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TOXIC HAZARDS DIVISION

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ELECTROPHORETIC SEPARATIONS ON ACRYLAMIDE GELS: DISC ELECTROPHORESIS

LEANDRO RENDON

DECEMBER 1965

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Foreword

This work was performed under Project No. 6302, "Toxic Hazards of Propellants and Materials," Task No. 630202, "Pharmacology – Biochemistry," from August 1963 to June 1965 in the Toxic Hazards Branch, Physiology Division, Biomedical Laboratory, Aerospace Medical Research Laboratories, Wright-Patterson Air Force Base, Ohio. The assistance and suggestions of 1/Lt Duncan E. McVean, and the assistance of TSgts. W. D. Leonard, G. W. Craig, and W. F. Hunt, Jr., who evaluated the procedure, are gratefully acknowledged.

This technical report has been reviewed and is approved.

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Abstract

Disc Electrophoresis, a new method for fractionating serum proteins and enzymes developed by Ornstein and Davis (Mt. Sinai Hospital, New York, N. Y.), possesses great sensitivity, speed and reproducibility while requiring a sample as little as 3 microliters in routine separations. The technique as adapted and standardized for use in the Toxic Hazards Branch, Aerospace Medical Research Laboratories, for serum protein and Lactic Dehydrogenase (LDH) isozyme separations is presented along with suggestions for making the apparatus needed to perform disc electrophoresis.

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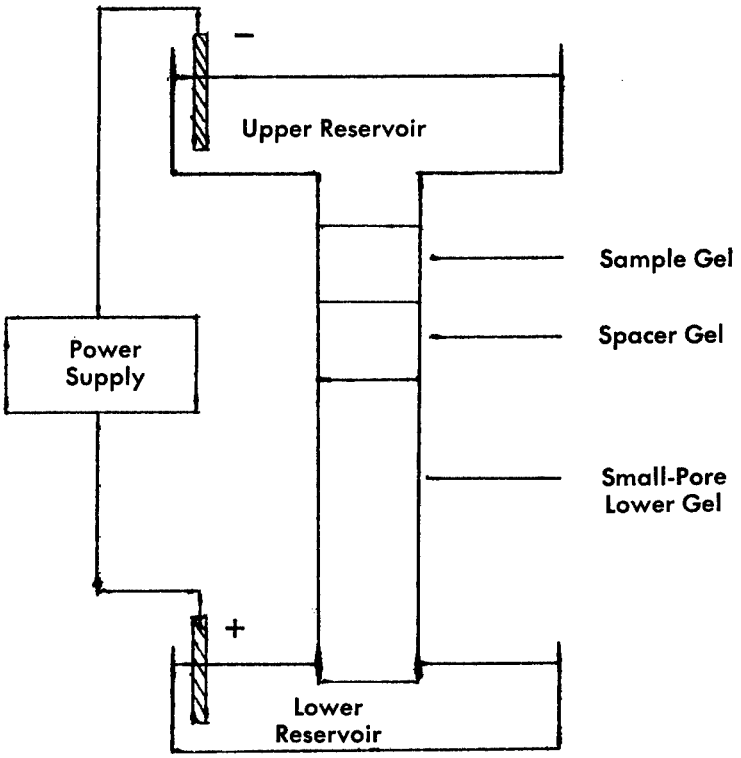

(frontispiece) Disc Electrophoresis

SECTION I

Introduction

New, powerful, analytical tools, and refinements in long-known principles such as electrophoresis, have been produced as the result of recent advances in biochemical methodology. These techniques provide new insight into the many protein fractions present in biological tissues and especially, enzymes. Electrophoresis provides a most effective method for the separation of ionic components of a mixture, since the differences in electrophoretic mobilities of the component ions are usually great enough to achieve separation. However, when the differences are small, separation may not be possible or satisfactory. To improve the separation, electrophoresis has been accomplished using various support materials, e.g., granular and solid media, and many techniques have been devised.

The resolving qualities of the various electrophoretic methods are quite different. The number of protein fractions usually separated from serum using paper electrophoresis is from 5 to 7. The best resolution occurs when the frictional properties of gels are employed, in addition to electrophoretic mobilities, to aid in the separation. Discontinuous electrophoresis, described by Ornstein and Davis (ref 5), is an effective method for fractioning serum proteins and enzymes; some 20 to 25 fractions can be separated from serum.

The technique derives its name from its dependence on *disc*ontinuities in the support medium where the separation occurs and from the form in the gel column in which the separated fractions appear, i.e., a stack of flat discs. In this new method, the pore size of the gel may be varied by merely changing the concentration of the inert polymer, polyacrylamide $(CH_2CHCONH_2)_n$. A sieving effect is obtained that produces separation of fractions, not only on the basis of differences in electrical mobilities but in molecular size as well. Thus separations of as many as 20 distinct and concentrated protein fractions in serum samples are possible on a routine and reproducible basis.

Disc electrophoresis is of particular interest both in research and clinical analysis, because it lends itself to the use of small quantities of biological sample. For serum protein separation studies, three microliters may be used routinely. This particular feature makes disc electrophoresis readily adaptable to use in clinical studies involving all kinds of laboratory animals (ref. 4).

Several methods have been described for accomplishing disc electrophoresis on acrylamide gels (refs 1, 2, 3, 4, 6). Each is designed for some specific purpose, and may include variations in the composition of the gels and the buffer solutions, size of sample used, differences in pH, amount of current used, and in the running time for electrophoresis, etc. This report outlines an optimum standardized procedure for disc electrophoresis that has had extensive use in studies involving the evaluation of serum protein and isozyme separation patterns as possible early indicators of nonspecific chemical stress in animals exposed to various chemicals and propellant materials. The procedure is simple, and with a small amount of practice, one can readily and routinely perform reproducible electrophoretic separations. The necessary materials and equipment are available from commercial sources, or can be "home made."

The electrophoretic separation is accomplished in a column of acrylamide gel consisting of three distinct layers (see frontispiece): (1) the large-pore upper gel which contains the sample; (2) a large-pore spacer gel in which electrophoretic concentration and fraction alignment takes

place; and (3) the small-pore lower gel in which the actual separation occurs. The rest of the arrangement includes an upper and lower buffer reservoir, electrodes for each reservoir, and a constant power supply source. Electrophoresis is performed in a vertical position, with the gel containers attached to the upper reservoir and the lower ends immersed in the buffer solution of the lower reservoir. The electrodes are arranged so that the upper one is the cathode and the lower one the anode. In this manner, the migration of sample ions is toward the small-pore gel in the lower section of the gel column. Current is applied for a specific period of time, after which the gels are rimmed out of the containers and placed in special staining solutions for location of the separated fractions.

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SECTION II

Equipment

(See fig. 1) The buffer reservoirs can be purchased from commercial sources or can be made from inert, nonconductive materials. Round plastic (polystyrene) dishes, 4 to 6 inches in diameter and 2 to 2½ inches deep, or pie-plate covers of similar type plastic material, can be used. These are generally available at local housewares stores.

Holes, approximately ¾-inch in diameter, are made in the bottom of one of the plastic dishes by heating the end of a brass cork borer of the proper size and touching it to the dish. The holes are made at equal intervals along a circumference and equi-distant from the electrode located in the center of the dish. Rubber electrical grommets, 1¼-inch-inside diameter, are then inserted to fit tightly into the holes. The grommets should have holes that fit snugly around the tubular con-

Figure 1

Parts of Equipment for Disc Electrophoresis. Power Supply and support for the upper reservoir not shown.

tainers for the gels. In use, the upper buffer reservoir, fitted with electrode and grommets, is supported above the lower reservoir by means of a tripod or support ring.

The electrodes are made from thoroughly cleaned cylindrical graphite poles removed from flashlight batteries, and are attached at the center of each reservoir by inserting into a flat-top rubber test tube cap (B-D Vacutainer cap) cemented to the bottom of the dish. The leads to the electrodes should be made of insulated wire soldered to the metal caps of the electrodes. Platinum wire electrodes can also be used. A simple way to make a suitable electrode is to wind the platinum wire around a 1¾-inch length of rigid polyethylene tubing with a ¼-inch-outside-diameter. After soldering the electrode leads to the platinum wire, the electrode can be inserted into the flat-top rubber test tube cap cemented to the bottom of the dish.

The tubular containers for the gel columns are made from glass tubing of a 5-mm-inside diameter and cut to 63-mm lengths. The ends of the tubes are square cut but not fire polished, since this may reduce the size of the opening. Sharp edges are removed with an emery cloth or fine-grade sandpaper. While the diameter of the tubes is not critical, all tubes of one set should be cut from the same piece of tubing to eliminate differences in electrical resistance from cylinder to cylinder.

A stand or rack to hold the tubes during filling and polymerization of the gels is made by cementing flat-top rubber test tube caps to a flat piece of wood or plastic. The open end of the cap fits snugly around a tube to prevent leakage of the gel solution.

The power supply source should provide a constant current to a maximum of 200 to 250 milliamperes. Current must be maintained with little or no change when an electrophoretic run is being conducted, to avoid introducing variations in the electrical mobility of the fractions being separated.

SECTION III

Materials

REAGENTS

The reagents in the form of pre-mixed, standardized solutions are available from Canal Industries Co., ("Canalco"), 4935 Cordell Ave., Bethesda, Md. They can also be obtained from Distillation Products Industries, Division of Eastman Kodak Co., Rochester 3, N. Y., and Matheson Coleman and Bell (MCB), 2909 Highland Ave., Norwood (Cincinnati) 12, Ohio, as reagent grade compounds.

1. *Acrylamide (Eastman #5521; MCB #AX330)* ($CH_2CHCONH_2$). This reagent should be kept in a cool, dark place to slow down polymerization. Its shelf life in the crystal form lasts for years.

2. *N,N'-Methylenebisacrylamide (Eastman #8383) ("BIS")* ($CH_2(NHCOCHCH_2)_2$). "BIS" is a crystalline solid that should be stored in a cool, dry, dark place.

3. *2-Amino-2-(hydroxymethyl)-1, 3-propanediol (Eastman #4833) ("TRIS") (Tris Buffer* — Sigma Chemical Co., 3500 DeKalb St., St. Louis 18, Mo.).

4. *N,N,N',N'-Tetramethylethylenediamine (Eastman #8178) ("TEMED") (MCB #TX405).*

5. *Riboflavin (Eastman #5181; MCB #VX 165).*

6. *HCl, reagent grade, 1 N.*

7. *Ammonium persulfate, reagent grade (MCB #AX 1340).*

8. *Glycine (ammonia-free) (Eastman #445; MCB #GX 205).*

9. *Glacial Acetic Acid (Eastman #763).*

10. *Amido Schwarz; also known as Naphthol Blue Black, Aniline Blue Black, C. I. No. 20470 (MCB #AX 1485).*

11. *Bromphenol Blue (Eastman #752; MCB #BX 1415).*

STOCK SOLUTIONS

(The following solutions are identified in a manner similar to that used by Ornstein and Davis.)

(A)	1 N HCl	-	48.00 ml	(B)*	1 M H_3PO_4	-	25.60 ml
	TRIS	-	38.30 grams		TRIS	-	5.70 grams
	TEMED	-	0.23 ml		TEMED	-	0.46 ml
	H_2O to	-	100.00 ml		H_2O to	-	100.00 ml
	(pH 8.9)				(pH 6.7)		

*To make 1 M H_3PO_4, dilute 68.3 ml of 85%, or
67.0 ml of 86% acid to make 1 liter.

(C) Acrylamide - 30.0 grams
BIS - 0.8 grams
H_2O to - 100.0 ml

(D) Acrylamide - 10.0 grams
BIS - 2.5 grams
H_2O to - 100.0 ml

(E) Riboflavin - 4.0 grams
H_2O to - 100.0 ml

The stock solutions are kept in amber glass or plastic bottles at temperatures around 10°C and have a shelf life of several weeks.

WORKING SOLUTIONS

These are also stored in plastic or amber glass bottles. The shelf life is up to several weeks with the exception of Small-Pore Solution #2 which must be prepared weekly.

Small-Pore #1 (15% Gel)
1 Part Stock Solution A
2 Parts " " C
1 Part H_2O
(pH 8.9±.1)

Small-Pore #2
Ammonium Persulfate 0.14 gram
H_2O to 100.00 ml
(Prepare weekly)

Large-Pore Solution (2½% Gel)
1 Part Stock Solution B
2 Parts " " D
1 Part " " E
4 Parts H_2O
(pH 6.7)

Buffer Solution for Reservoirs
TRIS 6.0 grams
Glycerine 28.8 grams
H_2O to 1 liter
(pH 8.3)

Dilute the buffer solution 1 part plus 9 of distilled water for use in the reservoirs. The solution can be used for 24 samples before being discarded. However, do not mix up the solutions once they have been used. If they are to be re-used, they should be kept in separate containers.

Fixative - Stain Solution
Amido Schwarz - 1.0 gram
7% Acetic Acid - 100.0 ml
(by volume)

After filtering, store in a reagent bottle. This solution can be re-used several times.

Bromphenol Blue Solution
Bromphenol Blue 0.01 gram
Dist. H_2O to 1 liter

Wash Solution for Destaining and Storing Gels
Glacial Acetic Acid 70.0 ml
Dist. H_2O to 1 liter

**Polyacrylamide Solution for Destaining*
Acrylamide - 6.00 grams
Riboflavin - 0.50 mgm
TEMED - 0.05 ml
H_2O to - 100.00 ml

*After the ingredients have been completely dissolved, the solution is placed in a 100-ml cylinder and exposed to a fluorescent light with the bulb at a distance of about 3 inches from the cylinder and parallel to it. The riboflavin is generally bleached in about 1½ hours. When this has been accomplished, the solution is mixed with an equal volume of distilled water and stored in a brown glass or plastic bottle.

SECTION IV

Procedure

POLYMERIZING THE GELS

1. Insert the glass tube in which the gels are to be polymerized into the rubber caps on the polymerization stand, carefully setting them in a vertical position. The tubes should be pushed into the holders so that they rest firmly against the base (see fig. 2).

2. Since polymerization rate is affected by temperature, the gel Working Solutions should be permitted to warm to room temperature before using. For each set of 8 gels, mix 5 ml each of Small-Pore Solution No. 1 and No. 2 in a small Erlenmeyer flask or test tube. Blend thoroughly but avoid excessive aeration. Using a pipette, fill the polymerization tubes with this mixture without delay to within ½-inch of their tops.

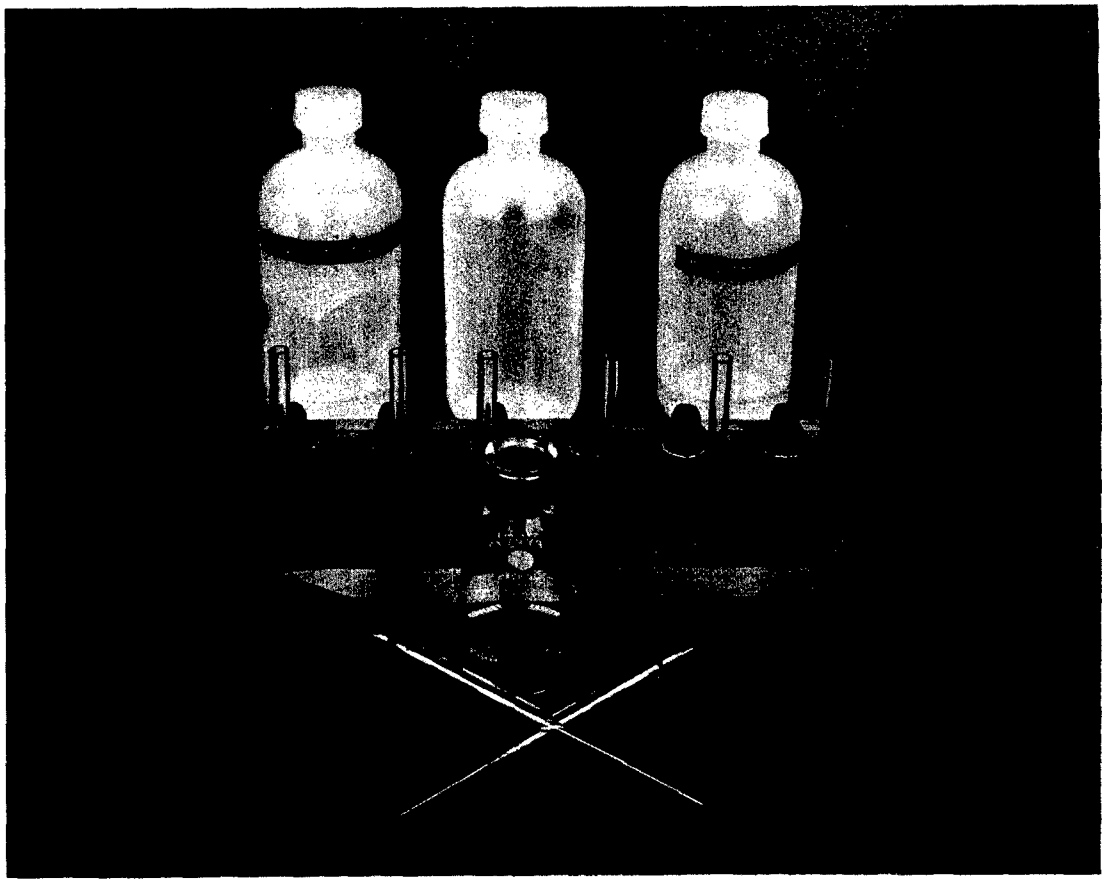


Figure 2

Gel Containers in Place on Polymerization Stand.

3. Very carefully overlay the solution in each tube with a ¼-inch column of distilled water. A 25-gauge needle, ⅝-inch long, attached to a 2-ml syringe and bent about 20° can be used for this purpose. Fill the barrel with enough distilled water so that surface tension prevents formation of a drop at the end of the needle. When the needle is laid against the inside wall of the tube, water will ooze out evenly. Disposable pipettes with rubber bulbs are most practical for this step because the long pipette tips permit reaching to the level of the gel solution to overlay with water. The water should drip down the sides of the tube but should not "bomb" the lower gel. This water overlay should produce a sharp refractile boundary at the interface (see fig. 3). Discard any tube in which swirling or mixing is apparent.

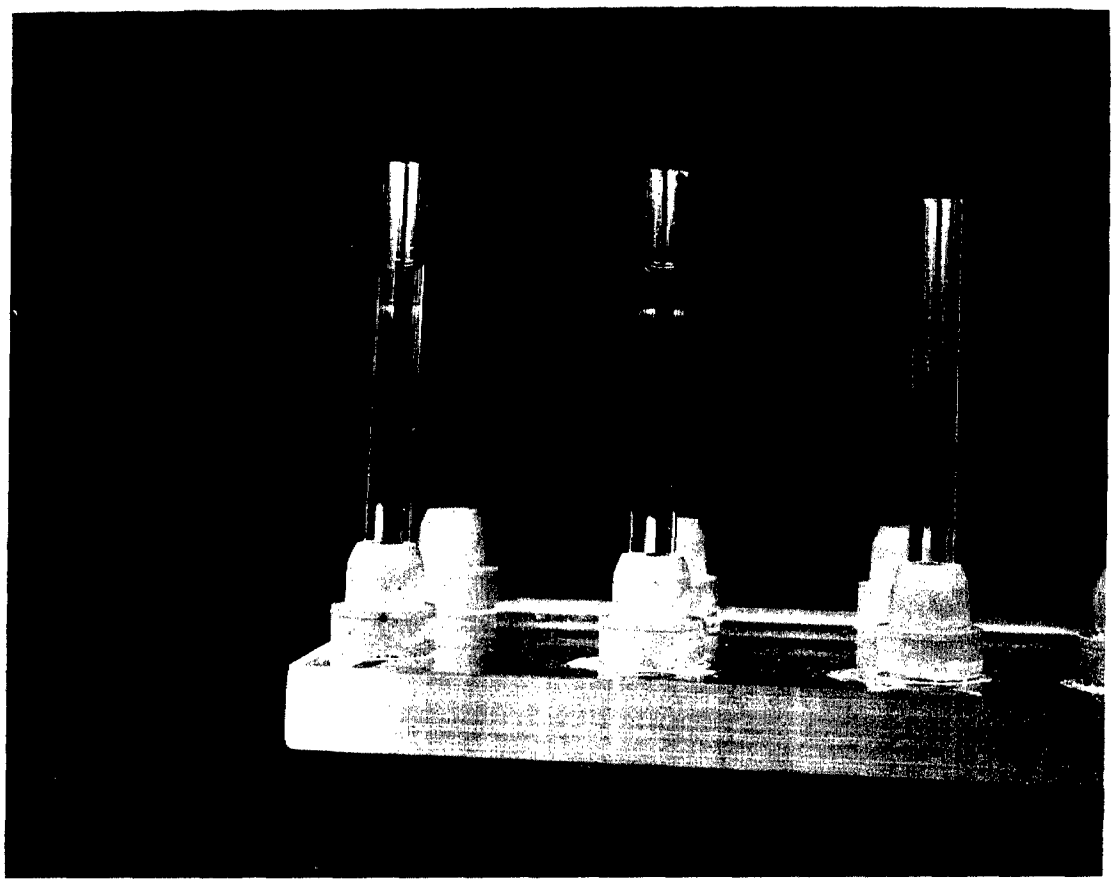


Figure 3

Refractile Line at Boundary of Lower Gel and the Water Overlay.

4. Allow gels to polymerize for about 40 minutes at room temperature. The first refractile line will disappear due to diffusion and a second will appear, about 3 mm below the location of the first, in about 25 minutes. This will mark the junction of the clear gel polymer. At the end of the 40 minutes, remove the water and unreacted polymer solution by inverting and shaking the tubes gently and draining. A disposable pipette with rubber bulb can also be used to aspirate the water and unreacted polymer from the tube.

5. Then, using a pipette, rinse the inside of the tubes gently with the Large-Pore Solution and discard the rinse. Add about ⅜-inch of Large-Pore Solution to each tube and overlay with about ⅛-inch column distilled water.

6. Place the base with the tubes about 3 inches away from a 15-watt flourescent lamp. (This procedure may also be used in step 4.) Care should be taken not to disturb the water-gel interfaces when moving the base to the lamp. The clear solution becomes increasingly opaque after about 5 minutes of exposure (see fig. 4) and total polymerization occurs in about 15 minutes. The polymerized Large-Pore gel forms the "spacer" section of the gel column.

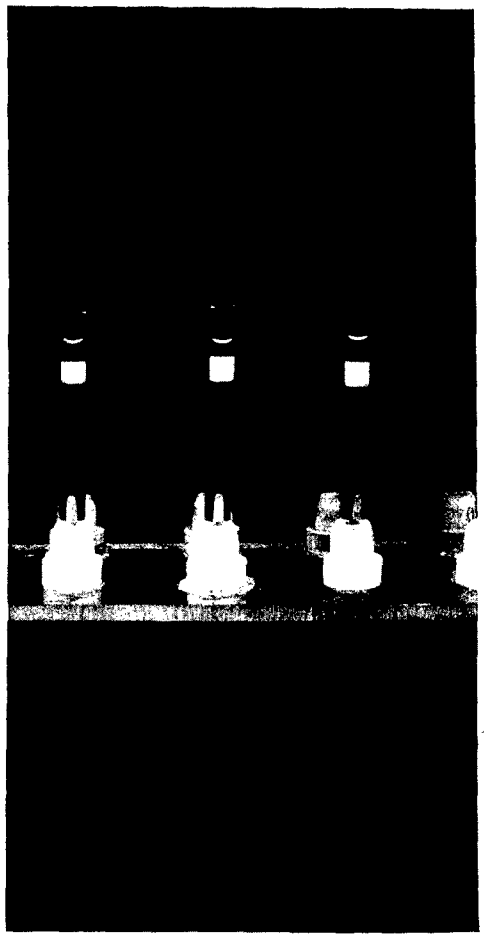


Figure 4

The "Spacer" Gel becomes Opaque. The water overlay can be seen above the opaque section of the column of gel.

7. Decant and shake off the water layer over the "spacer gel." Next, add to each tube a thoroughly but gently mixed solution containing 0.15 ml of Large-Pore Solution and 3 microliters of sample for serum protein separation. A disposable plastic tray of the type used for spot tests is ideal to use for mixing the gel solution and the sample. Hamilton syringes, 10 microliter size, can be used to measure volumes of the sample. After thorough mixing, the resultant solution can be transferred to the tubes by means of a disposable pipette (see fig. 5). No water overlay is required for this step. For lactic dehydrogenase (LDH) isozyme separations, more readily identified fractions are obtained when 6 microliters of sample are used. In the case of rat serum LDH isozyme separations, 15 microliters of serum should be used. Set support stand with tubes in front of the fluorescent lamp for 20 minutes for photopolymerization of the upper gel.

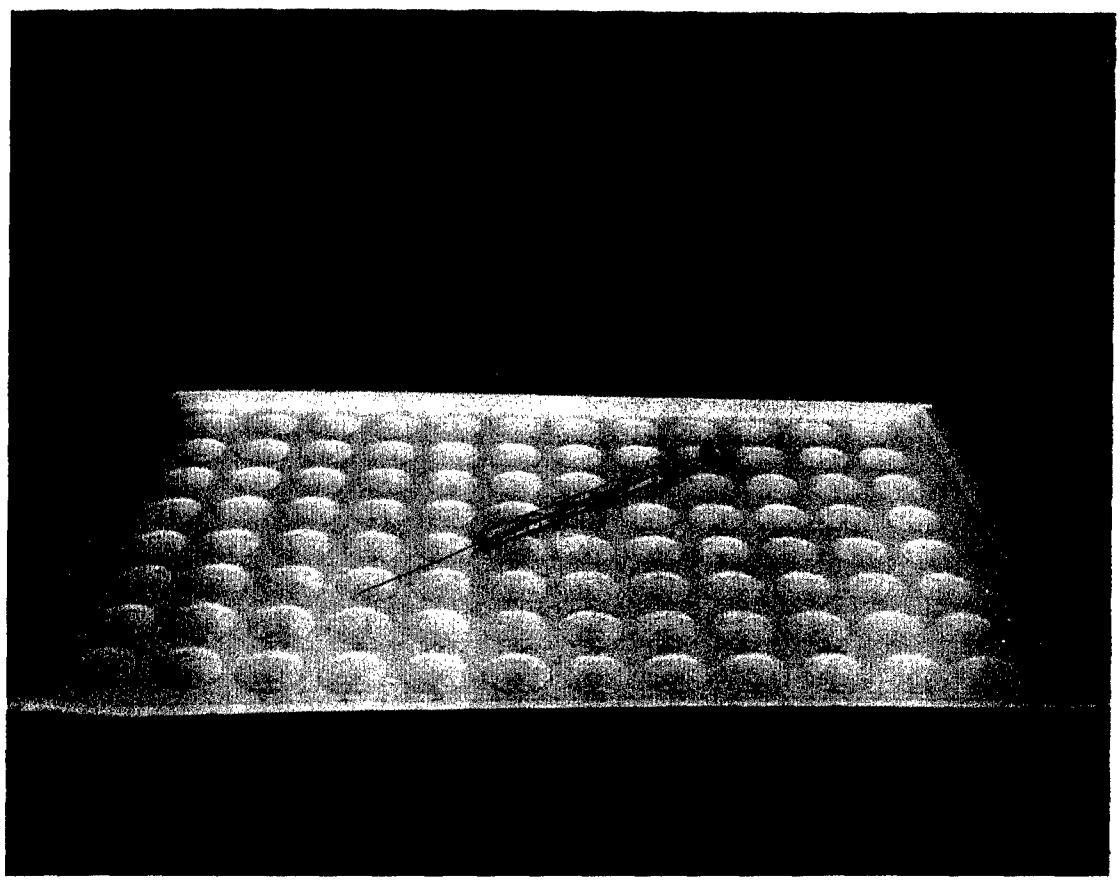


Figure 5

Mixture of Large-Pore Solution and Sample being Transferred to Tubes. The three tubes on the right show complete photopolymerization of the acrylamide gels.

8. When polymerization has been completed (upper gel becomes opaque), remove the tubes from the base by tilting sharply to break any seal that might have been formed (see fig. 6).

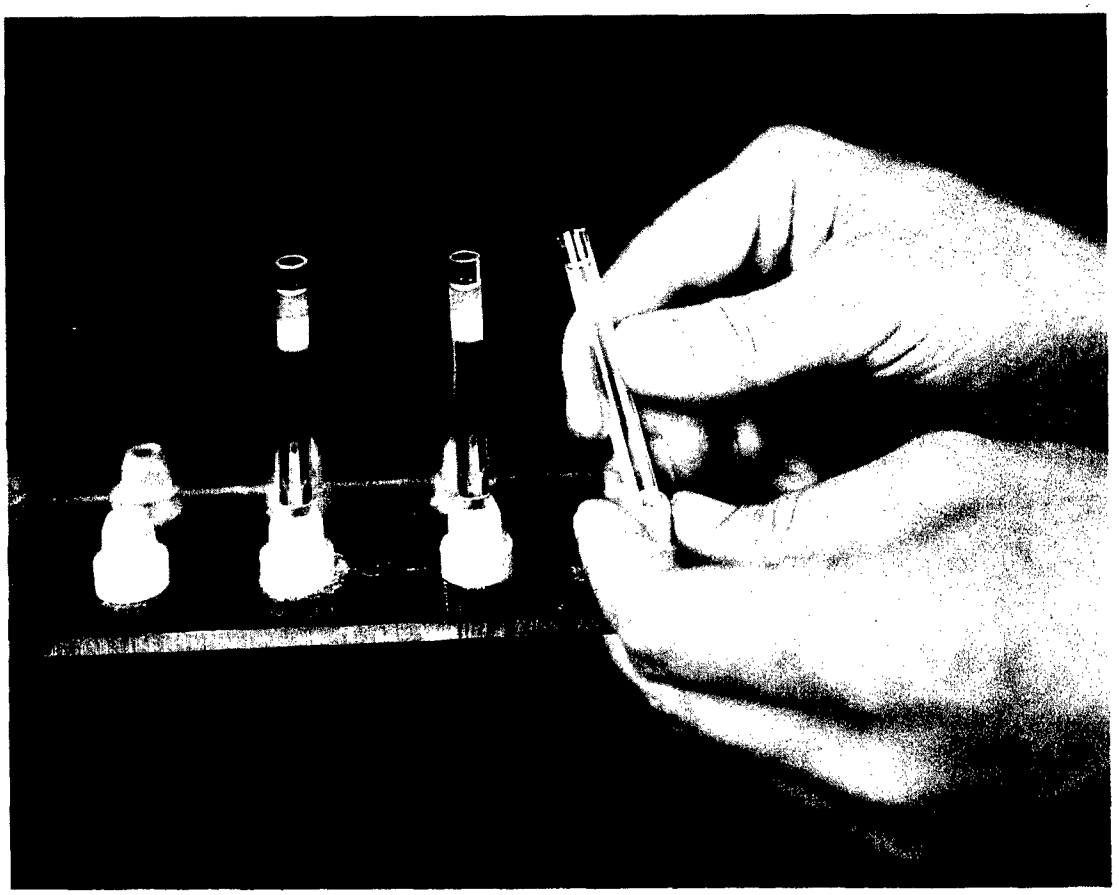


Figure 6

Removing Gel Container from Polymerization Stand.

ELECTROPHORESIS

1. The electrophoresis is started within an hour after the "spacer" gel has been polymerized. Delays beyond this period result in the gel becoming more tightly packed, and variations in the movement of the fractions in the gel column will occur. Fill the lower reservoir with buffer to about ½-inch from the top of the dish.

2. Insert the tubes, upper gel at the top, into the grommets of the upper reservoir (see fig. 7).

3. Next, place a hanging drop of buffer at the bottom end of each tube by means of a pipette to prevent trapping of bubbles.

4. Lower the reservoir so that the bottoms of the tubes are immersed at least ¼-inch in the buffer of the bottom reservoir.

5. Fill the remaining empty spaces above the gel tubes with buffer by means of a pipette and then fill the upper reservoir with enough buffer to reach a level of about one inch from the bottom of the dish.

6. Add a few drops of Bromphenol Blue Solution to the upper reservoir and stir to mix the dye thoroughly. This dye is added to act as an indicator of electrophoretic movement. The dye molecules move faster than the protein fractions which form behind the dye as it passes through the gels.

7. Connect the power supply, cathode to the upper and anode to the lower reservoir (see fig. 8).

a. For serum protein separations, a current of 2½ ma per tube for 45 minutes (or until the dye has moved at least 25 mm into the lower gel) should be used. An exception to this is human sera in which best results are obtained after 25 minutes.

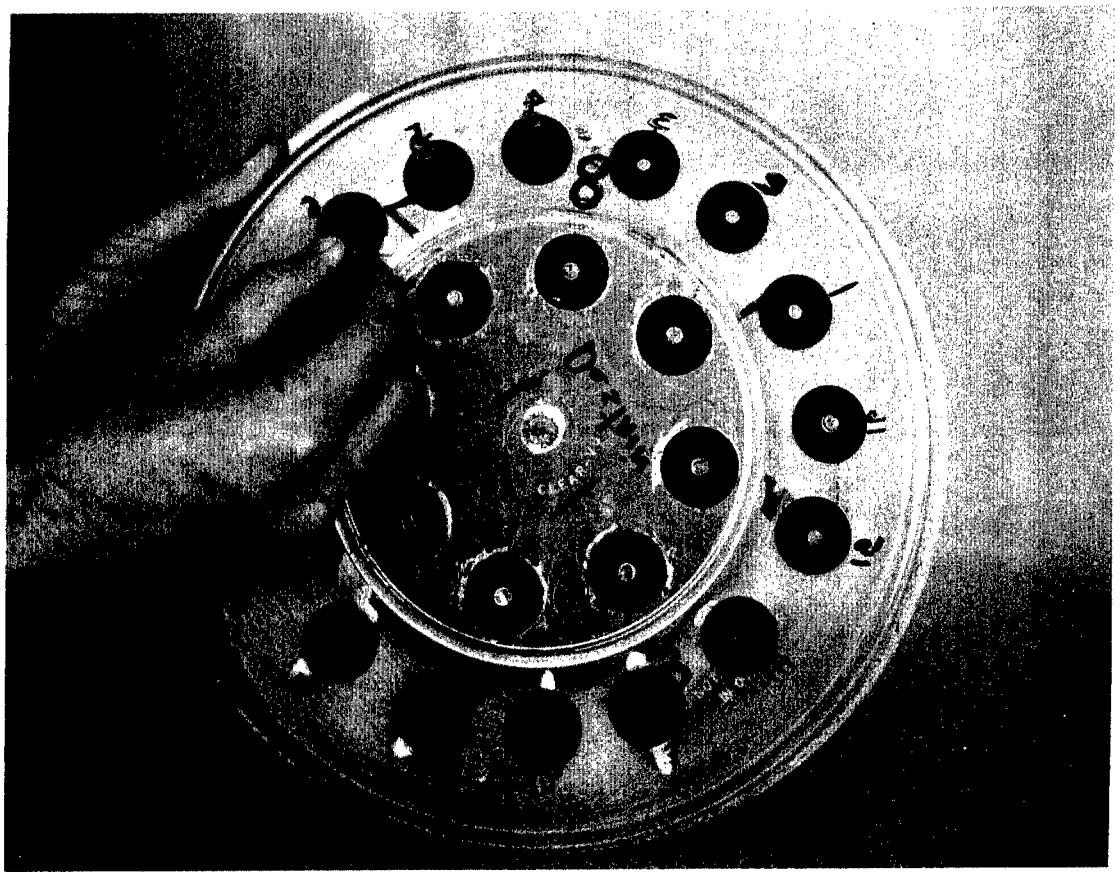


Figure 7

Inserting Tubes into Grommets of Upper Reservoir.

Figure 8

Arrangement of Equipment for Disc Electrophoresis.

b. For lactic dehydrogenase (LDH) isozyme separations, a current of 2½ ma per tube for 60 minutes should be used except in the case of rat serum in which instance 2½ ma per tube for 2½ hours should be used.

Currents higher than 5 ma per tube should be avoided since heating may produce artifacts. When electrophoresis is conducted at low temperatures (1° to 15°C) to reduce enzyme inactivation, the current is reduced to 1 ma per tube and the time is extended to about 2 hours.

8. Within 5 minutes after the current is applied, a thin ring of Bromphenol Blue will be seen migrating into the gels. If it does not appear, the current should be disconnected and the assembly thoroughly checked. The following possible sources of this difficulty should be investigated:

a. Electrodes not properly connected as specified in step 7;

b. Power supply not functioning properly;

Figure 9

First Step in Removal of Gel from Glass Tube following Electrophoretic Run.

c. "Spacer" or sample gel too tightly packed due to being too concentrated or too old; room temperatures above the average range of 22° to 25°C may also accelerate rate and extent of polymerization;

d. Tubes not extending into the lower buffer solution.

Concentration of the separated proteins is completed in the lower end of the spacer gel. The proteins are visible at this point as a thin refractile band just behind the dye band. Then, as they enter the lower (small-pore) gel, the proteins will begin to separate from one another and from the dye. As they separate, the fractions can often be seen as thin refractile bands. The free dye appears bluish-red and will be easily visible. Since the dye is bound by albumin, the deep blue band represents the location of the albumin fraction.

The electrophoresis is performed for the time indicated depending upon the purpose of the separation or until the albumin band has moved about 25 mm into the lower gel. By that time, the free dye will have moved some 6 mm ahead of the albumin band.

9. When the electrophoretic run is completed, turn off the power supply and decant the buffer solution. Remove the gel tubes from the upper reservoir.

10. Next, release the gels from the tubes by rimming under water. A simple method of doing this consists of introducing a teasing needle or wire into the top end of the tube between the gel and the wall of the tube for a distance of several millimeters while rotating the tube (see fig. 9). The needle or wire is pulled out and introduced into the bottom end of the tube in the same manner as just described. As the needle or wire is withdrawn, a slight pressure applied against the gel will bring the gel out about 5 mm beyond the end of the tube (see fig. 10). Now, attach a medicine dropper rubber bulb filled with water on the top end of the tube. As the bulb is squeezed, the hydrostatic pressure is uniformly applied against the gel which is easily forced out without sustaining damage.

STAINING AND DESTAINING GELS — LOCATING PROTEIN FRACTIONS

1. After removal from the containers, place each gel in separate test tubes containing enough Fixative-Stain Solution to cover the gel completely. At the end of ½ hour, the solution is decanted and the gels rinsed for a few minutes in a wash solution of 7% Acetic Acid. The gels can be left overnight in this wash solution if necessary.

2. The excess dye is removed by use of the same apparatus as for the electrophoresis. Destaining tubes are easily made from "disposable" pipettes cut to the appropriate length. The gels, upper gel section at the top, are placed in the destaining tubes which are about 1 mm in diameter larger than the gels. Medicine dropper tubes are also suitable for this purpose. The gels slide down in the tubes and wedge firmly against the constructed ends of the tubes.

3. Add enough 7% Acetic Acid solution to the lower reservoir so that a level about one inch from the bottom of the dish is obtained.

4. Insert the destaining tubes into the grommets of the upper reservoir. To prevent back flow of the free dye up the tube, add Polyacrylamide Solution for Destaining to each tube by means of a pipette. No bubbles should remain in the tube.

5. Place a hanging drop of the 7% Acetic Acid solution at the bottom end of each tube.

6. Now, lower the reservoir so that the bottoms of the tubes are immersed at least ¼-inch in the solution of the bottom reservoir.

Figure 10

Second Step in Removal of Gel from Glass Tube following Electrophoretic Run.

7. Fill the upper reservoir with enough 7% Acetic Acid solution to reach a level about one inch from the bottom of the dish.

8. Connect the electrodes, cathode to the upper reservoir. The unbound dye migrates down the gels and into the lower reservoir. Generally, the destaining is accomplished in about 2 hours using current at the rate of 5 ma per tube.

9. When destaining is completed, turn off the power supply and discard the destaining solution in both reservoirs. Transfer the gels to small test tubes containing 7% Acetic Acid. The gels can be preserved for long periods (at least 2 years) in this solution with negligible loss of dye intensity.

10. Sample Storage Sets (Model A) manufactured by R. P. Cargille Labs, Inc., Cedar Grove, New Jersey, provide a simple and efficient method of storing gels (see fig. 11).

STAINING GELS FOR LDH ISOZYME FRACTIONS

1. After the gels are removed from the containers following the electrophoresis procedure, they should be rinsed in distilled water prior to immersing in the following LDH isozyme staining solution modified in this laboratory from Allen's (U. of Michigan) procedure described in the "Canalco" Information Center Newsletter:

TRIS Buffer (0.05M) (pH 8.3)	11.00 ml
KCN (pH 8.3)	3.00 ml
Sodium Lactate	1.25 ml
Nitro-Blue Tetrazolium (NBT)	7.00 mg
b-Diphosphopyridine Nucleotide (b-DPN)	10.00 mg
Phenazine Methosulfate (2 mg/ml in dist. water)	0.15 ml

In the above solution, all of the ingredients except the Phenazine Methosulfate can be mixed within 15 to 20 minutes of use if kept in a cool, dark place. The Phenazine Methosulfate is light sensitive and breaks down very rapidly. It should be added just before the gels are immersed in the solution.

2. A special tray can be designed to hold each gel in a separate compartment (see fig. 1, upper left corner) or the gels can be placed in separate test tubes. Each gel should be immersed in the solution and incubated at 37°C in the dark for a minimum of 2 hours but not more than 2½ hours. This will assure uniform staining of the isozyme fractions. The fractions will appear as purple-colored bands, equi-distant from each other. The fastest moving fraction, LDH-1, appears in the region of the albumin and $alpha_1$ globulin bands, while the slowest moving fraction, LDH-5, remains near the top of the small-pore gel in the region of the gamma globulins. The other isozyme bands appear in the region between the LDH-1 and LDH-5.

3. LDH-1 is associated with cardiac muscle activity; LDH-2 and LDH-3 with kidney, pancreas, erythrocytes, normal serum and lung; LDH-4 with skeletal muscle and LDH-5 with liver injury or damage. Aberrations from the "normal" isozyme separation pattern can provide infor-

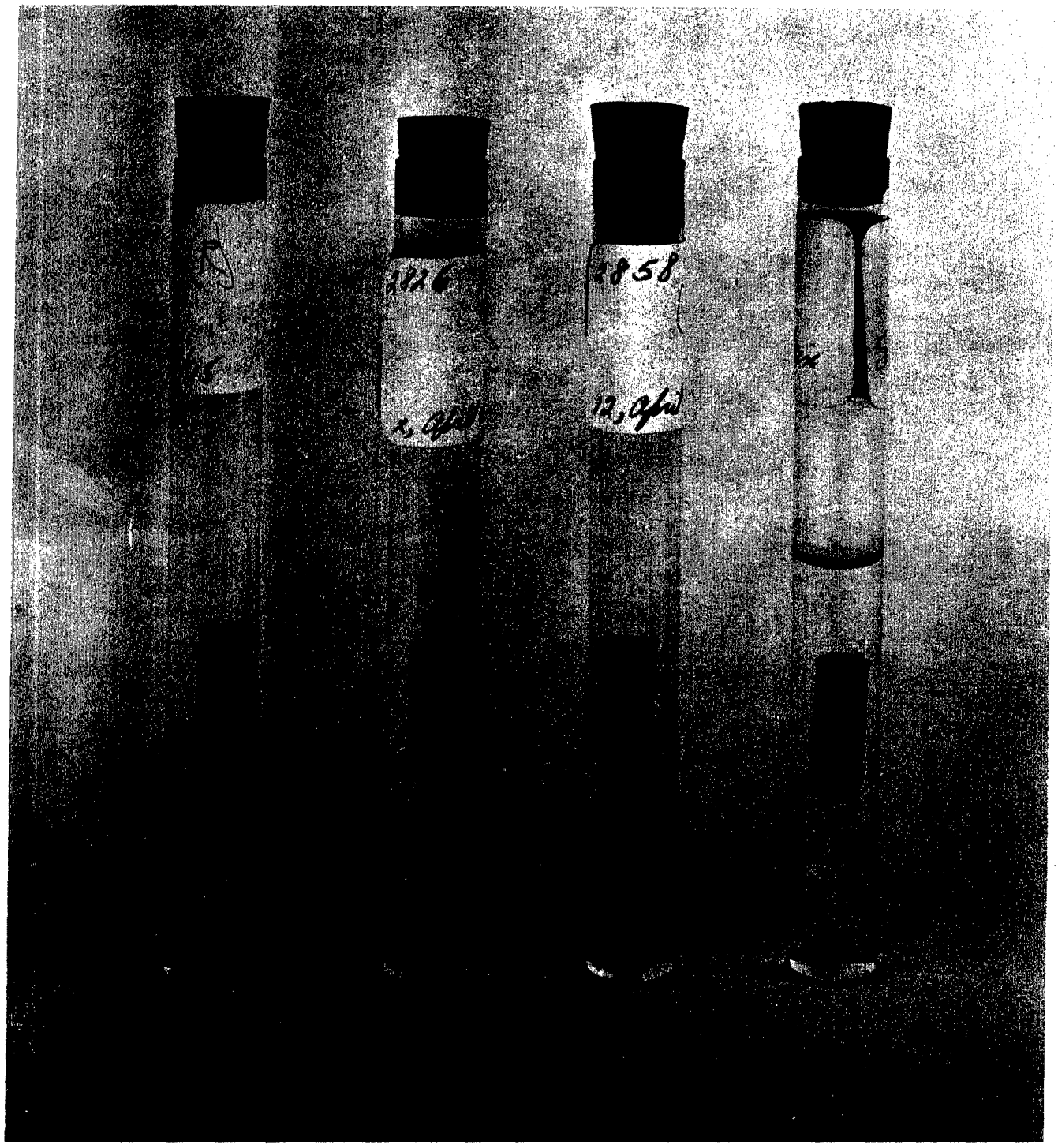


Figure 11

Acrylamide Gels in Storage Tubes. Typical serum protein separation patterns for (l to r): rat, dog, monkey, and human. Only the major fractions are visible in the photograph.

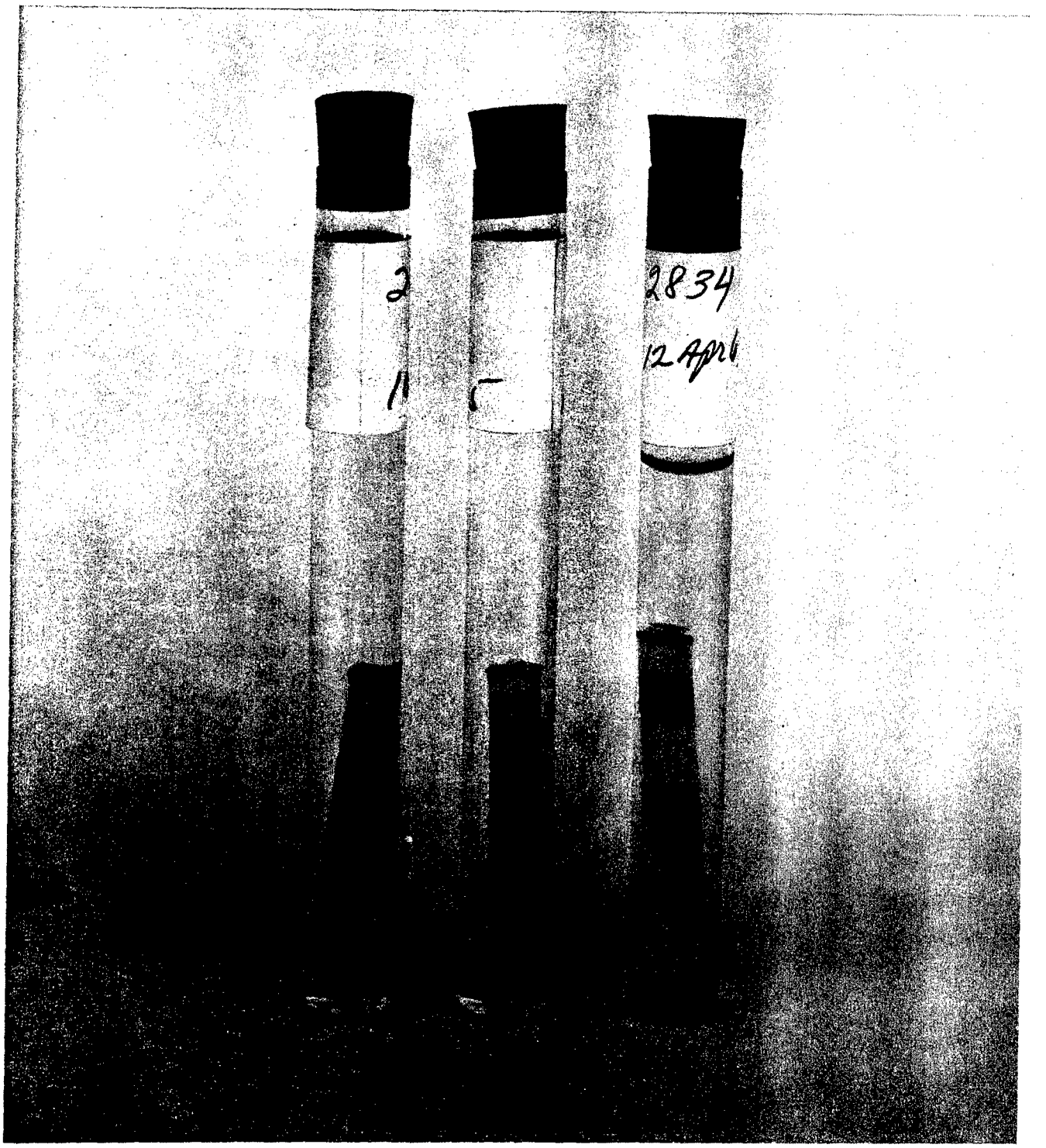


Figure 12

Normal Serum LDH Isozyme Separation Patterns in (l to r): Human, Monkey, and Dog (Beagle). The isozymes visible are LDH-1, LDH-2, and LDH-3.

mation pertaining to specific organ damage and as possible indication of nonspecific chemical stress (see fig. 12).

4. To prepare above individual reagent solutions:

0.05 M TRIS Buffer (pH 8.3):

TRIS	5.98 gms
1 N HCl	25.00 ml
Dist. water to	1 liter

0.06 M KCN (pH 8.3):

KCN	0.39 gms
"Tris" buffer to	100.00 ml

0.5 M Sodium Lactate (pH 8.3):

Sodium Lactate	5.60 gms
"Tris" buffer to	100.00 ml

These stock solutions should be kept in amber glass or plastic bottles at temperatures around 10°C. The Nitro-Blue Tetrazolium (NBT), d-Diphosphopyridine Nucleotide (b-DPN), also known as Cozymase and Coenzyme-1, Sodium Lactate, and Phenazine Methosulfate are available from the following commercial sources:

Sigma Chemical Co.,
3500 DeKalb St.,
St. Louis 18, Mo.

Nutritional Biochemicals Corp.,
21010 Miles Avenue
Cleveland 28, Ohio

The gels can be preserved in 7% Acetic Acid for periods of at least 2 years with little loss of dye intensity.

A. A. THOMAS, M.D.
Chief, Toxic Hazards Division
Biomedical Laboratory

APPENDIX

Helpful Hints in Disc Electrophoresis Methodology

Cleaning glass tubes: Glassware must be cleaned very carefully and thoroughly. Cotton-tipped applicator sticks can be used to remove gel residue and clean the inner walls of the gel containers after immersion in a detergent solution. After any remaining gel material, especially upper gel, is removed from inside the tubes, the tubes are rinsed and immersed in dichromate-sulfuric acid cleaning solution overnight. This step should be followed by a thorough rinse in distilled water, and drying in an oven.

"Curved" bands: These can be produced by pulling the tubes off the base cap too abruptly after the gel has been polymerized and just before electrophoresis without first breaking the seal by permitting air to leak into the cap. Overheating as a result of using too much current may also produce "curved" bands. Overage gel or old and improperly stored serum can also produce "curved" bands.

Sample Concentration: The usual amount of sample (3 microliters in protein fraction separations and 6 microliters for LDH isozyme separations) mentioned in the general instructions sometimes may result in a faint pattern after staining. If this happens, the amount of sample relative to upper gel should be increased until the desired intensity is obtained.

Protein staining reaction: If the protein bands appear blackish rather than blue, it is probably time for fresh stain. The persulfate carries over from the lower gel and after the stain has been used several times, the accumulated persulfate causes degradation of the Amido-Schwarz.

Number of serum bands: Ordinarily, it is possible to separate the proteins in serum into some 20 or more bands. If only a few bands are obtained, the possible causes may be:

1. "Swirling" or mixing of the overlaid water with the lower gel, in other words, "bombing" of the lower gel;
2. Blood not fresh or allowed to clot too long. Serum should be removed from cells and used within 2 hours after the blood has been withdrawn.

Effect of storage and age of serum: In working with over 4000 serum samples, personnel in this laboratory have observed that serum frozen within 2 hours after blood has been drawn and stored can be used for LDH isozyme separation. But once thawed and then re-frozen, the sample may not successfully reproduce the same band intensity as before. Storage itself in the frozen state will result in a gradual loss in band intensity. Some protein bands disappear after 2 hours even if the serum is frozen during the interval before electrophoresis is performed. In reporting electrophoretic data, the length of time sample has been stored and the temperature at which stored should be stated. If the samples are not frozen, the time interval and temperature between time blood is drawn and sample actually used should be noted. Rapid freezing to temperatures –20°C or below and rapid thawing have the least effect on serum samples.

Breaking gels: Sometimes gels break upon removal from the glass tube. This usually occurs at points of heavy protein concentration. The protein-laden gel tends to swell and adhere to the glass walls. To eliminate this, less sample material should be used. It is also helpful to chill the tubes before removal of the gels and to make certain that water is permitted to enter the tube during the rimming operation to act as a lubricant.

Removing gels from glass columns: (Dr. R. Reisfeld, NIH) Attach a blunted 22 to 25 gauge hypodermic needle to a water source by means of rubber tubing. Control the flow of water to a very slow trickle. Rim the gel with this needle from the front end of the gel, to a depth of about ½-inch. The water forces its way between the gel and the glass tubing, lubricating the gel and causing it to slide smoothly out. The needle works equally well on a water-filled syringe, but this is slightly less convenient to handle.

Gel removal: It has been reported in the literature that propylene glycol is an even better lubricant than water for use in removing gels with a hypodermic needle as described above.

Sample data sheets: Figures 13 through 15 are sample data sheets for recording densitometric results of scans of acrylamide gels.

Sample Data Sheet for Recording Densitometric Results of Scans of Acrylamide Gels

RECORD OF ELECTROPHORETIC SEPARATIONS

Animal Number:_____

Termatrex Accession Number:_____

Animal Species:_____

Control — Experimental (Encircle one)

Age of Sample:_____ Temperature at which stored:_____

Sex: Male Female (Encircle one)

Age: Young Adult Old (" ")

Body Fluid:_____

Contaminant:_____

Duration of Exposure:_____

			Percentage Distribution of Activity														
			Proteins								LDH						
Date	Baseline	Experimental	Albumin	a_1-Globulin	a_2-Globulin	b-Globulin	g-Globulin	Lipoproteins	A/G Ratio	Prot. Access. No.	Band 1	Band 2	Band 3	Band 4	Band 5	LDH Access. No.

Figure 13
Sample Data Sheet

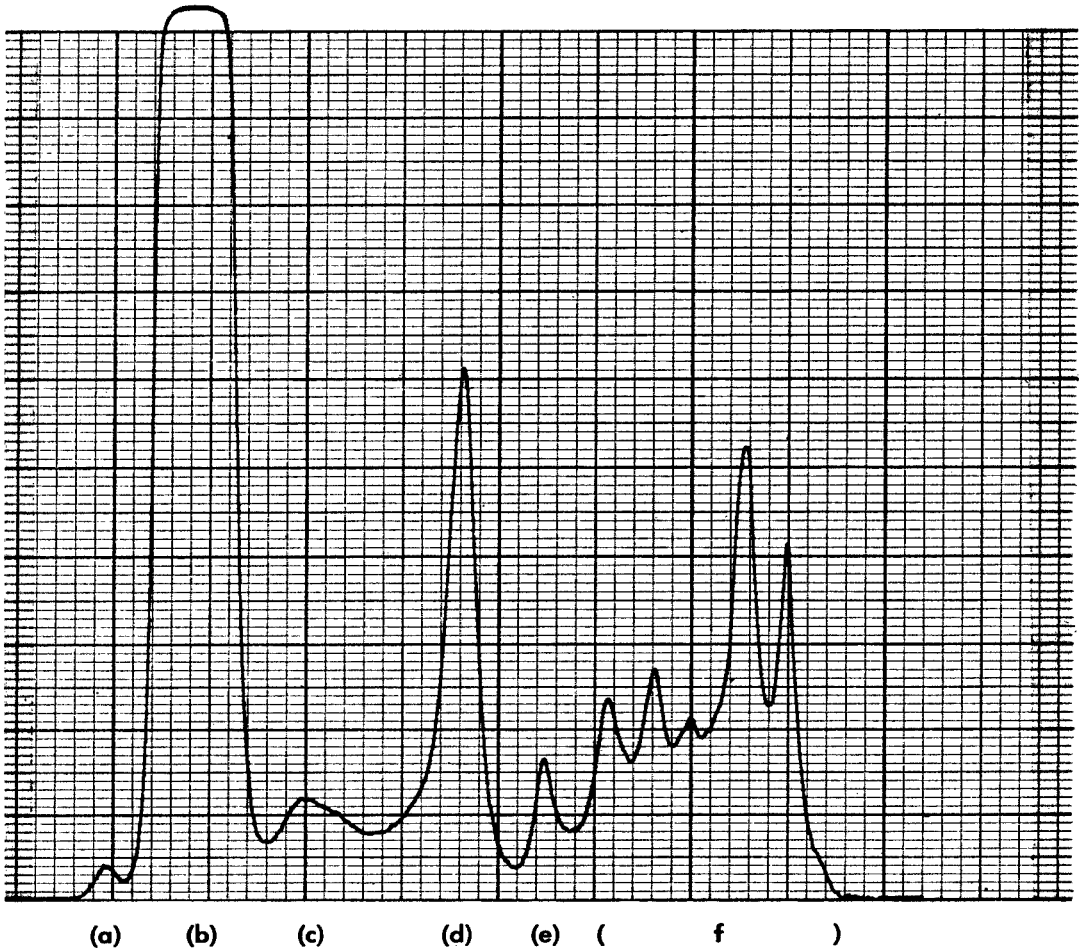
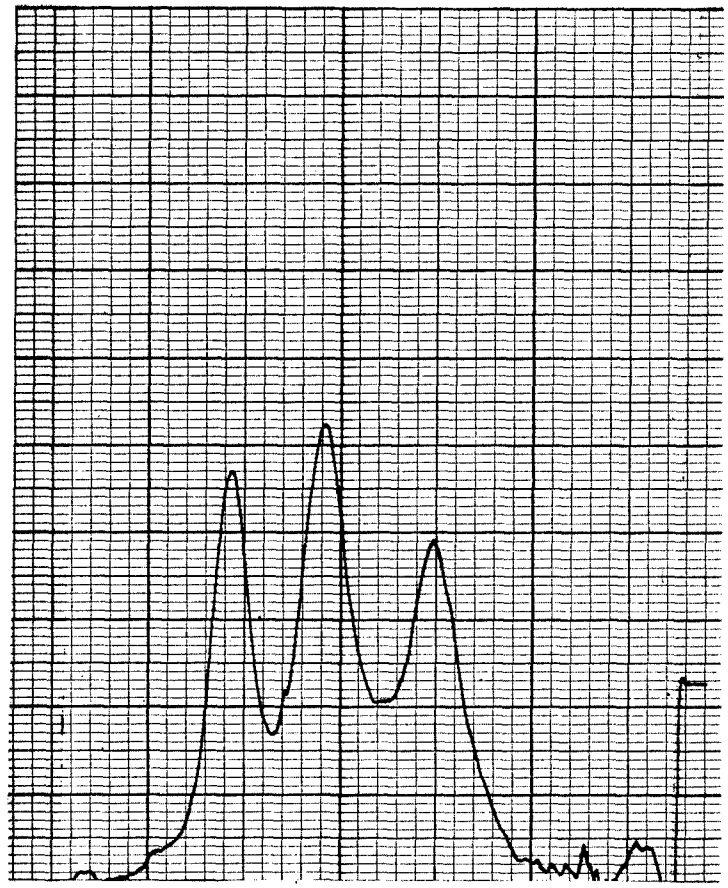


Figure 14

Sample Densitometric Scan of Human Serum Protein Fractions Separated on Acrylamide Gel. Main visible protein fractions are: (a) pre-albumin; (b) albumin; (c) alpha$_1$; (d) alpha$_2$, (e) beta; (f) gamma globulins. Many bands separated on the gel are not detected by the densitometer.



(—1) (—2) (—3)

Figure 15

Sample Densitometric Scan of Human Serum LDH Isozyme Fractions Separated on Acrylamide Gel. The bands observed are (l to r): LDH-1, LDH-2, and LDH-3. (Bands LDH-4 and LDH-5 not present in this sample.)

References

1. Broome, J., "A Rapid Method of Disc Electrophoresis," *Nature,* Vol. 199, No. 4889, p. 179, 1963.

2. Caldwell, Robert C. and Ward Pigman, "Disc Electrophoresis of Human Saliva in Polyacrylamide Gel," *Arch. Biochem & Biophysics,* Vol. 110, p. 91-96, 1965.

3. "Gel Electrophoresis," *Annals of the New York Academy of Sciences,* Vol. 121, Art. 2, 1964.

4. Narayan, K. Ananth, Suhasini Narayan and Fred A. Kummerow, "A Modified Disc Electrophoretic Method for Animal Blood Serum Proteins," *J. Chromatog.,* 16, 187-193, 1964.

5. Ornstein, L. and B. J. Davis, "Gel Electrophoresis," *Annals of the N. Y. Acad. of Sciences,* Vol. 121, Art. 2, p. 321 and p. 404, 1964. Also, "Disc Electrophoresis," preprinted by Distillation Products Industries, Division of Eastman Kodak Co., Rochester 3, N. Y.

6. Reisfeld, R. A., U. J. Lewis and D. E. Williams, "Disc Electrophoresis of Basic Proteins and Peptides on Polyacrylamide Gels," *Nature,* Vol. 195, No. 4838, pp. 281-283, 1962.

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13. ABSTRACT

Disc Electrophoresis, a new method for fractionating serum proteins and enzymes developed by Ornstein and Davis (Mt. Sinai Hospital, New York, N. Y.), possesses great sensitivity, speed and reproducibility while requiring a sample as little as 3 microliters in routine separations. The technique as adapted and standardized for use in the Toxic Hazards Branch, Aerospace Medical Research Laboratories, for serum protein and Lactic Dehydrogenase (LDH) isozyme separations is presented along with suggestions for making the apparatus needed to perform disc electrophoresis.

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Serum proteins						
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