance of about 3 inches. Photopolymerization is allowed to proceed for about 11/2 hours. The polymer solution is then diluted with an equal volume of distilled water, thoroughly mixed, and stored in a brown glass bottle. FIGURE 3 illustrates a minimum set of apparatus components.

PROCEDURE

(l) The stock solutions and small-pore solution #2 are removed from the refrigerator and permitted to warm to room temperature before use.

(2) A sample gel solution composed of about 0.15 to 0.20 ml. of large-pore solution and

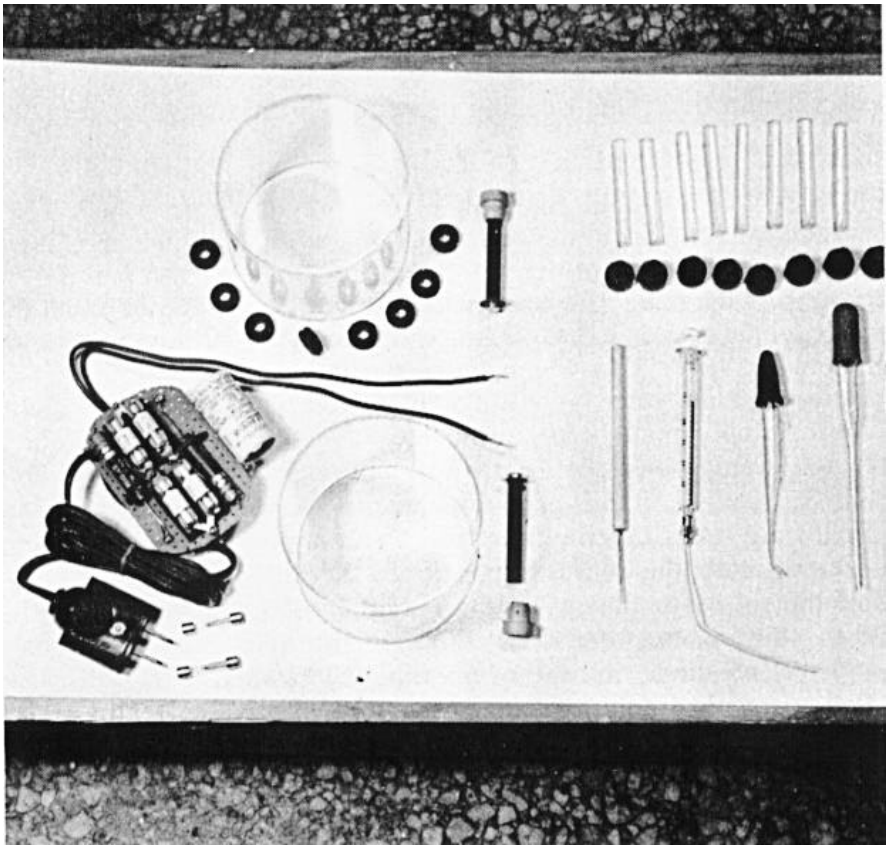

FIGURE 3. Parts of apparatus with power supply shown in A of FIGURE 2.

about 3 to 4 μl. of serum sample (about 200 gamma of protein) are gently but thoroughly mixed. Each sample is mixed in an individual container and protected from strong light. After all the sample mixtures are prepared, each is then added to the well of a rubber stopper attached to the tube stand previously described.

The total amount of protein in the sample volume should not usually exceed 200 gamma. Tuberculin syringes and needles, the latter fitted with lengths of small-bore polyethylene tubing, serve as useful devices for measuring, mixing, storing, and delivering the sample gel solutions into the stopper wells. A short column of fluid, e.g. serum, about 10 to 15 mm. Iong, in tubing of comparatively uniform bore, can provide an easily measured and fairly reproducible volume in the microliter range. If a more dilute protein sample is used, i.e. a protein concentration down to about 0.3 mg. per cent, a more concentrated large-pore solution is prepared, and the sample gel solution is made by dilution with the protein sample. If the concentration of the protein is less than 0.3 mg. per cent, the volume of the sample gel is increased to accommodate an amount of protein solution containing about 200 gamma, and correspondingly longer gel tubes are used. In order to prevent the occasional inhibition of gelation, the concentration of human serum in the sample gel should usually not exceed 1 part in 30 by volume.

(3) After the stopper wells have been filled with the sample solutions, the gel tubes are inserted into the wells, care being taken that the tubes are vertically positioned.

Prior to use, the gel tubes are cleaned in a detcrgent solution, and the inner walls vigorously cleaned with a cotton-tipped applicator stick. The tubes are then rinsed, first in distilled water and then in a solution of distilled water containing 1 part in 200 of Kodak Photo-Flo solution.* The gel tubes are drained and allowed to dry. Rapid drying can be accomplished by attaching the gel tubes to a vacuum line. This cleaning procedure must always be repeated prior to reuse of the gel tubes.

(4) A water layer is now placed on top of the gel solution. This is accomplished by means of a syringe barrel or pipette previously described. The needle with the attached syringe containing 1 to 2 ml. of distilled water is introduced into the top of the gel tube so that the needle rests against the wall and the hub against the top of the tube. The water will flow slowly and evenly down the inner wall of the gel tube and will layer smoothly on top of the denser sample gel solution. A water layer 3 to 4 mm. in height is adequate.

If the tubes are not clean or adequately coated with Photo-Flo solution, the water-layering step cannot be performed readily. The water, instead of flowing smoothly and evenly down the glass walls, will tend to collect into a bolus that will then drop rapidly and thus penetrate into, and dilute, the sample gel solution.

(5) Following water layering, the tube stand is placed directly under a daylight fluorescent bulb; the bulb is so positioned that it is about 1/2 to 1 inch above the tips of the gel tubes. The sample gel solutions are exposed to this light for about 30 to 45 minutes. After about the first 5 minutes light scattering can be seen in the gel solutions, indicating photopolymerization.

(6) Following photopolymerizatian, the tube stands are removed from the light. They are inverted so that the water layer and the adjacent small fraction of inhibited gel solution flow down the walls of the tubes to the open ends. A piece of absorbent cloth or paper, preferably as lint free as possible, is touched to the open ends of the tubes to absorb the liquid. The inner

* Eastman Kodak Company, Rochester, N. Y.

walls of the tubes are now rinsed with a large-pore solution in which the monomer (D) and the sucrose (F) stock solutions have been replaced by distilled water; that is the tubes are one-half filled with these solutions by means of a pipette and the tube stands rocked gently several times so that all areas of the walls are washed. The tube stands are then inverted and drained as described. The wash procedure is performed twice.

(7) A spacer gel is prepared by adding about 0.15 ml. of large-pore solution to the gel tubes. This large-pore solution is water layered as described in step (4). The tube stands are again placed under a fluorescent light, as described in step (5), and exposed to light for about 20 to 30 minutes.

In instances when a very dilute protein sample is used and the sample gel volume, and therefore height, are increased, the height of the spacer gel column must be increased proportionately.

(8) Following photopolylmerization of the spacer gel the tube stands are removed from under the lamp. The tube stands are then inverted for about one minute with the gel tubes resting on absorbent cloth or paper so that the water layer and adjacent inhibited large-pore solution flow down the tube walls and onto the absorbent material. While the tubes are draining, small-pore gel solution is prepared by mixing equal volumes of small-pore solutions #l and #2. A total final volume of about 20 ml. is sufficient for washing and filling 12 gel tubes. Promptly after this solution is prepared all of the gel tubes are half filled by means of a pipette, and the tube stands are rocked and tilted gently several times so that the surface of the spacer gel and all areas of the tube walls are washed. The small-pore gel solution is removed by inverting the tube stands, permitting the solution to drain down and out onto absorbent material. The wash procedure is performed twice. The gel tubes are now completely filled and an excess of small-pore gel solution is added so that a "bead" of solution rests on top of the upper ends of the gel tubes. The tubes are capped by placing on top of each, by means of forceps, a previously cut 10 mm. square of Saran Wrap* film. Sufficient excess of solution is added so that on capping a tube, a small amount of solution runs down the outside of the tube wall and no air bubble is trapped under the film.

The gel tubes are protected from strong light and permitted to stand undisturbed for about 30 minutes, at the end of which time electrophoresis can be performed. The gel time of this small-pore gel mixture is about 15 to 20 minutes. The time between the preparation of the small-pore gel solution and the capping of the tubes should not exceed 10 minutes, and the gel solution should reside undisturbed in the gel tubes for at least 5 to 10 minutes prior to the onset of gelation.

If the gel time exceeds the stated limits when prepared from fresh reagents, it can be corrected by making minor adjustments in the concentration of TEMED.

AETERNATIVE PROCEDURE

In certain instances the use of a sample gel may not be possible, that is, the protein sample may contain inhibitory substances that prevent the formation of a sample gel. In other instances, the incorporation of the sample into a solution undergoing vinyl polymerization may not be desirable. In both instances an alternative procedure can be employed in which the formation of a sample gel is bypassed and the sample is, instead, layered on top of a spacer gel just prior to electrophoresis.

*The Dow Chemical Company, Midland, Mich.

The procedure is identical to that just described except for the following differences (A) In the method outlined, the sample gel solution is replaced by a 40 per cent sucrose solution on top of which the spacer gel solution is layered and then polymerized. (B) Since the surface of the spacer gel formed against the sucrose solution is rarely flat and smooth, it is necessary, following the polymerization of the small-pore separation gel, to insert the gel tubes, separation gel down, into a tube stand and to drain off the sucrose solution. After several brief washes with the large-pore solution to remove excess sucrose, additional largepore solution is added on top of the spacer gel. A column about 4 mm. in height of large-pore solution is pipetted on top of the spacer gel. This gel solution, when water layered and photopolymerized, forms a smooth, flat surface upon which the sample is placed just prior to electrophoresis.

(9) When polymerization is complete, following step (8), the gel tubes are removed from the tube stand, caution being exercised to avoid stressing and distorting the gel column to prevent inadvertent separation from the gel tube wall. Removal is accomplished by pressing and tilting the tube against one side of the flexible cap wall so as to provide a space through which air can enter the hollow of the cap as the gel tube is removed (see FIGURE 4). Polymerization of a zone, 0.5 to 1.0 mm. in height, of the sample gel adjacent to the base of the stopper well is generally inhibited. This watery solution is removed by inverting the gel tubes, sample gel down, and touching the open end to absorbent material.

(10) Electrophoresis should preferably be started within 1 hour after the separation gel has been prepared. The tubes, sample gel uppermost, are inserted into the grommets of the upper buffer reservoir, and this reservoir is filled with about 200 ml. of the stock buffer solution diluted to 1/10 strength with distilled water. One ml. of 0.001 per cent Bromphenol Blue in water is stirred into the upper buffer. Any air spaces in the gel tubes above the sample gel are displaced with buffer by means of a pipette. Next a hanging drop of buffer is placed on the

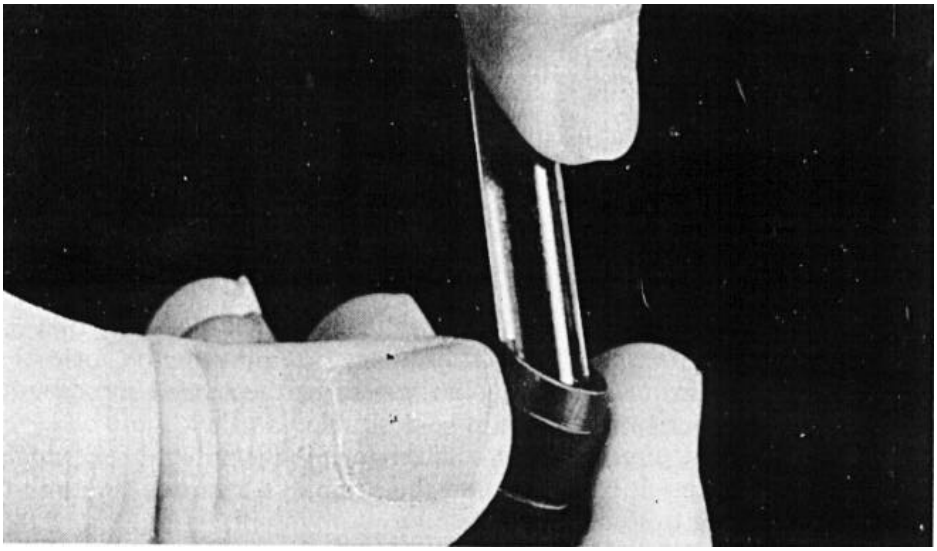

FIGURE 4. The release of the gel tube from stopper following polymerization.

bottom of each gel tube to prevent trapping of bubbles, and the upper reservoir is then lowered so that the bottoms of the gel tubes are immersed about 1/4 inch in the buffer (also 1/10 strength) of the lower reservoir. The lower reservoir should be filled to within 1/2 inch of the top (See FIGURE 1) .

In the instance when a sample gel is not used, the volume of the tube above the spacer gel is first filled with buffer, the tubes are attached to the upper reservoir, and the upper reservoir is filled with buffer. The sample solution is picked up in a pipette and the pipette introduced through the buffer solution and into the gel tubes to a point about 4 mm. above the surface of the spacer gel. The sample is gently expelled and permitted to layer between the top of the surface of the spacer gel and a *less dense* buffer solution above. The gel tubes must be carefully positioned so that the protein solution is evenly distributed across the face of the spacer gel.

(11) The power supply is connected, cathode to the upper reservoir. The current is adjusted to about 2 to 5 m. Amp. per tube. (If the power supply used is the one described in FIGURE 2A, and the buffer reservoirs are 4 inches in diameter, the required current is obtained by diluting the stock buffer to half strength rather than 1/10 strength.) Currents higher than 5 m. Amp. per tube should be avoided since excessive ohmic heating may result in the formation of pattern artifacts. Electrophoresis is carried out until the front of the light blue albumin disc has migrated about 25 mm. into the separation gel and the free Bromphenol Blue dye has therefore migrated about 30 mm.; with a current of about 5 m. Amp. per tube, the time for electrophoresis is about one-half hour. In the instance when the sample solution is layered above the spacer gel, the current should not initially exceed 2 m. Amp. per tube so as to prevent conveclive losses of the sample into the upper reservoir. After the sample has entered the spacer gel, the current is then increased to 5 m. Amp. per tube.

When electrophoresis is carried out at room temperature with the apparatus, reagents, and conditions described here, the temperature within the separation gel containing the sample fractions is in the range of about 35° to 40° Centigrade. If the sample to be separated contains heat labile substances, e.g. enzymes, which may be inactivated at the temperature of the operating conditions described, it may be necessary to reduce both the ambient temperature and the current. At a current of 1 m. Amp. per gel tube, heating within the sample volume of the separation gel is negligible; but, for the same distance of migration, the time for separation must be increased to about 2 l/2 hours.

(12) At the completion of electrophoresis, after the power supply is turned off, the buffer solutions are decanted. If fewer than 12 samples are run, the buffer solutions may be reused (see Reagent section), but the upper and lower buffer solutions must not be pooled or positions reversed in the apparatus in future runs since this would result in contamination of the cathodic buffer reservoir by gel catalyst substances and chloride ions which migrate into the anodic reservoir during electrophoresis.

(13) The gel tubes are removed from the upper reservoir and the gels removed from the tubes by rimming under water. The water lubricates the gel surface and prevents mechanical damage to the gel by the rimming wire or needle. The wire is slowly introduced into the bottom of the gel tube between the small-pore gel and the tube wall for a distance of about 5 to 10 mm. while continuously rotating the gel tube (see FIGURE 5). The wire is then withdrawn with a slight pressure against the gel, stretching the gel so that it protrudes about 2 mm. beyond the end of the tube (see FIGURE 6). The needle is then withdrawn completely. It is then introduced into the other end and a continuous rimming action is again applied as the

needle advances until the gel slips out of the tube (see FIGURE 7).

(14) Each gel is immersed in at least 2 ml. of fixative-stain solution for a minimum of 1 hour. At the end of 1 hour, the fixative-stain solution is decanted and the gels are rinsed for a few minutes in tap water.

(15) Electrophoretic destaining is performed in the same apparatus. The gels, sample gel uppermost, are placed in the destaining tubes, which have been attached by their wide ends

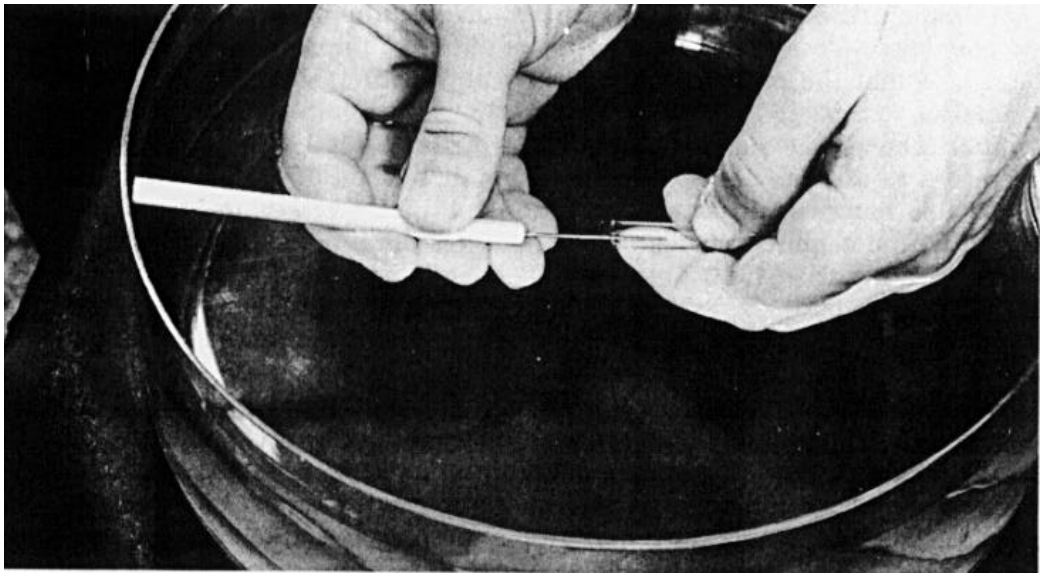

FIGURE 5. Removal of gel from gel tube following electrophoresis (first step).

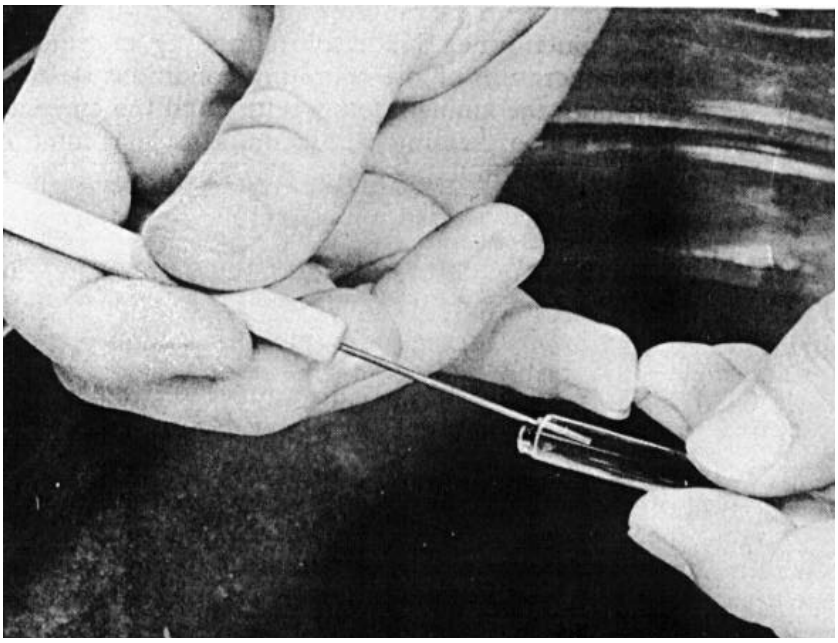

FIGURE 6. Removal of gel from gel tube following electrophoresis (second step).

to the upper buffer reservoir. The gels slide down in the tubes and should wedge firmly against the constricted ends of the tubes. In order to reduce convective disturbances and backflow of free dye up the tube, polyacrylamide solution for destaining may be added to each tube by means of a pipette up to the top of the sample gel. No air bubbles should remain in the tubes after adding polyacrylamide solution. The space above the gel in the destaining tube is carefully filled with wash solution by means of a pipette. If the difference between the i.d. of

FIGURE 7. Removal of gel from tube following electrophoresis (third step).

the destaining tube and that of the gel is 1 mm. or less, no polyacrylamide solution is necessary; instead, a small cotton plug should be inserted into the upper end of the destaining tube after filling with wash solution. About 200 to 300 ml. of wash solution are then added to each reservoir, and 20 to 30 microliters of fixative-stain solution are mixed into the upper reservoir. The electrodes are connected, cathode to the upper reservoir. On applying a voltage, the unbound dye migrates down the gels and into the lower reservoir. Destaining is completed within about I hour, using the power supply described in FIGURE 2A. This time can be reduced to about 20 minutes if the current is adjusted to about 15 m. Amp. per tube by using a higher voltage power supply such as that outlined in FIGURE 2B.

(16) At the completion of destaining, the power supply is shut off, the wash solutions are decanted, and the gels are transferred to small test tubes containing wash solution for storage.

RESULTS

The set of Amido Schwarz stained gels illustrated in FIGURE 8 shows a typical distribution of proteins of normal adult human serum haptoglobin type 2-2 when subjected to disc eleclrophoresis according to the conditions described. All the samples were obtained from the same serum specimen, and all eight patterns were electrophoresed simultaneously.

A ppearance of the Pattern and Distribution of Proteins

The composition of the sample and spacer gels has been modified recently in order to improve the physical qualities of the gels. The modification has resulted in minor changes in the pore sizes.

In the present as well as in the earlier formulation of the sample and spacer gels, these gels have bcen designed to exert as minimal a frictional resistance to the passage of proteins as is possible. In both versions the amount of protein that fails to penetrate these gels is negligible compared with the amount that migrates through them and into the separation gel. Since, at the time this paper was prepared, investigations on the localization of protein in these new gels was not completed, the following description must be considered tentative. Still another composition of the sample and spacer gels has been used by Kochwa *et al.*,[9] and the reader is referred to this paper for the identification and sites of proteins in these sample and spacer gels. These latter gels are of somewhat smaller pore size than those used in the procedure reported in the present paper.

The sample gel is homogeneously stained a pale blue. Although it is not yet definitely known in this latest formulation, this portion of the gel column probably contains some of the macroglobulins. In a,ddition it contains protein aggregates derived from cellular debris (red and white cells). A thin disc of stained protein is visible at the lower end of the sample gel adjacent to its junction with the spacer gel. At least some, but not necessarily all, of the proteins in this disc are probably 19-S gamma globulins.

The spacer gel is free of visible dye except at its lower end. (The diffuse increase in density of this gel, in comparison to the protein-free areas of the separation gel, is due to the photographic reproduction of the light scattering of the spacer gel and not to the presence of colored material.) A stained disc about 1 mm. in thickness is noted at the lower end of the spacer gel. (The origin of the separation gel is indicated in FIGURE 1, Ornstein.[8]) This protein has not as yet been identified.

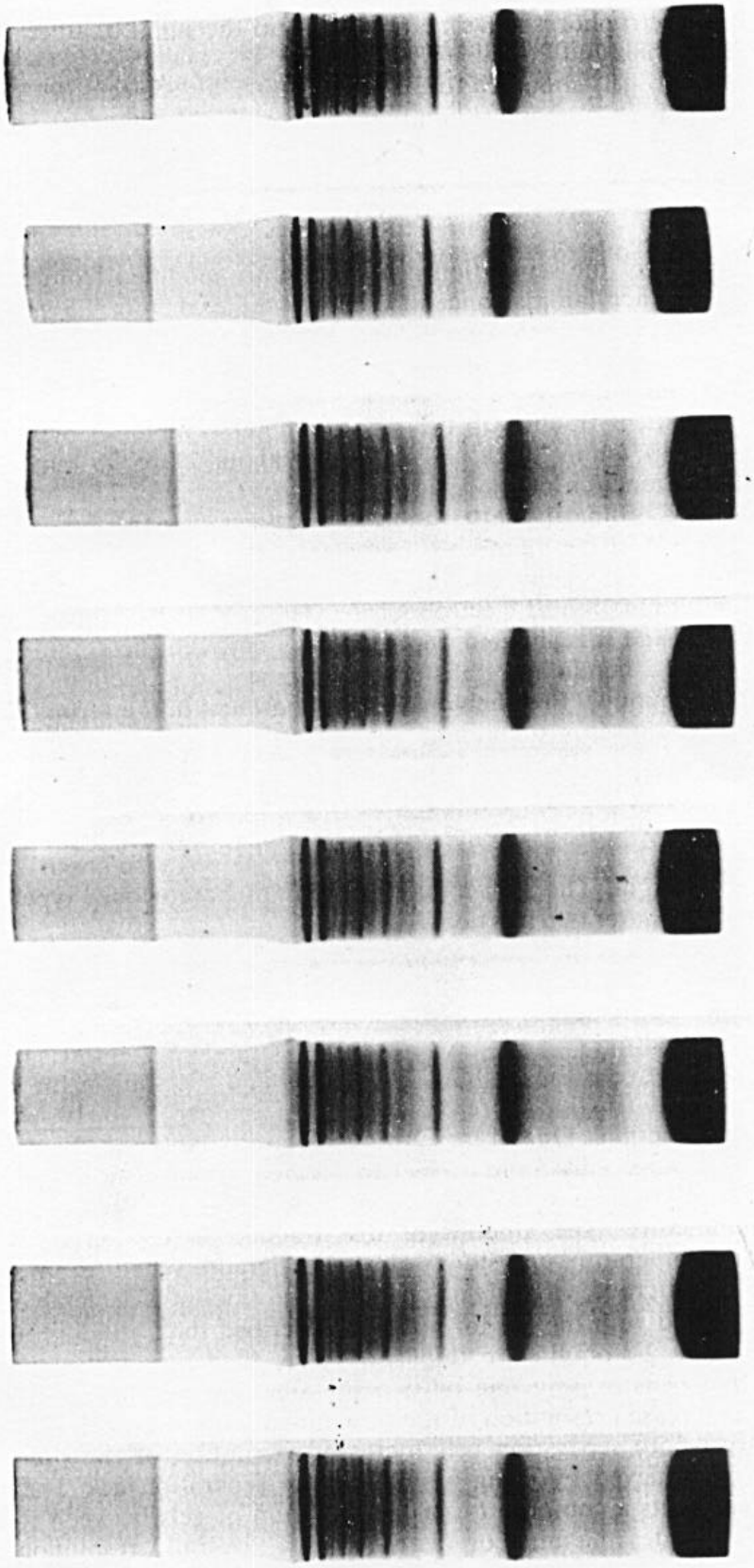

FIGURE 8. Electrophoretic patterns of normal human serum (haptoglobin type 2-2). One sample run simultaneously in eight tubes.

A correlation between the distribution of proteins in Smithies' starch gel technique and disc electrophoresis has been made, and the nomenclature employed here is that of Smithies and Poulik.[1,2,10]

In the separation gel, about 20 to 30 protein discs are detectable. The major source of variation in numbers is due to the genetic variation in haptoglobin proteins among different individuals. In general, the discs of the slower moving proteins are diluted less by diffusion spreading and therefore stain more intensely and their faces are more sharply demarcated than those of the faster proteins.

The composition of the separation gel was slightly modified from that of the earlier version. The acrylamide concentration was reduced from 7.5 gm. per cent to 7.0 gm. per cent and the BIS from 0.2 gm. per cent to 0.184 gm. per cent. The modification was made in order to increase the separation and resolution of the slower moving proteins.

The most prominent fraction in the separation gel is albumin, which is seen as a disc about 3 to 4 mm. thick and is located about 25 to 30 mm. from the origin, that is, the junction of the spacer and separation gels. The front face of the albumin disc is ordinarily flatter and more sharply demarcated than that of the rear face. Ahead of the albumin is one clearly evident pre-albumin, situated adjacent and to the rear of the position that the free Bromphenol Blue dye disc occupied at the terminatibn of electrophoresis. The second most prominent fraction is transferrin, and it is situated about midway between the albumin and the origin. Between the albumin and transferrin are some 4 to 8 fractions collectively identified as postalbumins. Free hemoglobin A, if present in the serum, is located in the region of the slower postalbumins.

Between the origin and the transferrin there are a number of fractions. At the origin and just within the separation gel is an intensely stained disc containing, among other constituents, the beta 1-lipoprotein. The S alpha 2-globulin (19S alpha 2 glycoprotein) is visible as a thin, intensely stained disc about 1.25 mm. distant from the origin. The slower haptoglobins of haptoglobin type 2-2 are located between the latter two fractions.

The faster 2-2 haptoglobins are found in the first 1/3 of the distance from the S alpha 2-globulin to the transferrin. These are seen as four fractions. This region also shows a pale diffuse background stain and is the volume occupied by the 7-S gamma globulins.

The gel region between the S alpha 2-globulins and the transferrin contains, in addition to those fractions already described, another 5 to 10, as-yet-unidentified protein fractions. One of these fractions, presumably the one designated "p" protein by Poulik and Smithies,[10] and located about 3 mm. behind the transferrin, is barely visible in the patterns when serum is analyzed within a few hours of venipuncture. The amount of this fraction in the serum increases rapidly at room temperature and slowly at 5° Centigrade. When serum is stored at about -15° Centigrade, virtually no change in the amount is detectable.

It should be noted with the gel formulation described that, although the discs behind the S alpha 2-globulin are thin and sharp, they are very close to one another and that usually only one or two pre-albumins are resolved. Further separation and increased resolution of the slow moving fractions can be achieved by using a larger-pore separation gel. Increased resolution of the pre-albumin fractions can be achieved by using a smaller-pore separation gel. The present formulation represents a compromise in the spectrum of gels between these two extremes that provides maximal or near maximal "overall" resolution of the human serum protein spectrum.

DISCUSSION

The present technique represents a comparatively minor modification of earlier versions, and the rationale of the method remains within the framework of disc electrophoresis theory as previously developed. For this reason the discussion is designed to cover both the older and newer variations of the procedure.

Gels

The monomer, acrylamide and comonomer (crosslinking agent), N,N'-Methylenebisacrylamide (BIS) concentrations and their ratios are varied to achieve the desired properties of (l) anticonvection in the case of the sample and spacer gels and (2) sieving as well as anticonvection in the case of separation gels.

The sample and spacer gels, designed solely to serve as anticonvection media, are prepared with concentrations and ratios of monomer and comonomer that yield gels of maximal pore size and also possess adequate strength and elasticity. It is in general highly desirable that these gels remain intact and attached to the separation gel during the entire procedure so that they may be examined following staining for the presence of retained protein.

Large-pore gels are prepared by using the lowest possible concentrations of monomer and comonomer that reproducibly yield gels with adequate gross mechanical properties. It has been found that at concentrations below about 2.0 gm. per cent acrylamide and about 0.5 gm. per cent BIS, gelation usually does not occur. Slight increases of one or both of these ingredients, however, result in the reproducible formaton of gels with satisfactory mechanical properties.

In the present procedure, sucrose is added to both the sample and spacer gel formulations. Such gel solutions can be polymerized more rapidly, and the gross mechanical properties of such gels are improved.

The incorporation of the sample into a gel, as opposed to layering it on a gel surface, is more convenient and yields somewbat higher resolution. If possible denaturation of a particular protein during polymerization of the sample gel is a source of concern, parallel runs can be made (one with the protein incorporated into the sample gel, the other with the sample layered on top of the spacer gel) and the stained (for proteins or enzymes, for example) patterns compared.

Sieving gels for the separation of the sample species can be prepared from a wide range of concentrations and ratios of monomer and comonomer. (For large proteins, e.g. 19S gamma globulins, the large-pore gels just described can serve as sieving gels.) We have examined gels prepared from concentrations of monomer ranging from 1.5 to 60.0 gm. per cent and comonomer ranging from 0 to 0.625 gm. per cent. A few useful generalizations can be made. As the concentration of monomer is increased, the concentration of comonomer should be decreased. Increases of both components tend to produce gels of increasing stiffness and friability, while decreases of both tend lo produce gels of increasing softness and elasticity. At both extremes, the gels are difficult to manipulate. Gel pore size can be regulated by varying either component. The efficiency of utilization of monomer and comonomer and the percentage yield of polymer are functions of the concentrations of both monomer and comonomer, and therefore pore size and frictional resistance are not necessarily a simple function of the concentrations of acrylamide and BIS (see FIGURE 2 of Ornstein's paper[8] .

The concentrations of monomer and comonomer suggested in the "Materials and Methods" section of this paper are in a sense rather arbitrary. The particular concentrations and ratios must be found by experiment to meet the requirements of adequate separation of

the protein mixture being analyzed. As for the separation of a complex mixture of proteins, it is unlikely that any one combination of monomer and comonomer will adequately separate all the fractions. Thus the separation gel presently employed resolves a number of discrete and easily visualized fractions between the S alpha 2-globulin and the albumin while those proteins behind the S alpha 2-globulin are crammed close together and, at the other end of the pattern, those ahead of the albumin are poorly resolved. It is not to be inferred that the individual fractions resolved between the S alpha 2-globulin and the albumin are "pure" (densitometric traces confirm the existence of considerable overlap of fraction with fraction as can be seen in FIGURE 1 of Ornstein's paper[8]). These can often be further resolved by alternative gel recipes.

Catalyst Systems

In selecting catalyst systems, i.e. catalyst and accelerator, for the preparation of the gels, it is desirable to keep the concentration of these extraneous ions to a minimum so as to permit maximum flexibility and freedom for establishing the desired Kohlrausch condition.

Two different catalyst systems are used: riboflavin[11,12]-TEMED for the sample and spacer gels and persulfate-TEMED for the preparation of the separation gels. Both thermal polymerization and photopolymerization, which require an ultraviolet rather than visible light source, were examined and found less convenient than those presently used.

Both riboflavin-TEMED and persulfate-TEMED were examined for the preparation of the sample and spacer gels. At given concentrations of monomer and comonomer, both catalyst systems yielded gels with quite similar gross mechanical properties. However, comparison of the gels in disc electrophoresis of serum proteins showed that the persulfate-TEMED catalyzed gels had somewhat smaller pores than those catalyzed with riboflavin-TEMED, if the gels were used shortly after preparation. It was found on further investigation that if a riboflavin-TEMED catalyzed gel is exposed to light (riboflavin-TEMED is a photocatalyst system) for a sufficient length of time, its pore size may become virtually indistinguishable from that of a persulfate-TEMED catalyzed gel. Since the pore size of a riboflavin-TEMED catalyzed gel is larger than that of a persulfate-TEMED catalyzed gel, when such gels are used within the period of time stipulated in the procedure, the former catalyst system was selected for the preparation of the sample and spacer gels. Riboflavin catalyst systems possess two additional virtues. First, gelation can be effected using low concentrations of riboflavin,[11,12] i.e. in the one mg. per cent range. Second, the gel time is conveniently varied since gelation is initiated by exposure to strong light, in contrast to the case of chemical initiation in which the gel time, for practical purposes, is set by the concentrations of the catalyst system ingredients.

Riboflavin has been used in this laboratory without the addition of accelerator for the preparation of large-pore gels. The results were, for the most part, satisfactory; but the addition of TEMED to the system permits gelation, which in some instances failed to occur, particularly of gel solutions containing protein mixtures. The addition of sucrose to the large-pore gel solution further increases the reproducibility of gel formation and the mechanical strength of the gel without appreciably altering the pore size. The effect of solutions containing sucrose on polymerization is presently under investigation.

A persulfate-TEMED catalyst system is used for the preparation of separation gels

primarily because persulfate, as employed in the present procedure, yields gels of reproducible pore size and, for practical purposes, gelation can be considered complete within about 30 minutes after its onset.

A number of accelerator substances were tested. The inorganic reducing agents were discarded after brief investigation because of either their high electrical conductivity in aqueous solution or because of difficulty in controlling the gel time in their presence. For these reasons the aliphatic tertiary amines were investigated. Among these TEMED* was selected because of its high efflciency, that is, its capacity in low concentrations to accelerate polymerization. Triethanolamine was somewhat less efficient. Beta dimethylamino-propionitrile was less efficient than either of the above.

In an earlier version of the procedure, a small amount of potassium ferricyanide was added to the separation gel solution, in addition to persulfate and TEMED, in order to increase control of the gel time. Such control is very convenient, especially since it permits the user to prepare a large number of separation gels simultaneously. It has been found, however, that the presence of the ferricyanide can be correlated with occurrence of gel inhomogeneities, which are reflected as small local variations in gel pore size. A less pronounced correlation was also noted between the gel time and inhomogeneities; the longer the gel time, the more frequent the occurrence of local pore-size variations. The resolution of sample species migrating through such zones of variable size is frequently reduced. Therefore in the latest version of the procedure, the ferricyanide has been deleted. In addition the gel time was reduced to 15 to 20 minutes. Since ferricyanide has been deleted, control of gel time is most satisfactorily achieved by adjusting the concentration or ratios of the persulfate and TEMED. In the present version, in comparison with the earlier one, the persulfate concentration is the same, but the TEMED concentration is reduced.

Buffers and Salts

Glycine was picked as the source of the trailing or slow ion because its pK and mobility of glycinate are such that the effective mobility can be set at a value below that of the slowest known proteins of net negative charge in the pH range in which most pf the serum proteins are anionic. The minimum pH of this range is somewhere around 8.0.† The effective mobility of glycine can also be adjusted for the separation step to values exceeding that of the fastest proteins in a pH range in which many, but not all, proteins retain their biological activities.

The characteristics of two other weak acids usable as the trailing ion clarify the reasons for the above selection. The pK and mobility of borate are such that the effective mobilities of boric acid differ only slightly from those of the slowest proteins even around pH 8.0. Minor variations in pH, in fact, may reverse the relative mobilities with the result that the slowest proteins will not undergo Kohlrausch stacking and concentration. On the other hand the pH of the medium in which separation occurs need not be as high as with glycine (pH about 9.0 and pH about 9.5, respectively) with the particular separation gel described in this paper.

In contrast to boric acid, phenol has a pK and phenolate has a mobility which readily

* Suggested by H. W. Holly, American Cyanamid Company, *New* Yorlc, N. Y.

†While this paper was in press an error in the theorctical developmont of the technique was detected which indicates that the specific formulation of reagents described here does not provide for the stacking of the slowest known proteins. For details see footnote to APPENDIX E of Ornstein's paper[8] (this *Annal*).

permit one to set up conditions for stacking and concentration of the slowest proteins. In the separation step, the pH must be set at a value close to or slightly above that of the glycine system. Since glycine is more compatible with most proteins than is phenol, the former is selected as the trailing ion.

The foregoing remarks are made to illustrate the basis for selection of the trailing ion for disc electrophoresis of serum proteins in the gel described. Different degrees of freedom or limitation in the selection of the trailing ion will be encountered depending on the characteristics, i.e. molecular dimensions, mobilities in the different pH ranges, and stabilities of the sample species. If, for example, the range of the molecular dimensions of the sample species is reduced relative to that of the serum proteins, the pore size can be decreased. It follows then that the pH in the separation gel can also be lowered since the increased sieving will diminish the mobilities of the sample species.

The chloride ion was selected as the fast or leading ion, since its mobility relative to those of proteins is high. The reagent, hydrochloric acid, is comparatively inexpensive and obtainable in relatively pure state. Sulfate and phosphate have also been used successfully.

The counter ion, i.e. the ion of sign opposite that of the slow, fast, and sample ions, serves as a buffer to set initially and then to regulate the various pH's behind the moving anion boundary. TRIS was selected since it is a reasonably satisfactory buffer in the range required.* TRIS is also a relatively innocuous substance to most proteins.

TECHNIQUE

The technique of disc eleclrophoresis differs in a number of respects from other techniques using sieving gels, and it is with these differences in mind that the technique will be discussed.

Rigid, transparent, circular cross-section tubing of glass is used. The individual samples are each electrophoresed in separate tubes.

The type of tubing is limited to materials to which the gels adhere with sufficient strength to remain firmly attached during electrophoresis, while not so tightly bonded to prevent easy removal of the gel column following electrophoresis. It has been found that glass is most satisfactory, although lucite, styrene, and cellulose acetate-butyrate can also be used. Tubing of circular cross section is preferred because, among other reasons, a symmetrical cross section exerts minimal strain and stress on the gel and thereby reduces the likelihood of separation of the gel from the wall.

By using a gel with a small cross sectional area rather than a thick slab, heat production is correspondingly reduced. Thermal gradients across the tube are, in addition, minimized since the gel tube wall serves as a good heat insulator. Thermal gradients across the gel are also more symmetrically distributed in a gel with a circular cross section in contrast to a slab. By reducing the amount of heat produced and by reducing and making more symmetrical thermal gradients, higher voltage gradients can be imposed and the electrophoretic running time correspondingly reduced to achieve a given level of resolution.

Transparent tubing is used since the sample and spacer gels are only moderately light-scattering and the sieving gel is highly transparent, permitting the investigator to observe the electrophoretic migration of proteins. The proteins are visible by virtue of their color or the

* Recent work shows that a TRlS-phosphate buffer can replace the TRIS-chloride in the sample and spacer gels. In these gels, the pH is set slightly below 7.0, and thus TRIS-phosphate serves as a more satisfactory buffer than TRIS-chloride.

refractive index differences between them and the surrounding gel medium. Thus the stacking and concentration of the protein ions can be seen, and during separation as many as 12 to 15 serum protein fractions are easily visible to the unaided eye. In instances when the optimum running time is not known, examination of the separating fractions during the run permits a useful estimate to be made. Visualization during the run also can be of great value in the identification of the site or source of pattern artifacts.

It can be argued that it is preferable to apply a number of samples to a single gel slab rather than to introduce each sample into a separate gel tube since in the former case all the samples are migrating through a single and therefore more homogeneous gel. An examination of reproducibility series (FIGURES 5 and 8 of Ornstein's paper[8]) suggests that reproducibility of the separate gel tube technique can be comparable to that of the slab technique. (If gel pore size is uniform from gel to gel, the relative distances of migration of the various sample species will be the same for different running times and/or voltages. For this reason consecutive or simultaneous runs can be compared without error.)

One of the most critical steps in the preparation of gel matrices is the formation of a gel surface that serves as the origin or site of onset of electrophoretic separation. This surface should be as smooth and as flat as possible and free of defects because appreciable changes in sieving effects occur abruptly as proteins pass through this surface. Gel surface imperfections may thus be imprinted on the sample species as they enter this gel during electrophoresis.

A number of experiments were performed to find a simple means of forming a satisfactory gel surface. Smooth-faced plugs and caps of a variety of materials were used as surfaces against which the gel origin was formed. None was completely satisfactory. Many materials caused variable inhibition of gelation and therefore localized variations of pore size; or the process of removing the device after gelation marred the surface.

Water layering, on the other hand, is much more successful. A small portion of the gel solution adjacent to the water layer does not gel, but about 1 to 3 mm. below the water interface gelation with the formation of a flat, smooth gel surface occurs consistently. The inhihited zone provides a "buffer" volume that reduces diffusion of salts and water between the water layer and the forming gel.

In earlier versions of the procedure, the separation gel solution was added to a gel tube, the bottom of which was stoppered and the origin was formed by water layering. The spacer and sample gels were then formed consecutively on top of this gel.

Although the surface of the separation gel was flat and smooth, the separation gel in the vicinity of the origin was not always homogeneous. Experiments were performed that indicated that the volume of gel near the origin, although visually homogeneous, was in fact frequently composed of two regions of different pore sizes. In brief, as polymerization takes place the volume of gel solution decreases. It was noted that the forming gel often bonds to the wall *before* most of the volume decrease occurs; since the walls and the stopper are rigid, any shrinkage that occurs is manifest by a distortion of the gel at the open water-layered end. As a result, the first formed gel boundary, initially flat, becomes progressively distorted, convex down. At the same time that this distortion takes place, gel solution (the polymerization of which is partially retarded) directly above the distortion goes on to polymerize forming a second smooth, flat surface. Since this additional volume of gel is quite small, its volume loss during polymerization is so slight as to be virtually undetectable on visual examination.

The additional gel layer, however, is usually more porous than the original gel first formed and for this reason a gel pore size discontinuity is often present at this junction. Any curvature of this junction is imprinted on proteins which are sieved appreciably.

The present procedure bypasses this problem by adding the separation gel solution on top of a previously water-layered and therefore, smooth faced spacer gel. The open end of the gel tube is covered with a flexible Saran Wrap film. In this instance distortion of the separation gel due to volume loss during polymerization is restricted to a gel region distant from where the sample species migrate.

Staining and Staining Artifacts

The proteins in the gels are simultaneously fixed (insolubilized) and stained in 7 per cent aqueous acetic acid containing concentrated Amido-Schwarz dye.

The acetic acid solution itself is only moderately effective in "fixing" the serum proteins. However, the inclusion of the Amido-Schwarz enhances fixation as well as stains the proteins, and reasonably satisfactorily stained patterns are obtained. Other, more effective, fixing agents are presently being examined.

Rapid removal of unbound dye can be achieved by electrophoretic destaining. Two cautionary remarks, however, must be made. If electrophoretic destaining is begun before fixation is complete, artifacts in the pattern will be generated since the proteins will migrate in the electrophoretic field. Such artifacts are typically more pronounced in the central portion of the gel since fixation proceeds from periphery to center. Pattern artifacts of this type are recognized by the fact that the peripheral portion of a protein disc is flat and its faces comparatively distinct, while the central portion of a disc is thickened, blurred, and curved.

Prolonged electrophoretic destaining can result in the reduction of the amount of dye bound to a protein. The dye is not irreversibly bound to protein but in equilibrium with very low concentrations of free dye. Continuous removal of free dye displaces the equilibrium with consequent leaching of bound dye. This loss can be prevented by adding a fixed, very low concentration of dye to the cathodic reservoir. Free dye thus continuously migrates through the destained volume of the pattern and the loss of bound dye is minimized.

SUMMARY

The technique of disc electrophoresis has been presented, including a discussion of the technical variables with special reference to the separation of protein fractions of normal human serum.

ACKNOWLEDGMENTS

Since the theoretical aspects of disc electrophoresis were predominantly the work of Doctor Leonard Ornstein and the development of the technique preponderantly the work of the author (although there was considerable overlap), the authorship of the two papers was decided on this basis. The obvious debt to Doctor Ornstein is accordingly acknowledged.

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