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# Two-Dimensional Gel Electrophoresis of Ribosomal Proteins from Thermophilic and Mesophilic Bacteria

Walter C. Pickett

# TWO-DIMENSIONAL GEL ELECTROPHORESIS OF RIBOSOMAL PROTEINS FROM THERMOPHILIC AND MESOPHILIC BACTERIA

by

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# INTRODUCTION

Since Miquerel's isolation of the first thermophilic bacterium (1), three theories for thermophilic stability have been postulated. The first of these suggests that thermophily results from the protective action of lipids (2). A second theory ascribes thermophily to a special metabolic state involving high rates of synthesis and degradation (3). The third postulates that thermophilic stability may be a result of the physical-chemical properties of important macromolecules of the organism (4). This investigation was undertaken in order to examine the applicability of the latter theory with respect to the physical-chemical differences of the ribosomal proteins of thermophilic and mesophilic bacteria as assessed by comparative gel electrophoresis.

The heterogeneity of the ribosomal proteins was demonstrated by Waller and Harris in 1961 (5). Acid extracts of ribosomes contained a number of protein fractions with distinct electrophoretic mobilities. This finding led to speculation and experimentation as to the role of these proteins in protein synthesis and/or the structural integrity of the ribosome.

Primarily three problems, however, are hampering the formidable task of elucidating the structural and functional roles of these proteins in the ribosome: 1. The difficulty of purifying ribosomes and ribosomal subunits;

2. The existence of relatively few well defined systems of ribosomes; 3. The paucity of effective techniques for separating a large number of closely related proteins.

The first problem includes not only the actual problem of preparing ribosomal fractions but also the development of reasonable criteria to distinguish purified ribosomes from contaminating material. Emphasis has been placed here on the key function of the ribosome, that is its role in protein synthesis. With respect to "in vitro" protein synthesizing systems, ribosomes can be assessed by comparing the protein synthesizing capacity of 70s ribosomes, 30s and 50s subunits and smaller fragments at various stages of purification. Purified ribosomal subunits have enhanced protein analysis considerably as the great number of proteins was drastically reduced. However, the preparation of ribosomal subunits and smaller fragments has led to some criticism (6). Purified ribosomal fractions and subunits are more likely to be artifactual than the intact ribosome.

Related to the purification problem is the second problem that has plagued the analysis of ribosomal proteins. Relatively few well defined systems exist where the preparation of purified ribosomes can be achieved and even fewer where that of ribosomal subunits can be achieved. This has severely limited comparative studies. The ribosomal proteins of the thermophilic bacterium, Bacillus

stearothermophilus, for example, have been compared with ribosomal proteins from Escherichia coli rather than with those from mesophilic species of the genus Bacillus. Such studies cannot differentiate between differences of mesophiles and thermophiles versus intergeneric differences. In general intergeneric differences can obscure subtle differences unique to systems such as these.

The third problem, that of having effective techniques for separating ribosomal proteins, has also impeded progress in this field. Only very recently have advanced electrophoretic techniques been introduced. The traditional free boundary or Tiselius electrophoresis has been replaced by electrophoretic methods that offer separation due to particle size as well as due to charge. Ornstein and Davis' (7,8) introduction of polyacrylamide as an electrophoretic support allows this type of separation and it has been the most successful support in the characterization of ribosomal proteins. The introduction of a discontinuous buffer system ("disc electrophoresis") in conjunction with polyacrylamide electrophoresis was extremely useful in the characterization of the ribosomal proteins from ribosomal subunits or from ribosomal fractions containing only a few proteins. However, the concentrating effect in disc electrophoresis often results in protein aggregation when it is applied to the many proteins from

intact ribosomes. Zonal electrophoresis is, therefore, preferable to disc electrophoresis for the fractionation of proteins from intact ribosomes.

The purpose of this investigation was to design and construct an electrophoresis apparatus capable of comparing precisely the electrophoretic mobilities of complex protein mixtures and to use this apparatus for a comparison of proteins from intact ribosomes of both mesophilic and thermophilic bacteria.

## MATERIALS AND METHODS

### Organisms and Growth Conditions

Two mesophilic species and two thermophilic strains of the genus Bacillus were used in this study. The organisms are listed below:

| MESOPHILES | THERMOPHILES |
|---|---|
| B. licheniformis (NRS 243) | B. stearothermophilus (2184) |
| B. pumilus (NRS 236) | B. stearothermophilus (10) |

The cells were grown earlier (1969) according to the following procedure developed in this laboratory (9) and stored at -20°. The cells were grown in fermentors with a medium consisting of 1% Trypticase and 0.2% yeast extract; the medium also contained 1 ml of antifoam per 25 liters. Mesophilic cells were grown at 37° and thermophilic cells were grown at 55°. The cells were harvested during the logarithmic phase (Absorbance of 1.0 at 540 nm; approximately 2 g wet weight per liter). The culture was quickly chilled and the cells were collected by centrifugation at 30,000 x g using a continuous-flow system. The cells were washed with buffer I frozen in liquid nitrogen, and stored at -20°.

# Chemicals

Cell Growth:

Trypticase - Baltimore Biological Laboratory
Yeast extract - Difco
Antifoam - Union Carbide Corp., SAG-471
Agar - Difco

Ribosome Isolation:

Tris (hydroxymethyl)-aminomethane (Tris) - Sigma
Magnesium acetate - Matheson
Ammonium Chloride - Allied Chemicals
Spermidine trihydrochloride - Nutritional Biochemicals
2-Mercaptoethanol - Eastman Kodak
Sucrose - Mann
Deoxyribonuclease - Sigma

Ribosomal Protein Isolation:

2-chloroethanol - Eastman Kodak

Protein Determination:

Bovine Serum Albumin (BSA) - Sigma
Sodium Carbonate (Anhydrous) - Fisher
Copper Sulfate ($CuSO_4 \cdot 5H_2O$) - Fisher
Phenol reagent - Fisher
Sodium Potassium Tartrate - Fisher

RNA Determination:

Ferric Chloride (Anhydrous) - Fisher
Hydrochloric Acid - Fisher
Orcinol (recrystallized twice from benzene) - Fisher
Ribose - Eastman Kodak

Polyacrylamide Electrophoresis

Urea - Baker
Acrylamide - Eastman Kodak
N,N'-methylene bis-acrylamide (Bis) - Eastman Kodak
EDTA-$Na_2$ - G.F. Smith Chemicals
Tris - Sigma
Riboflavin - Sigma
Boric acid - Sigma

N,N,N',N' - tetramethylethylenediamine (TEMED) - Eastman Kodak
Ammonium peroxidisulfate (Per) - Matheson
Glycine - Sigma
Sodium dodecyl sulfate (SDS) - Sigma
Glycerol - Baker
Amido Black - Sigma

Construction of Apparatus

Plexiglass solvent - Cadillac Plastic

## Reagents

Ribosome Isolation:

A. Buffer I: 0.01 M Tris
0.01 M Magnesium acetate
0.06 M Ammonium chloride
adjusted to pH 7.4 with concentrated Hydrochloric acid

B. Buffer II: Buffer I plus 0.006 M 2-Mercaptoethanol and 0.006 M Spermidine trihydrochloride

C. Buffer TM/4: 0.01 M Tris
0.001 M Magnesium acetate adjusted to pH 7.4 with concentrated Hydrochloric acid

Ribosomal Protein Isolation:

A. Acetic acid method: 67% acetic acid

B. Modified acetic acid method: 67% acetic acid (0.032 M in Magnesium chloride)
0.1 M Magnesium chloride

C. Lithium chloride method: 4 M Lithium chloride

D. 2-Chloroethanol method: 2-Chloroethanol
Rinse Solution: 1 part TM/4 Buffer and 5 parts 2-Chloroethanol 0.06 M in HCl

Protein Determination: 2% Sodium Carbonate in 0.1 N Sodium Hydroxide
1% Copper sulfate
2% Sodium Potassium tartrate
Phenol Reagent, 1 N
Bovine Serum albumin (100 ug/ml)

RNA Determination: 1% Ferric chloride (anhydrous; in concentrated HCl)
Orcinol in ethanol (100 ug/ml)
Ribose (100 ug/ml)

Polyacrylamide Electrophoresis:

A. First Dimension

1. Separation gel, pH 8.6: 36.0 gm urea (36%)
   4.0 gm acrylamide
   0.133 gm Bis
   0.8 gm EDTA-$Na_2$
   3.2 gm boric acid
   4.86 gm Tris
   0.30 ml TEMED

   Distilled water is added to a final volume of 99 ml. The solution is polymerized with 1 ml of 7% Per.

2. Sample gel, pH 8.6: 60.0 gm urea
   4.0 gm acrylamide
   0.2 gm Bis
   0.085 gm EDTA-$Na_2$
   0.32 gm boric acid
   0.06 ml TEMED

   Distilled water is added to a final volume of 100 ml. The solution is polymerized with 100 ul of the following solution: 5 mg riboflavin and 50 mg of Per dissolved in 10 ml of water.

3. Electrode Buffer, pH 8.6: 2880 gm urea
   19.2 gm EDTA-$Na_2$
   76.8 gm boric acid
   116.4 gm Tris

   Distilled water is added to a final volume of 8 liters.

4. Starting Buffer (dialyzing buffer) pH 5.0:
   480.0 gm urea
   0.74 ml glacial acetic acid
   2.4 ml 5 N KOH

   Distilled water is added to a final volume of 10 liters.

B. Second Dimension

1. Separation gel, pH 4.6: 360 gm urea
   90.0 gm acrylamide
   5.0 gm Bis
   52.3 ml glacial acetic acid
   9.6 ml 5 N KOH
   5.8 ml TEMED

   Distilled water is added to a final volume of 970 ml. The solution is polymerized with 33 ml of 10% Per.

2. Electrode Buffer, pH 4.6: 140.0 gm glycine
15.0 ml glacial acetic acid
Distilled water is added to a final volume of 10 liters.

C. Staining Solution: 5.5 mg amido black
50 ml glacial acetic acid
Distilled water is added to a final volume of 1 liter.

## Apparatus

A. Fermentor: New Brunswick Scientific Co., model MF-128 S

B. French Press: Aminco, model 5-590

C. Homogenizer: Potter-Elvehjem

D. Centrifuges: High speed - Spinco, model L preparative ultracentrifuge
Analytical Ultracentrifugation - Spinco, model E analytical ultracentrifuge

E. Microliter pipettes: Eppendorf (Brinkmann)

F. Spectrophotometer: Zeiss, model PMQ-II

G. Lyophylizer - Virtis

H. Ultraviolet Lamp: Minerva, model R-51

I. Pump: Little Giant, model CP-5000

## Isolation and Purification of Ribosomes

Ribosomes from the four organisms were isolated according to the procedure of Stenesh and Schechter (9). All operations were carried out at 4° or in crushed ice.

The frozen cells (partially dehydrated) were reconstituted by gentle suspension in approximately 2 volumes of cold buffer I per weight of cells. The cells were collected by centrifugation at 12,000 x g for 20 minutes and suspended with gentle stirring in 2 volumes of buffer II per weight of cells.

When the cells were evenly suspended in the buffer, the solution was poured into a prechilled French pressure cell. The pressure was raised to 18,000 psi in approximately one minute and maintained at that level by a slight and continuous opening of the outlet valve. The disrupted cells were released at a rate of 10 ml per minute into a container surrounded by crushed ice. Deoxyribonuclease (200 ug/25 g of cells) was added and, after 5 minutes, the mixture was centrifuged at 30,000 x g for 30 minutes. The supernatant was removed with a pipette to within 1 cm above the pellet of cellular debris and was centrifuged at 105,000 x g for 2 hours. The resulting supernatant was discarded and the pellet was rinsed with Buffer II. The pellet represents the crude ribosomal preparation and appears as a reddish-brown gel varying slightly in appearance from

organism to organism.

The pellet was suspended in 25% the original volume of Buffer II by gentle homogenization in a Potter Elvehjem Homogenizer. The crude ribosomes were further purified by a low speed centrifugation (5 minutes at 10,000 x g) and a high speed centrifugation (2 hours at 105,000 x g). The resulting pellet is rinsed with Buffer II, suspended in 10% of the original volume of Buffer II to yield the final ribosome preparation which was frozen in liquid nitrogen and stored at -20°.

An attempt was made to purify the ribosomes by centrifugation through a sucrose solution. For this purpose, 1 ml of ribosomes was carefully layered over 30 ml of 30% (w/w) sucrose in Buffer II, centrifuged for 8 hours at 25,000 rev/min (Spinco, SW 25.1 rotor), and the pellet suspended in Buffer II. These ribosomes were compared with those prepared in the usual manner by means of analytical ultracentrifugation. It was found that sedimentation decreased the yield of ribosomes and did not decrease the amount of impurities. Hence, the sucrose purification step was omitted from subsequent preparations.

## Preparation of Ribosomal Proteins

Ribosomal proteins were prepared according to the method of Fogel and Sypherd (10) developed for E. coli. The ribosomes were thawed and dialyzed at 4° against 500 volumes of cold TM/4 buffer for at least 24 hours with five changes of buffer.

Using a prechilled glass homogenizer as a mixing vessel, five volumes of cold 2-chloroethanol were added dropwise with gentle stirring (Vortex mixer) for every volume of dialyzed ribosomes. Concentrated HCl was added dropwise with gentle stirring (Vortex mixer) to a final concentration of 0.06 N. The homogenizer was placed in crushed ice and the solution homogenized at fifteen minute intervals for at least 2 hours. The solution was transferred quantitatively to a centrifuge tube with Rinse Solution and the precipitated RNA was removed by centrifugation at 5,000 x g for 10 minutes, and the supernatant was carefully poured into a volumetric flask (50 ml for 3 ml of original ribosomes). The RNA pellet was resuspended in a volume of Rinse Solution equal to 30% of the original volume of ribosomes and chloroethanol. The RNA was again removed by centrifugation at 5,000 x g for 10 minutes and the supernatant was pooled with the first supernatant. The RNA pellet was rinsed with Rinse Solution which was also

pooled. The supernatant rinsings were brought to volume with rinse solution. Aliquots of this solution (2 ml) were used for protein determination in order to calculate the yield of ribosomal protein.

It is important to remove the aliquot for protein assay at this point, rather than later, since ribosomal proteins are soluble in chloroethanol but are largely insoluble when the chloroethanol is removed by dialysis.

The aliquots and the bulk of the chloroethanol solution were placed in dialysis bags and dialyzed against at least 80 volume of distilled water to remove the chloroethanol. After 3 days of dialysis, with 6 changes of water, the bulk of ribosomal protein is lyophilized and stored at -20°. The dialyzed aliquots (2 ml) were transferred quantitatively to small volumetric flasks (25 ml). The proteins are dissolved by the addition of NaOH (2 mmoles), the solution made up to volume and used for protein determination by the method of Lowry, et al. (11).

## Protein Determination

Protein was determined spectrophotometrically according to the method of Lowry, et al. (11) with BSA as a standard.

Equal volumes of 2% Na, K-tartrate and 0.1% $CuSO_4 \cdot 5H_2O$ were combined. To one ml of this solution were added 50 ml of 2% $Na_2CO_3$. Five ml of this reagent were mixed with 1.0 ml of the sample and allowed to stand at room temperature. After 10 minutes, 0.5 ml of 1 N Folin-Ciocalteau reagent was added with immediate mixing on a Vortex mixer. The absorbance was measured after 30 minutes at 750 nm versus a blank.

## RNA Determination

RNA was determined spectrophotometrically using the orcinol method (12). To the sample (3 ml) were added 3 ml of 0.1% $FeCl_3$ in HCl and 0.3 ml of the orcinol solution (100 ug/ml of 95% ethanol). The tubes were heated for 40 minutes in boiling water, cooled to room temperature and the absorbance was measured at 700 and 580 nm versus a blank. The absorbance at 580 nm was subtracted from that at 700 nm to minimize absorbance due to glucose.

# RESULTS AND DISCUSSION

## Construction of Electrophoresis Apparatus

### General Comments

The design and construction of the zonal polyacrylamide gel electrophoresis apparatus, adopted in part from Kaltschmidt and Wittman (13), is detailed in the following photographs. The primary aim of this design is to provide an electrophoresis apparatus capable of high resolution and suitable for precise comparison of multiple protein samples. High resolution is achieved by the use of two polyacrylamide concentrations and buffers of both acidic and basic pH. Precise comparisons are achieved by simultaneous electrophoresis of different samples using identical buffers and gel systems. Up to five different samples can be analyzed simultaneously in this apparatus.

The quality of resolution and comparison also depends on three fundamental electrophoretic properties and associated factors. These properties are the electric field strength, the frictional force of the medium, and the electrical charge of the particle. These are interrelated since, in electrophoresis, a charged particle is accelerated in an electric field until the electric force equals the frictional force of the particle's environment (14), at which point the particle maintains a constant velocity.

In order to control these factors as much as possible, the following features were incorporated into the design of the apparatus.

To assure a uniform electric field strength and minimal ohmic heating in the one dimensional (1D) apparatus, the electrode buffer is circulated and mixed in a neutral vessel. In the two dimensional (2D) apparatus where the buffer volume is much larger, a uniform electric field is maintained by uniform placement of the elongated electrodes. Ohmic heating is reduced in the second dimension apparatus by constructing buffer compartments in direct contact with the gel surface. Any fluctuations in the electric field were, of course, compensated for by simultaneous electrophoretic runs in both the first and the second dimensions.

Since the frictional force of a viscous medium such as polyacrylamide is undefined (14), care was taken to use identical gel solutions in all phases of this procedure. Time and temperature of polymerization were standardized to assure uniform structural characteristics of the polymer.

Differences in particle charge other than those related to the inherent structural differences were reduced by having buffer volumes that were either larger and/or efficiently circulated as well as by the uniformity of the electric field, the use of the identical gel, and the simultaneous electrophoresis of different samples.

The apparatus is constructed almost entirely of plexiglass. All edges are finely milled to provide water tight seals for the gel support compartments. Plexiglass solvent or chloroform is used for permanent seals.

One-dimensional apparatus

Figure 1. Intact one-dimensional apparatus.

| Code | Part | Material & Dimensions | Comments |
|---|---|---|---|
| 1. | Upper buffer container and tube support | Plexiglass | |
| 2. | Anode | Stainless steel | |
| 3. | Lower buffer container | Plexiglass | |
| 4. | Cathode | Stainless steel | |
| 5. | Tubes | | 3 cm from support plate |
| 6. | Upper buffer support | Plexiglass | |
| 7. | Circulation tubing | Tygon | circulation direction indicated |
| 8. | Circulation control valve | | |
| 9. | Mixing vessel | 4 liter | |
| 10. | Pump | | |

Figure 1. Intact one-dimensional apparatus

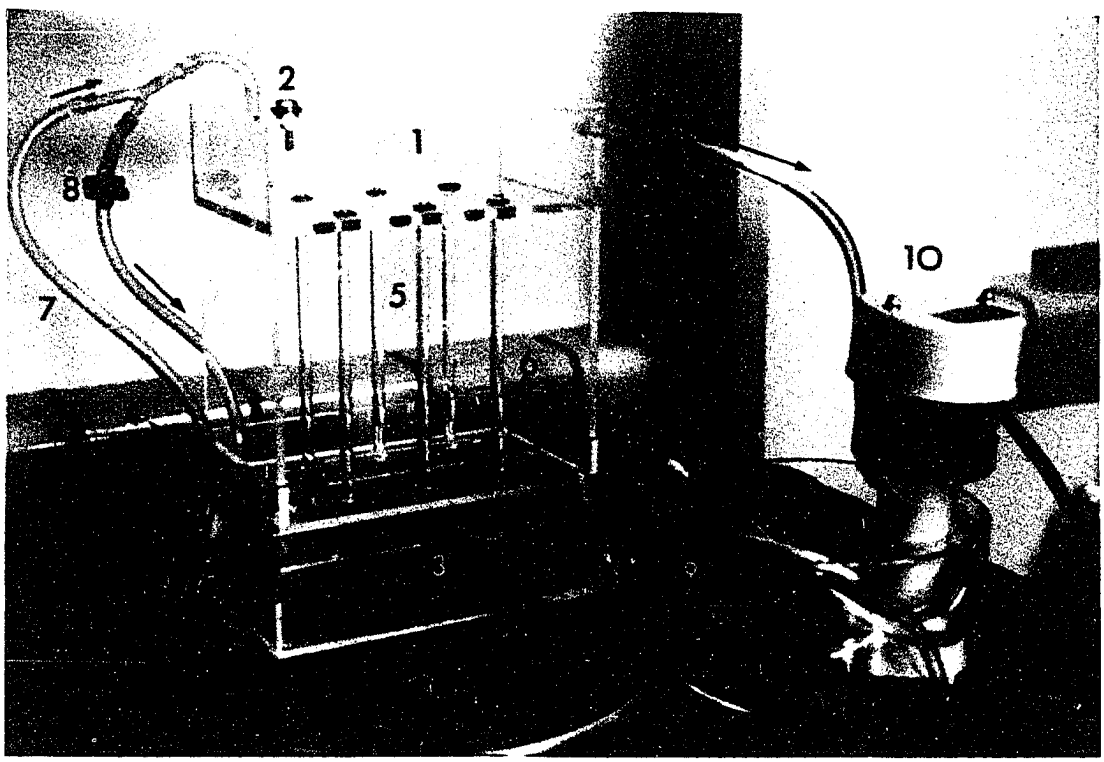


Figure 2. Acrylamide loading apparatus

| Code | Part | Material & Dimensions | Comments |
|---|---|---|---|
| 1. | Loading rack | | Obtained from Canalco |
| 1a. | Movable lock | | |
| 2. | Electrophoresis tubes | soft glass 180 x 6 mm i.d. | Outer edges fire polished |
| 3. | Loading caps | | Vacutainer caps |

Figure 2. Acrylamide loading apparatus

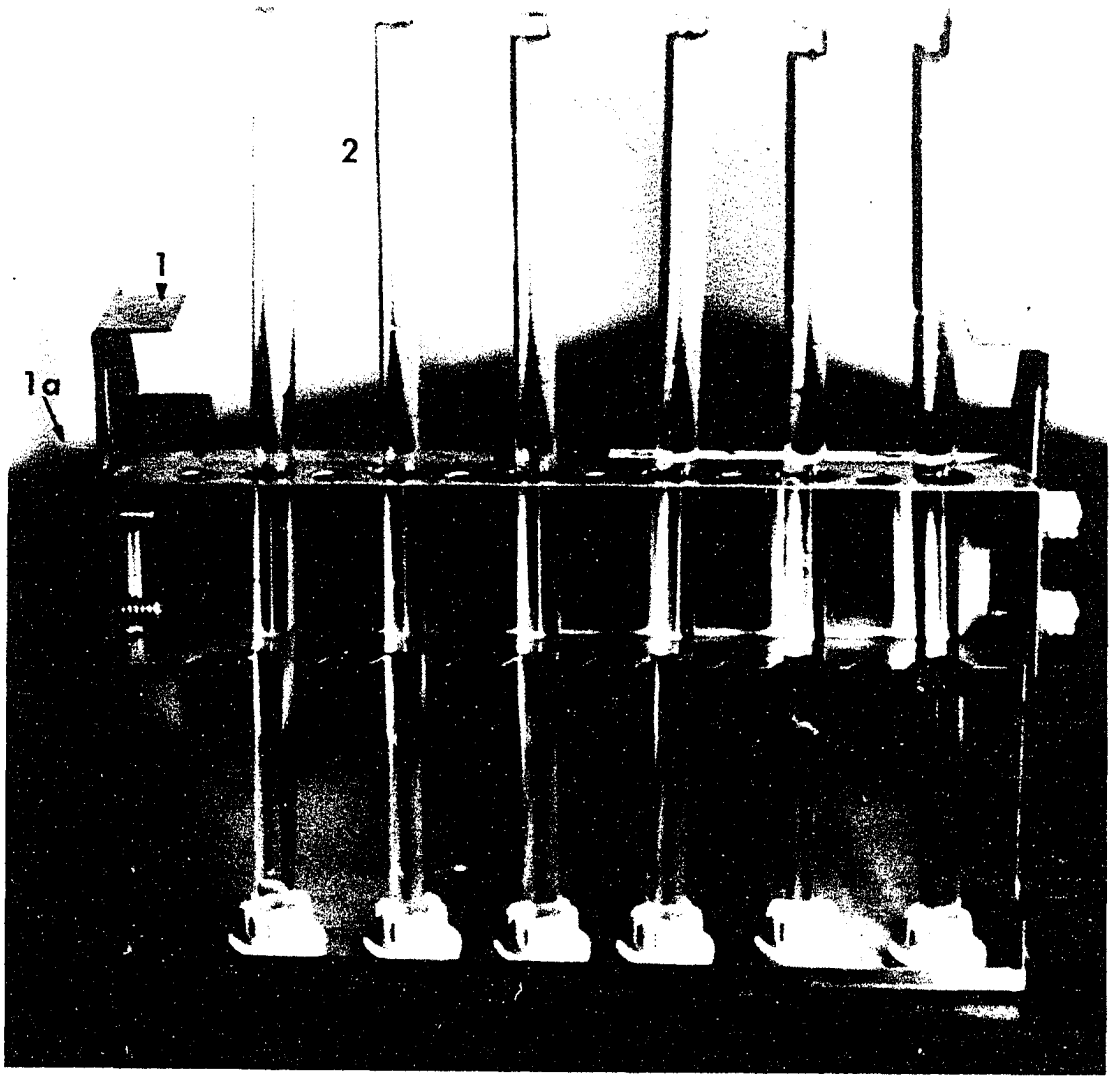


Figure 3. One-dimensional lower buffer container

| Code | Part | Material & Dimensions | Comments |
|---|---|---|---|
| 1. | End pieces | Plexiglass 5 x 4¼ x ¼" | |
| 2. | Bottom piece | Plexiglass 8¼ x 5 x ¼" | |
| 3. | Side pieces | Plexiglass 4¼ x 9 x ¼" | |
| 4. | Upper buffer support plate | Plexiglass 4¼ x 8½ x ¼" | |
| 5. | Support plate support | Plexiglass 4¼ x ½ x ¼" | |
| 6. | Support plate handle | Plexiglass 5 x ½ x ¼" | |
| 7. | Electrode (cathode) | Stainless steel ¼" bolt | |
| 8. | Electrode (wire) | Nichrome 29 guage | |
| 9. | Buffer circulation holes | | |
| 10. | Lower buffer exit to mixing vessel | | Exit above support plate |
| 11. | Buffer entrance from mixing vessel | | Enters beneath support plate |

Figure 3. One-dimensional lower buffer container

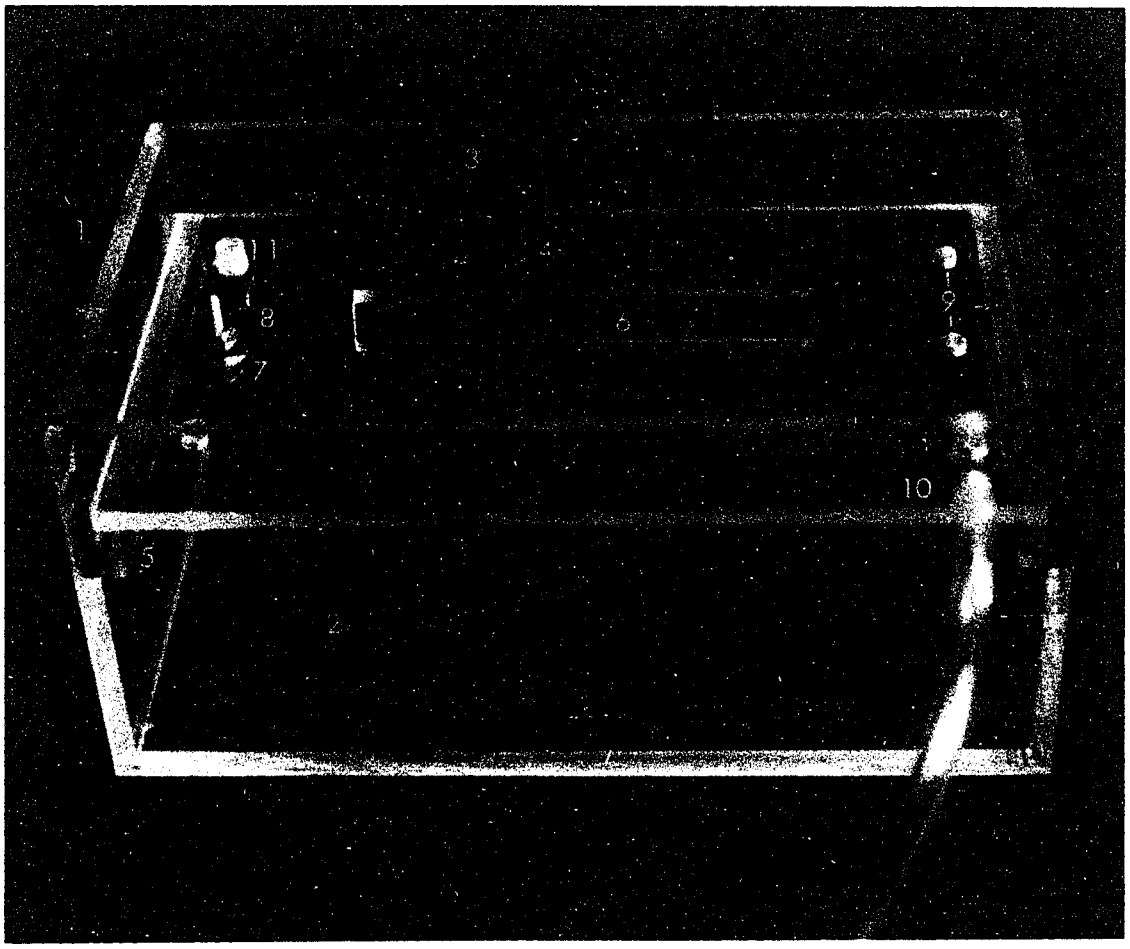


Figure 4. One-dimensional upper buffer container

| Code | Part | Material & Dimensions | Comments |
|---|---|---|---|
| 1. | End pieces | Plexiglass 5 x 9⅛ x ⅛" | |
| 2. | Side pieces | Plexiglass 8.75 x 2½ x ½" | Holes milled to support grummet |
| 3. | Bottom piece | Plexiglass 8½ x 4⅛ x ⅛" | |
| 4. | Rubber grummets | Rubber 6 mm i.d. | |
| 5. | Top plate | Plexiglass 8.75 x 4.75 x .125" | |
| 6. | Buffer entrance spout | Plexiglass | Hollowed from solid plexiglass rod |
| 7. | Buffer exit spout | Plexiglass | |

Figure 4. One-dimensional upper buffer container

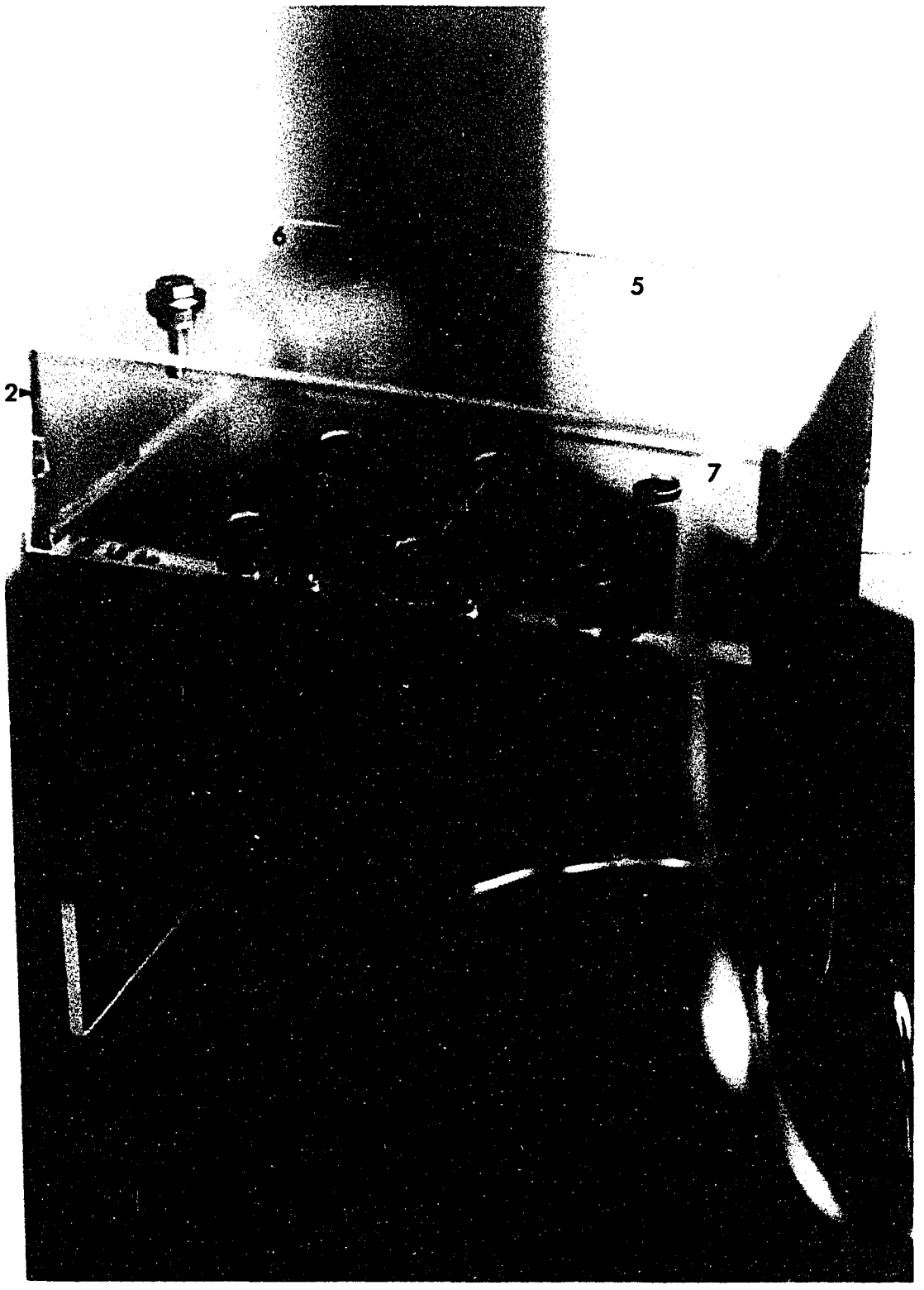


Figure 5. Hydrostatic gel removing system

| Code | Part | Material & Dimensions | Comments |
|---|---|---|---|
| 1. | 50 ml syringe | glass | |
| 2. | Rubber tube | | Wired to syringe |
| 3. | One dimensional gel support | styrofoam | |

Figure 5. Hydrostatic gel removing system

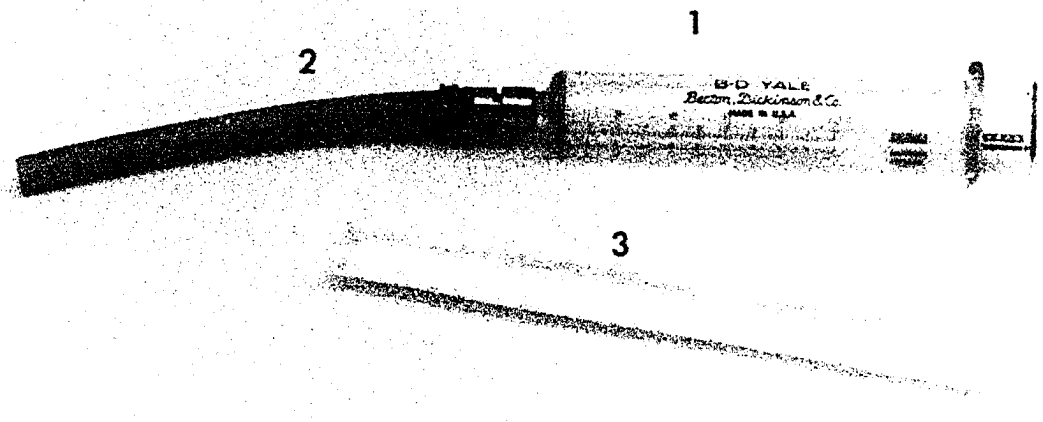


Figure 6. One-dimensional gel and resting plate

| Code | Part | Material & Dimensions | Comments |
|---|---|---|---|
| | Plate | Plexiglass 3/8" diameter | Milled into plate |

Figure 7. One-dimensional staining, destaining and dialysis rack

| Code | Part | Material & Dimensions | Comments |
|---|---|---|---|
| 1. | Rack frame | Plexiglass aluminum screen 10.625 x 9" | Screen melted into plexiglass |
| 2. | Rack frame (bottom) | Plexiglass aluminum screen 10.625 x 9" | |
| 3. | Gel dividers | Plexiglass 8 x ½ x ¼" | |

Figure 6. One-dimensional gel and resting plate

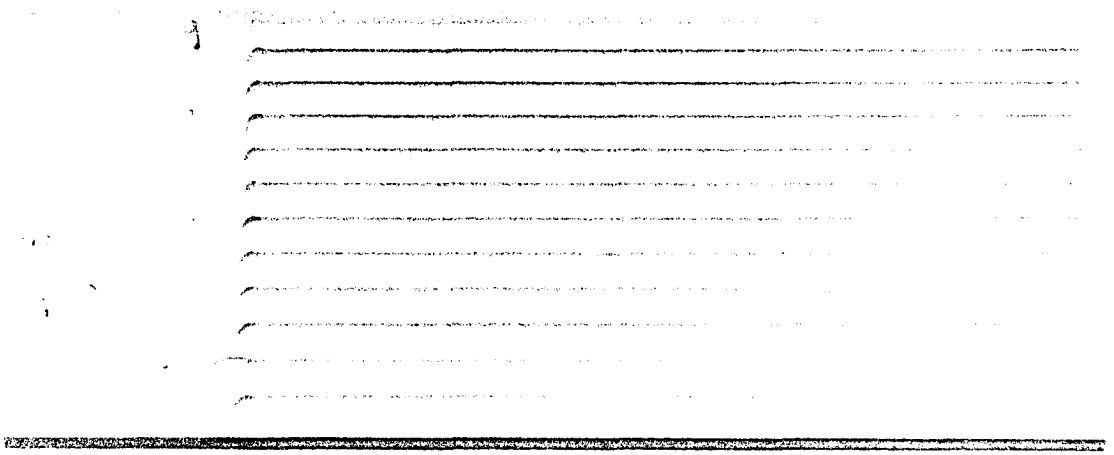
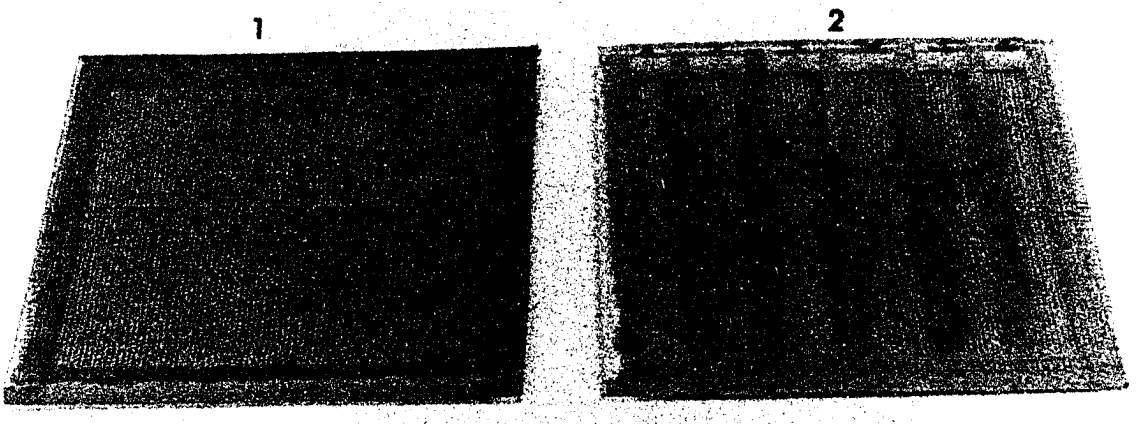


Figure 7. One-dimensional staining, destaining and dialysis rack



Two-dimensional apparatus

Figure 8. Intact two-dimensional electrophoresis apparatus

| Code | Part |
|---|---|
| 1. | Upper buffer & gel support |
| 2. | Lower buffer container |
| 3. | Top electrode plate |
| 4. | Power source attachment |

Figure 8. Intact two-dimensional electrophoresis apparatus

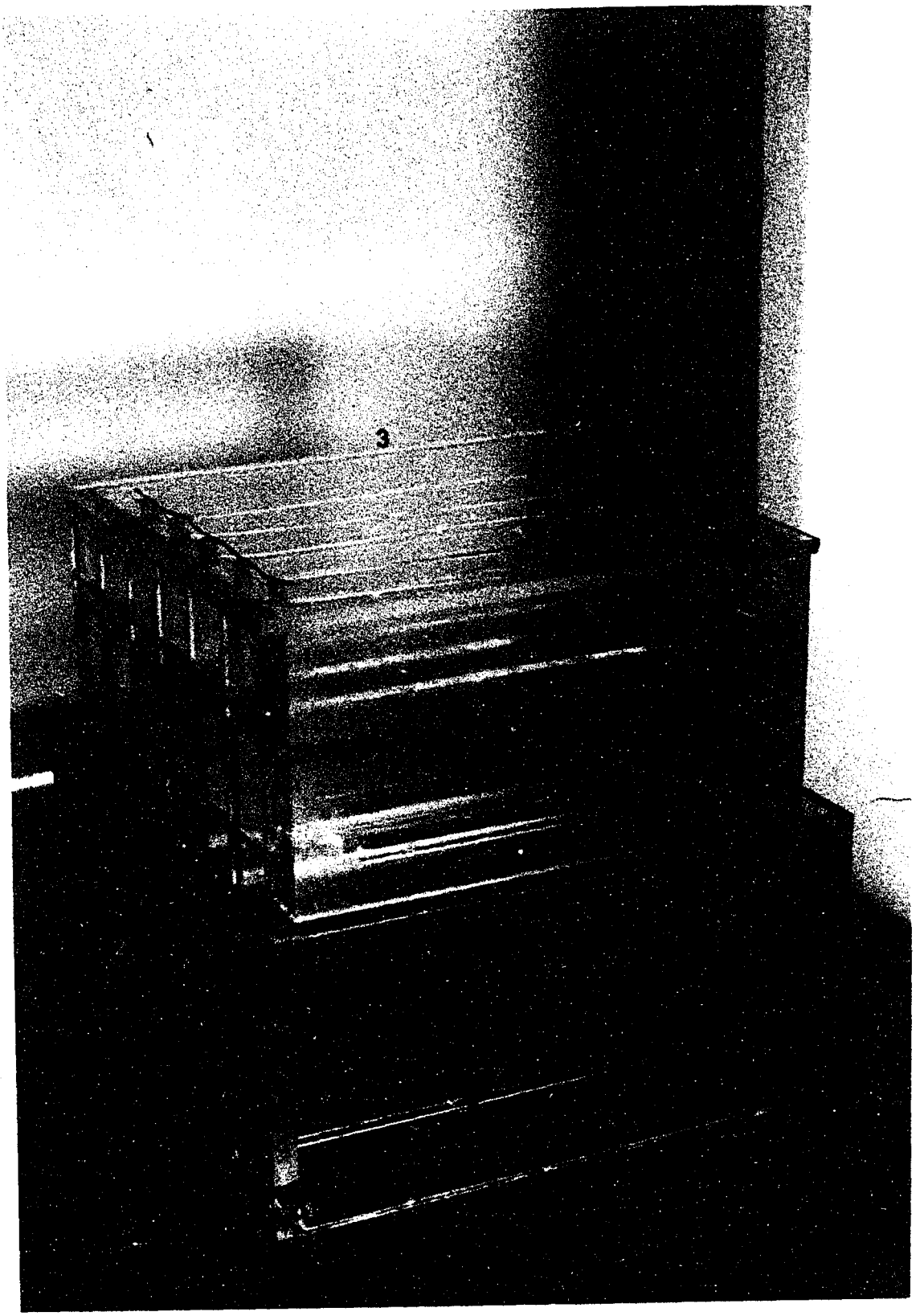


Figure 9. Two-dimensional lower buffer container

| Code | Part | Material & Dimensions | Comments |
|---|---|---|---|
| 1. | Side pieces | Plexiglass 11 x ¼ x 5½ x ¼" | |
| 2. | End pieces | Plexiglass 10 x 5½ x ¼" | |
| 3. | Bottom piece | Plexiglass 10½ x 11½ x ¼" | |
| 4. | Upper buffer supports | Plexiglass 9½ x 1½ x ½" | |
| 5. | Electrodes | Platinum 27 guage | 2" apart |
| 6. | External power source attachment | Copper 18 guage | Soldered to platinum electrodes |

Figure 9. Two-dimensional lower buffer container

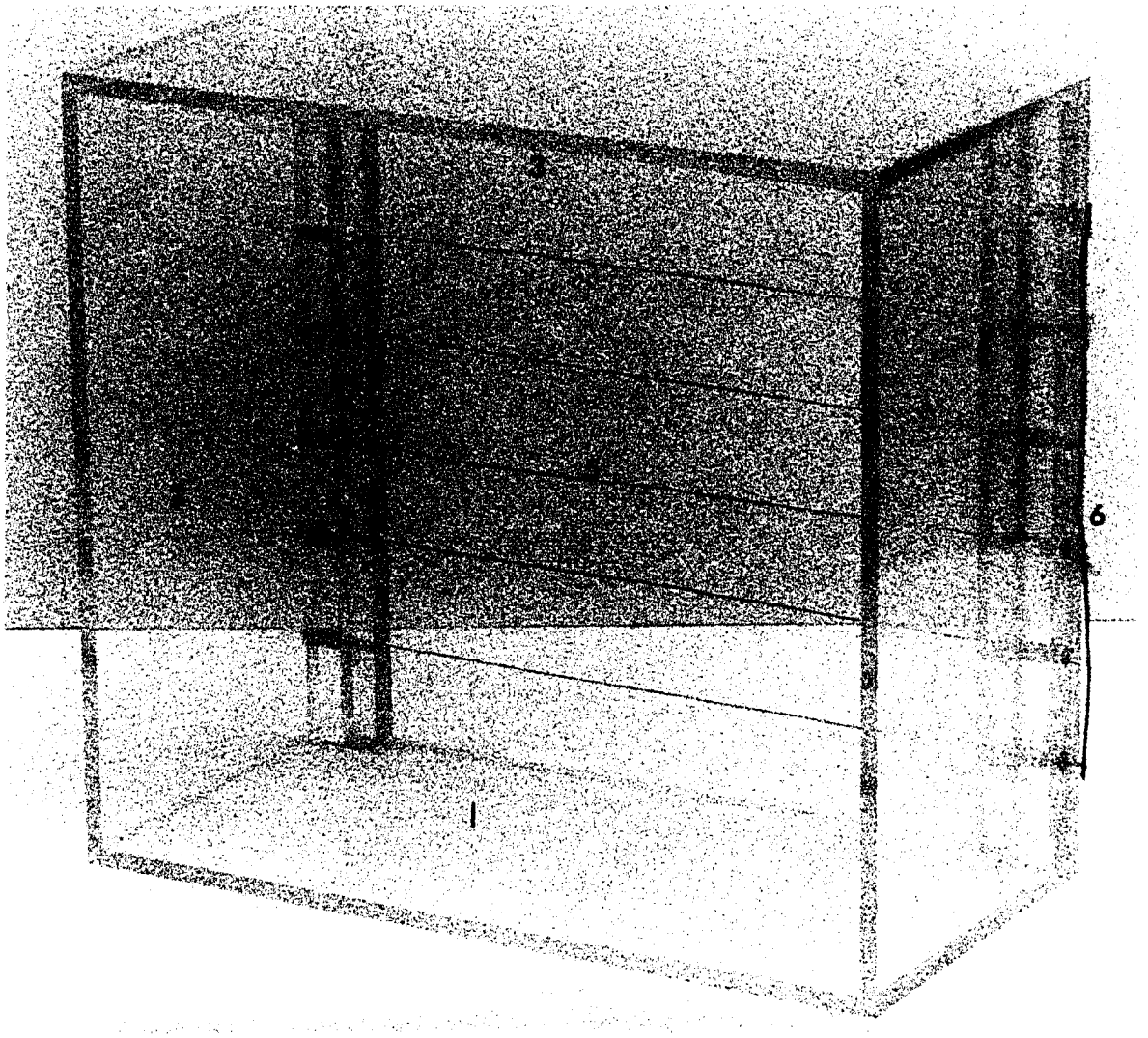


Figure 10. Upper buffer electrode plate

| Code | Part | Material & Dimensions | Comments |
|---|---|---|---|
| 1. | Electrode support | Plexiglass 10¼ x 8.75 x ¼" | |
| 2. | Electrode post | Plexiglass ½ x ¼" diameter | |
| 3. | Electrodes | Platinum 27 guage | |
| 4. | External power source attachment | Copper 18 guage | soldered to platinum |
| 5. | Placement blocks | | |

Figure 10. Upper buffer electrode plate

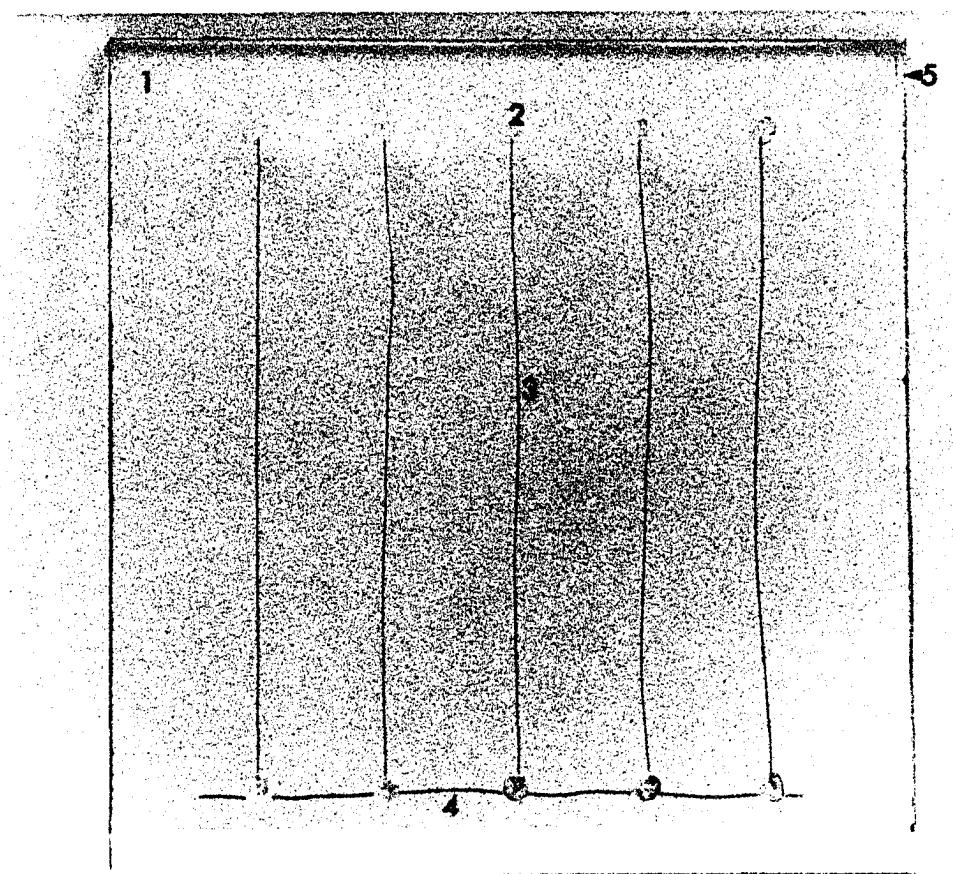


Figure 11. Semi-separated two-dimensional gel compartments and upper buffer container

| Code | Part | Material & Dimensions |
|---|---|---|
| 1. | Right & left end gel compartments | Plexiglass |
| 2. | Middle gel compartments | Plexiglass |
| 3. | Upper buffer container | Plexiglass |
| 4. | Tightening bolts | Stainless steel 3/16" |

Figure 11. Semi-separated two-dimensional gel compartments and upper buffer container

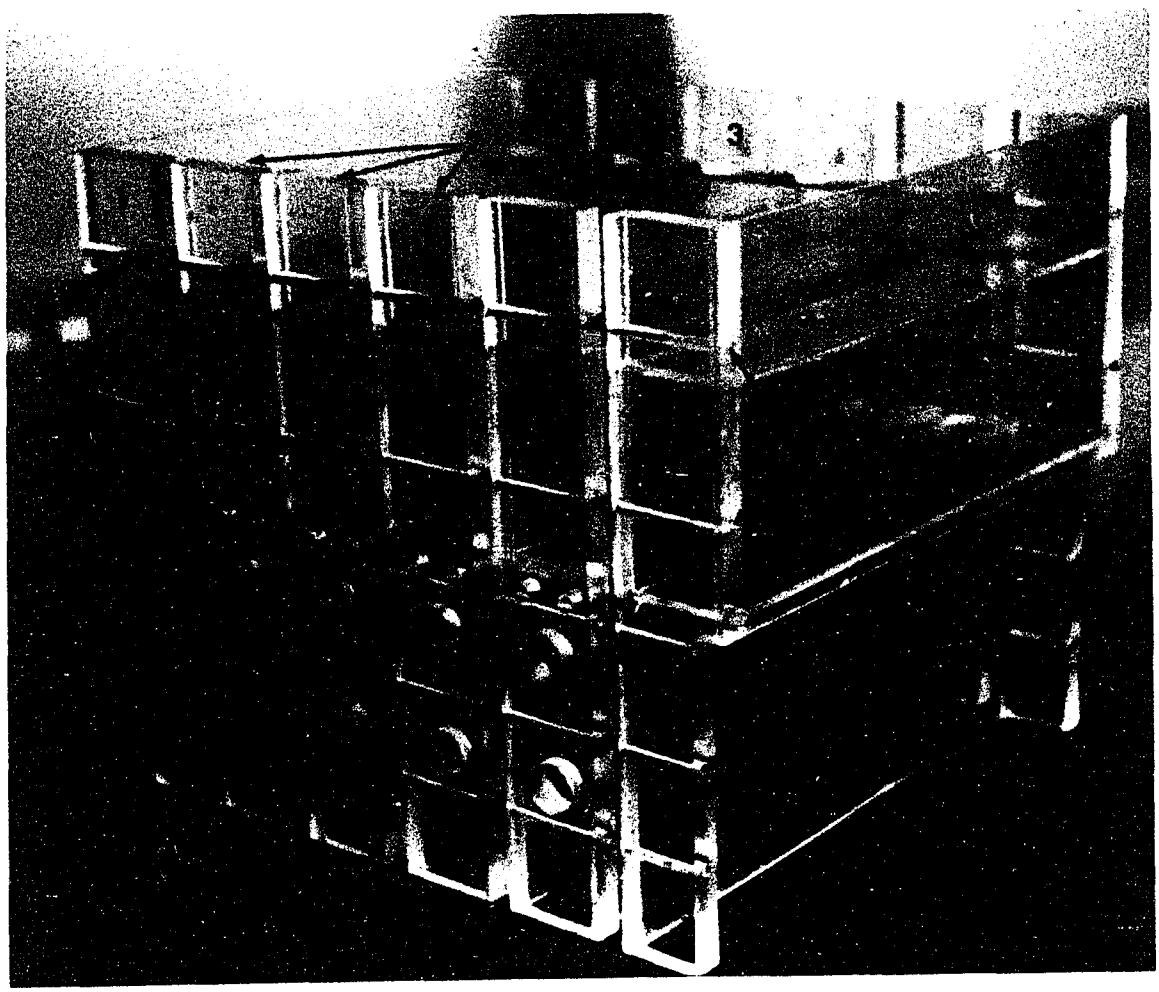


Figure 12. Two-dimensional right end compartment

| Code | Part | Material & Dimensions | Comments |
|---|---|---|---|
| 1. | End pieces | Plexiglass 9.625 x 1½ x ½" | |
| 2. | Back piece | Plexiglass 10 x 5 x .125" | |
| 3. | Gel contact surface | Plexiglass 7.75 x 9½ x .125" | |
| 4. | Holes for bolts | 1/8" | |
| 5. | Separation pieces | Plexiglass | |
| 6. | One half the one-dimensional gel support | Plexiglass | Forms "V"-shaped groove with other half |

Figure 12. Two-dimensional right end compartments

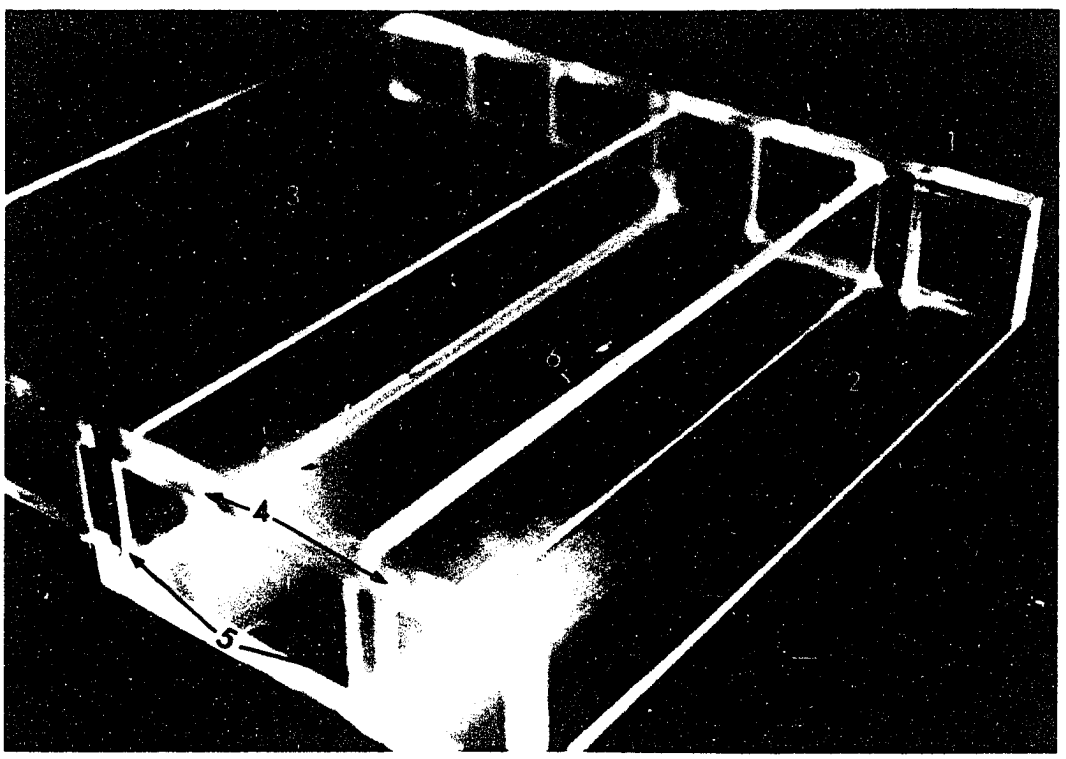


Figure 13. Combined right and left end compartments

| Code | Part | Material & Dimensions |
|---|---|---|
| 1. | Upper buffer compartment | Plexiglass |
| 2. | Gel compartment | Plexiglass 9.18 x 7.75 x .18" |

Figure 13. Combined right and left end compartments

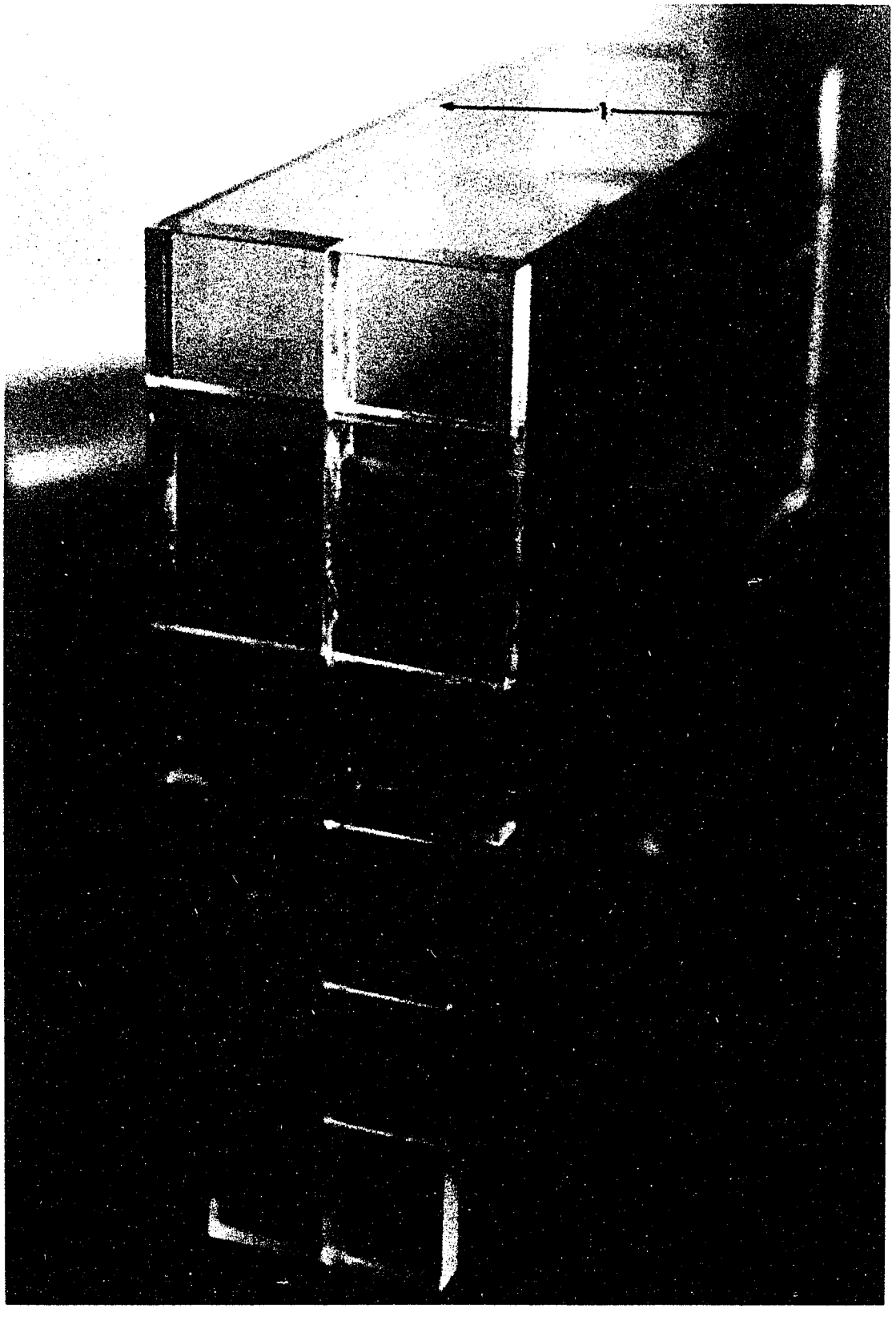


Figure 14. Middle and end gel compartments

| Code | Part | Material & Dimensions | Comments |
|---|---|---|---|
| 1. | End piece | Plexiglass 9.625 x 1½ x ½" | |
| 2. | End gel contact surface | 1/8" Plexiglass | Flush with end piece edge |
| 3. | Middle gel contact surfaces | Plexiglass 7.75 x 9¼ x .125" | Recessed 3/16" from edge |
| 4. | End compartment back | Plexiglass 10 x 5 x .125" | |
| 5. | End buffer dividers | Plexiglass 7.75 x 1½ x .125" | |
| 6. | Middle buffer dividers | Plexiglass 7.75 x 1.312 x .125" | |
| 7. | Beveled edge | | |
| 8. | Buffer circulation hole | 3/4" diameter | |
| 9. | Gas escape holes | 1/8" diameter | |
| 10. | Bolt holes | 3/16" diameter | |

Figure 14. Middle and end gel compartments

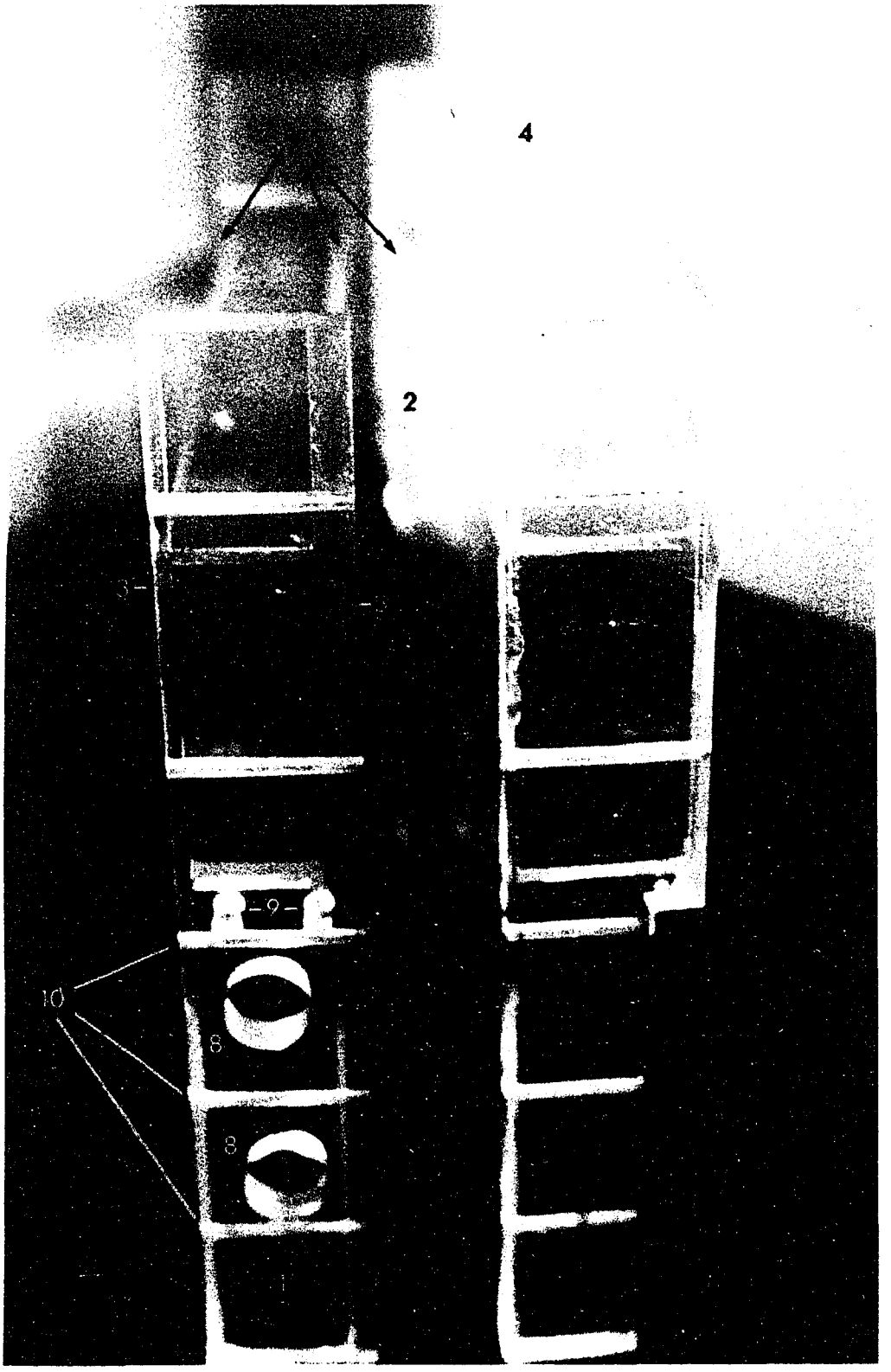


Figure 15. Multiple gel slab sealer

| Code | Part | Material & Dimensions |
|---|---|---|
| 1. | End pieces | Plexiglass 9 x 2 x ¼" |
| 2. | Side pieces | Plexiglass 11 x 2 x ¼" |
| 3. | Bottom | Plexiglass 9 x 9 x ¼" |

Figure 15. Multiple gel slab sealer

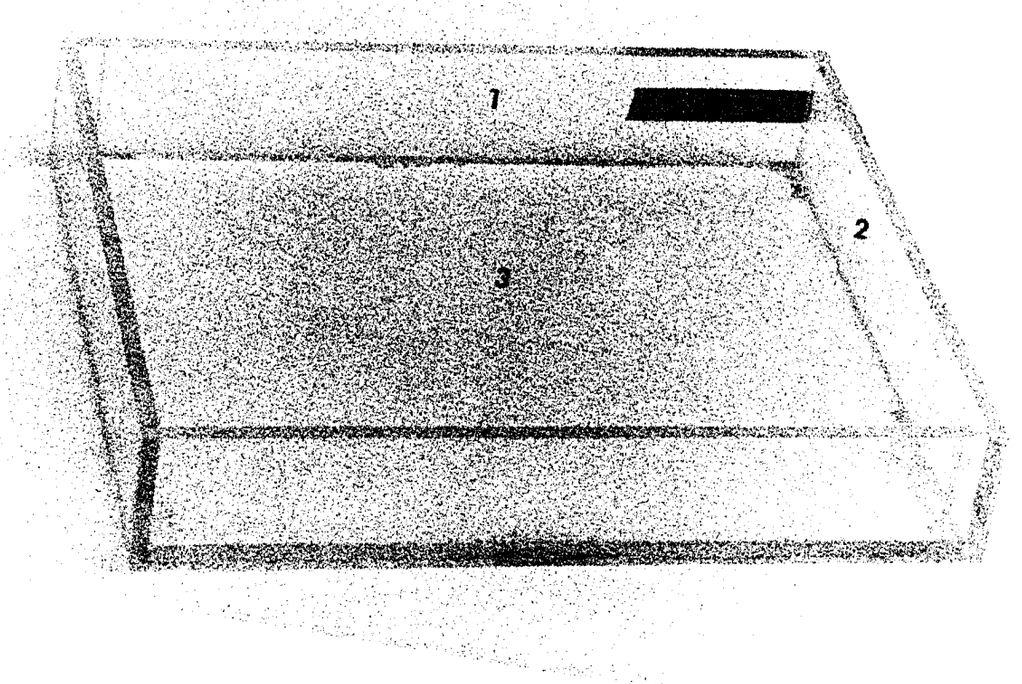


Figure 16. Single gel slab sealer

| Code | Part | Material & Dimensions |
|---|---|---|
| 1. | Base | paraffin |
| 2. | Groove | ½" deep |

Figure 17. Gel slab staining and destaining support

| Code | Part | Material & Dimensions |
|---|---|---|
| 1. | End cross pieces | Plexiglass 8¼ x ½ x ¼" |
| 2. | Uprights | Plexiglass 4.75 x ½ x ¼" |
| 3. | Side pieces | Plexiglass 10¼ x ½ x ¼" |
| 4. | Runners | Plexiglass 8.75 x ¼ x ¼" |
| 5. | Gel slab support | |

Figure 16. Single gel slab sealer

Figure 17. Gel slab staining and destaining support

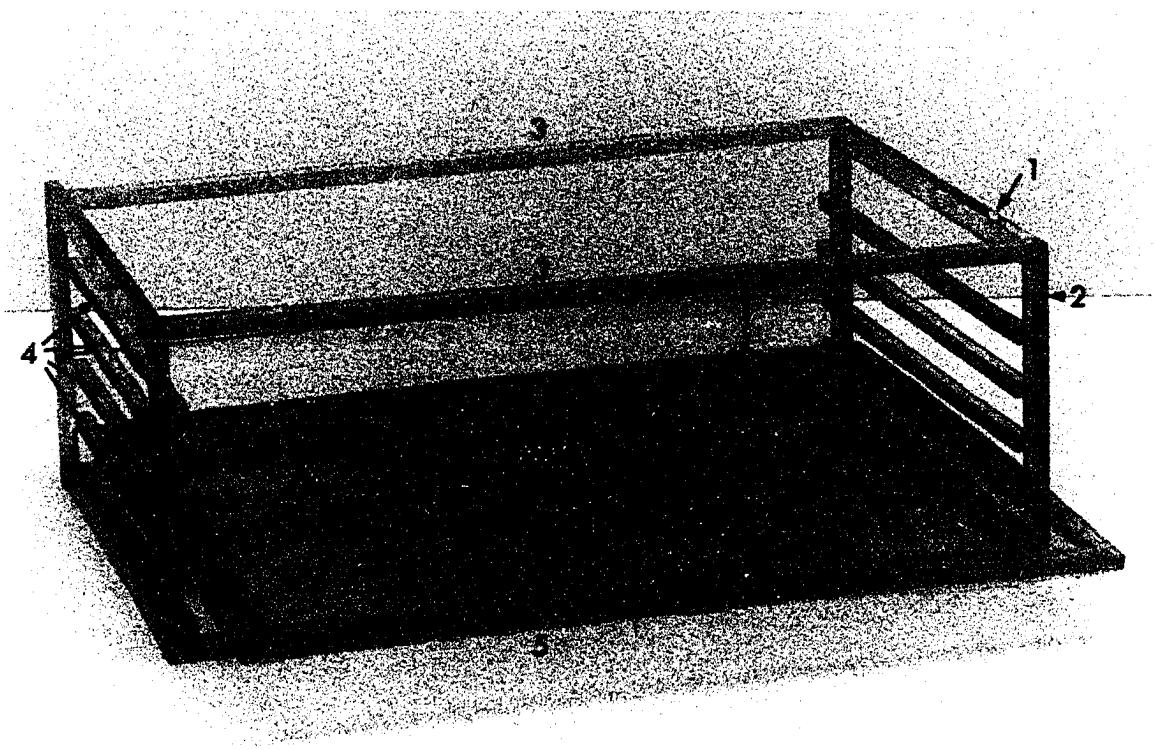


Figure 18. Gel slab support

| Code | Part | Material & Dimensions | Comments |
|---|---|---|---|
| 1. | End pieces | Plexiglass 9 x ½ x ¼" | |
| 2. | Side pieces | Plexiglass 10 x ½ x ¼" | |
| 3. | Screen | Aluminum 10 x 9" | Melted into plexiglass with soldering iron |

Figure 18. Gel slab support

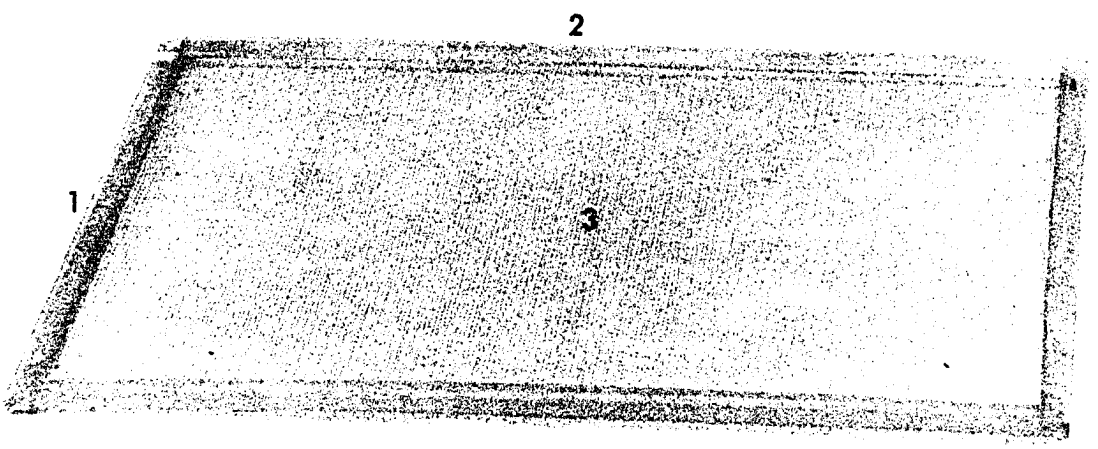
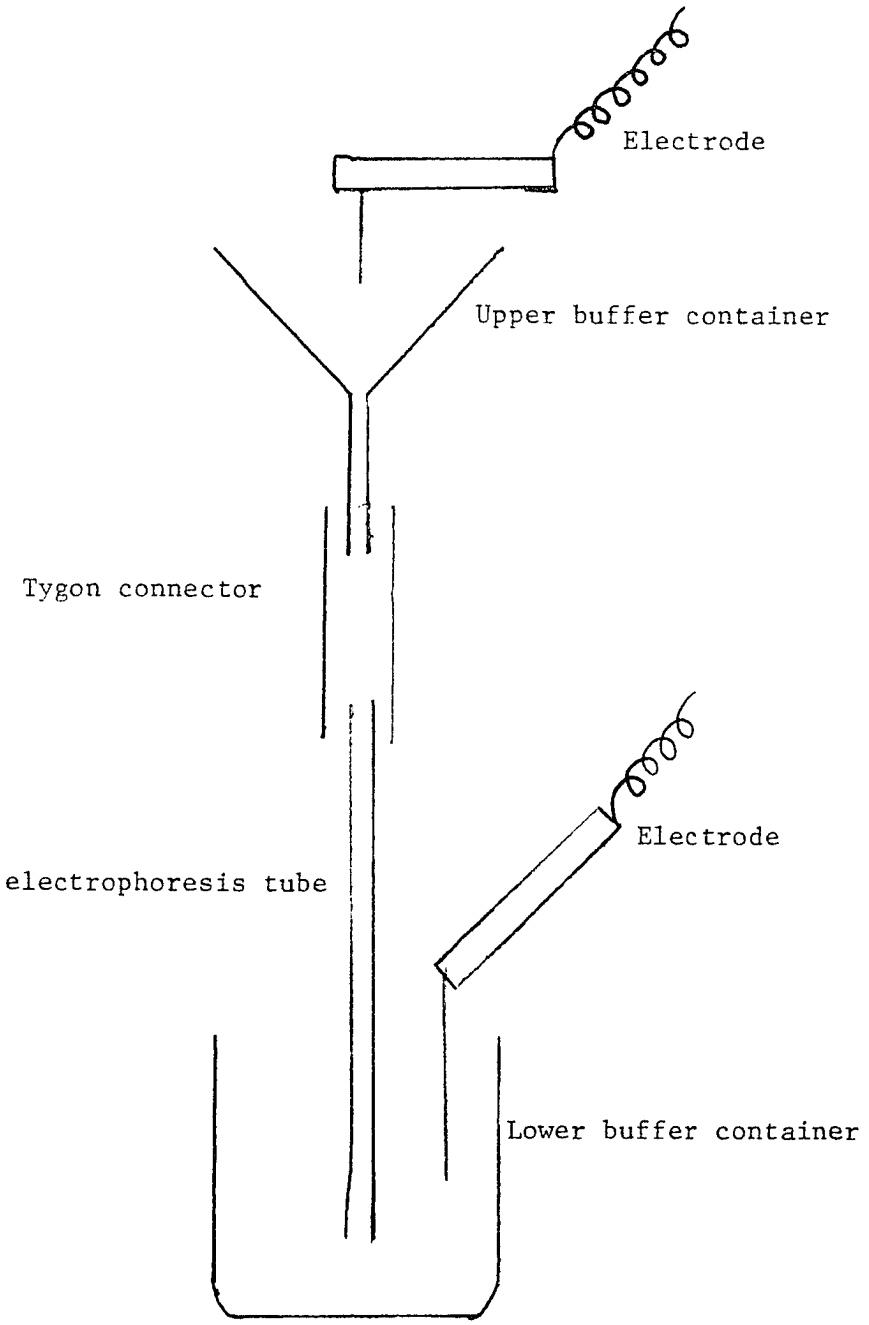


Figure 19. Small scale apparatus



## Electrophoretic Procedure

### General Comments

Electrophoresis is run at room temperature in both the first and the second dimensions and all solutions are at room temperature when used but gel solutions, buffers, and peroxides are stored under refrigeration. Solutions of monomers contain high concentrations of urea which tends to precipitate out at lower temperatures. Therefore, it is imperative that the urea be redissolved before the solutions are used.

Materials can be stored under refrigeration for the periods indicated below, as determined in this work:

| | |
|---|---|
| 1-D separation gel | 6 months |
| 1-D sample gel | 6 months |
| 7% and 10% Ammonium Persulfate | 6 days |
| Ammonium Peroxidisulfate-Riboflavin | 3 days (or until pH changes below 2.0) |
| Electrode Buffer | 1 month |
| Dialysis Buffer | 1 month |
| 2-D separation gel | 6 months |
| 36% and 48% urea | 1 month |

The ribosomal protein is polymerized in the middle of a 180 x 6 mm glass tube. The acrylamide concentration is 4% and it is buffered at pH 8.6. Proteins migrate both as cations and anions depending on their net charge. The 1-D

gel is then adapted for the 2-D electrophoresis by dialysis against the starting buffer. The 1-D gel is incorporated horizontally into the edge of a 200 x 200 mm slab, 9% in acrylamide monomer. Electrophoresis in the 2-D electrophoresis is then carried out at pH 4.6, a pH at which the proteins migrate as cations. The proteins are visualized by amido black staining.

## One-dimensional electrophoresis

### A. Tube Preparation

Polyacrylamide electrophoresis in the first dimension is carried out in soft glass tubes. The tubes are 180 mm in length with a 5 mm inner diameter. The tubes are fire polished to prevent tearing of the rubber grummets upon insertion into the 1-D apparatus. Caution is taken to prevent a reduction of the inner diameter as this would inhibit gel removal. The fire polished tubes are prepared for electrophoresis by soaking them overnight in a 1% solution of SDS.

Prior to addition of the 1-D gels, the tubes are removed from the SDS solution. The superfluous detergent is shaken off and the tubes are allowed to stand until dry. The SDS cleaning facilitates rapid removal of the gel from the tube and minimal scarring of the gel since the polymerized material is unable to cling to imperfections in the glass.

B. Gel Loading

The SDS cleaned tubes are closed on one end with parafilm or by conveniently placing the tubes in the small rubber caps (destaining caps) from a Canalco Disc Electrophoresis kit and then locking them into the loading rack.

The separation gel 4% (w/v) in acrylamide monomer and buffered at pH 8.6 is deaerated in a desiccator with a water aspirator for approximately 10 minutes to remove oxygen and to prevent the formation of bubbles. To the separation gel is then added 0.1 ml of 7% Per for every 20 ml of separation gel. The solution is swirled gently and then added to the tube to a height of 70 mm to form the catonic separation gel (the gel into which positively charged proteins migrate). The same separation gel solution is used in all tubes to be run simultaneously in order to ensure identical composition.

Immediately following the addition of separation gel to the tubes, the solution is overlaid with a few drops of 36% urea solution (the urea concentration in the separation gel). This layering is necessary: 1. To eliminate the meniscus that would otherwise be formed by the acrylamide clinging to the sides of the tube and to ensure the formation of a flat interface between the separation gel and the sample gel; 2. To prevent diffusion of urea from the separation gel.

Chemical polymerization occurs in approximately 10 minutes. Following the polymerization, the layered urea solution is removed from the tube by insertion of a small roll of filter paper taking care not to disrupt the gel surface during this process.

The sample gel is prepared while thb separation gel is polymerizing. One or two mg of lyophilized ribosomal protein is dissolved in 0.4 ml of sample gel. Ribosomal proteins from mesophiles were soluble in a sample gel containing 48% urea, but those from thermophiles required a urea concentration of 60%. Accordingly, the latter concentration was used throughout for the sample gel. Gentle swirling is usually sufficient to dissolve the protein. The sample gel is then deaerated for approximately 5 minutes. Two microliters of Per-Riboflavin solution are then added with a microsyringe. The sample is gently swirled and 0.2 ml of this solution (larger volumes must be avoided) is layered on the separation gel in the following fashion. A plastic tube (1 mm diameter, 8 cm long) is snugly fitted over a disposable polycarbonate Eppendorf pipet tip. Using a 100 ul Eppendorf pipet the desired amount of sample gel can be layered directly onto the cationic separation gel. This is accomplished with minimal contamination of the anionic portion of the tube by sample gel. Removal of the slight contamination by the sample gel is accomplished by wiping the tube clean with rolled filter paper. Any

bubbles that may be present in the sample gel are removed by using a Pasteur pipet.

The sample is then overlaid with enough 55% (w/w) urea solution (a solution slightly less dense than the sample gel) to eliminate meniscus formation by the sample gel. The use of 60% urea solution is avoided as it readily mixes with the sample. Even with this modification, the urea is gently run towards the side of the tube to prevent disruption of the sample gel. The use of cold urea causes precipitation of the protein in the sample and, therefore, must be avoided. Layering of distilled water, as suggested by Kaltschmidt (13), causes depletion of urea from the sample gel and precipitation of protein. A 55% urea layer solution offers a compromise between the depletion of urea and the disturbance of the sample gel.

The sample gel monomer is photopolymerized. The tube is held approximately 6 inches from the ultraviolet lamp (see apparatus) for 15 minutes. After polymerization, the urea solution is removed with rolled filter paper.

A second volume of separation gel (30 ml), to be used as the anionic separation gel, is deaerated as described. Polymerization is initiated with 1.5 ml of 7% Per and the tubes are then filled with the mixture. Polymerization occurs in about 15 minutes to form the anionic separation gel.

C. Electrophoresis

Following polymerization, the end of the tube containing the anionic separation gel is inserted into the upper buffer. This is assisted by wetting the end of the tube before inserting it into the rubber grummet of the upper buffer container (see figure 4).

Inserts not being used are plugged with plexiglass inserts. The glass tubes are adjusted such that the end containing the anionic separation gel is approximately 1 cm from the support plate housing the cathode (see figure 3).

The electrodes are connected to a Savant constant voltage power supply which is adjusted to 5 mA per tube and an initial potential of 90 volts (±4 volts). Running time for the one dimensional electrophoresis is 14 hours.

The buffer, approximately 8 liters, is circulated via a pump through a two liter mixing vessel depicted in figure 1. The circulation route is also shown in this figure. This circulation is necessary to prevent electrolyte shifting. The rate of pumping is 50 liters per hour. The rate of circulation to the upper and lower buffer is regulated by the clamp depicted in the same diagram.

D. Gel Removal

The gel is loosened by injection of glycerol between the gel and the glass tube using a syringe with a blunt

needle. The glycerol is injected into both ends of the tube. The needle is circled around the gel taking care not to scar or twist the gel. Any air bubbles that may have become trapped during this process can be removed by holding the tube vertically and slowly removing the needle.

After lubrication, the gel may slip from the tube when held vertically. If this does not occur, the lubricated tube is kept horizontally for 10 minutes. The gel is then removed hydrostatically with a water filled syringe (see figure 5). The water filled syringe has a short piece of rubber tubing attached in place of the needle. The diameter of the tubing is approximately 5 mm securing a tight fit over the 1-D electrophoresis tube. The application of a slight pressure with the syringe plunger will then remove the gel. Gel breakage is most likely to occur at the interface between the sample and separation gel. Therefore, minimal pressure must be applied when the sample gel emerges from the tube.

To prevent buckling of gels, a plexiglass plate with 3/8" grooves milled into it (see figure 6) was constructed. The grooves are wetted with starting buffer before the gel is placed into them. This provides lubrication when the gels are slid from the tubes onto the plate and ensures that no protein precipitation will occur in the gel.

At this point, the 1-D gel may be stained and photographed or prepared for 2-D electrophoresis.

Two-dimensional electrophoresis

A. Gel preparation

The 1-D gels are prepared for electrophoresis in the second dimension by dialysis against 750 ml of starting buffer. This is accomplished by carefully transporting the gel with a small styrofoam scoop (equal in size to that of the gel) from the plexiglass plate to the dialysis rack (see figure 7). After one hour, the buffer is replaced by fresh buffer. This is repeated twice.

The 2-D apparatus is assembled in a step by step fashion according to figure 11. None or up to a maximum of four middle sections (see figure 14) can be held between the right and left end pieces in a sandwich-like fashion. When the pieces are fitted together 200 x 200 x 5 mm channels are formed to hold the two dimensional separation gel.

In order to prevent leakage, the finely milled edges of the middle and end pieces are coated with a thin layer of inert silicone stopcock grease. Tightening the ten stainless steel bolts forms leak-proof channels for the addition of the two dimensional separation gel.

A liter of 2-D separation gel is filtered before

addition of Per and the mixture is then placed in a plexiglass box (see figure 15) of a cross section corresponding to that of the 2-D apparatus. The polymerizing gel is overlaid with 50 ml of water and the intact 2-D apparatus is set gently into the polymerizing gel. After 15 minutes the gel has polymerized and the bottom of the chambers in the 2-D apparatus are securely sealed. Water above the gel sealing the bottom of the four channels is removed with rolled filter paper.

A liter of 2-D separation gel and Per is then filtered and added to the four vertical chambers. The monomer is gently run down the side of the apparatus through a small funnel to prevent the formation of bubbles. The monomer is added until it reaches the V-shaped grooves (figure 12) at the top of the chamber.

At this point, the 1-D gels are removed from the dialysis rack (by means of a styrofoam scoop) and cautiously set in the V-shaped grooves. Additional 2-D separation gel monomer (from the same batch that filled the chambers)is added until the gel is surrounded by the 2-D separation gel. Additional gel is added in the event that sinking occurs (due to expansion of plexiglass during polymerization) in order to maintain the monomer level until polymerization is complete (about 15 minutes).

The 1-D gels are often inadvertently broken and consequently present a problem for accurate electrophoresis

in the second dimension. To eliminate this problem, an alternate procedure has been developed. This involves adding the monomer in the 2-D apparatus up to the bottom of the V-shaped grooves and, unlike in the normal procedure, the gel is allowed to harden. Broken gels are self-adherent and require only proper orientation to resemble the initial intact state. The 1-D gels are then placed atop the polyacrylamide slab in the V-shaped groove. Unpolymerized acrylamide is added until the level of the 2-D gel surrounds the 1-D gel. This requires approximately 20 ml. Occasional swirling prevents the gel from polymerizing while portions of the same gel in the 2-D chamber do polymerize. This procedure also permits the use of 1-D gels of small diameter without the need to redesign the 2-D apparatus.

Using either procedure, bubbles must be removed from under the 1-D gels before polymerization occurs. Gentle lifting of the 1-D gel with a spatula is sufficient to remove any bubbles trapped under the 1-D gel.

B. Electrophoresis

After the 1-D gels have been polymerized into the 2-D gels, excess polyacrylamide at the bottom of the apparatus is removed by rinsing with distilled water. The apparatus is then placed into the bottom electrode buffer container (see figure 9) such that the elongated platinum electrodes are between the polyacrylamide slabs.

This is necessary to prevent formation of bubbles at the bottom of the 2-D slabs from the electrolysis of the buffer. The cathode buffer container is then filled with the electrode buffer and the upper buffer container (see figure 8) is filled next. The upper electrode (see figure 10) is then placed directly above the slabs. The electrodes are designed to provide uniform ion distribution since the buffer is not being circulated.

An initial current of 400 mA (100 mA/slab) and a potential difference of 100 volts is applied from a constant voltage power supply. The amperage drops considerably (to 260 mA) during the run as the resistance increases due to elution of ions or ionic species from the gel. Best results are obtained when the electrophoresis is carried out for eighteen hours. A uniform rate of bubble evolution from the 5 platinum electrodes is a rough indication that connections are secure and ion distribution is uniform.

Following the electrophoresis, the 2-D apparatus is dismantled in a stepwise manner, and the polyacrylamide slabs are gently transferred to aluminum screens framed in plexiglass (see figure 18). The screens supporting the polyacrylamide slabs are slipped into a multi-level plexiglass storage frame (see figure 17).

C. Staining

A 13 liter staining vessel is filled with an aqueous

solution of 0.55% amido black and 5% acetic acid. The solution is mixed thoroughly before the polyacrylamide gel slabs are immersed. The slabs are stained for 20 minutes with constant stirring. As a precaution, a screen is laid over the top gel since the slabs often float to the surface.

Following the staining, the slabs are rinsed for 2 hours under tap water. The stained slabs are then destained in 13 liters of 1% acetic acid for approximately 5 days. The solution is constantly stirred to prevent the establishment of a dye concentration gradient. The solution is replaced with a fresh solution as the dye elutes from the gel.

The one dimensional gels can be stained in a like manner.

The polyacrylamide slabs can be stored for six or seven weeks in the destaining solution. After a month, the spot corresponding to the amido black-protein complex begins to fade. Restaining is unsuccessful and it, therefore, appears that the protein diffuses from the polyacrylamide gel after prolonged storage.

The gels must be kept covered with solution or deformation of the gel will occur in hours. Procedures for dry storage (15) are available, however, they were not employed in this study.

D. Photography

A cleaned plexiglass square (12 x 12 x 1/4") is

used to support the polyacrylamide slab for photography. The slab is cleaned of any residue left over from the destaining bath by carefully wiping it with a damp kimwipe. Bubbles formed between the gel and the plexiglass are removed by pressing the gel against the plexiglass and rubbing it with outward strokes. In order to bring out the stained protein spots most effectively, the plexiglass plate is set on a large piece of filter paper. Measurements of protein migration are facilitated by placing a translucent, plastic grid (6 x 6 mm in its smallest division) under the filter paper. The gel is then placed onto the plexiglass. The composite (gel-plexiglass-filter paper-grid) is set on a light box to illuminate the protein spots and to superimpose the grid onto the polyacrylamide slab. Side flood lights at a 45° angle to the surface of the slab are employed to enhance contrast from above. Photographs are taken with a Polaroid MP-3 Land Camera set up with black and white Polapax 200 speed, type 42 film. The best photographs are obtained by using an 11.3 lens setting, a 1/30 second exposure time, and a red filter to eliminate exposure due to the residual dye.

E. Applications

In order to determine the optimum running conditions, to perform pilot runs, and to check other variables, it is advantageous to carry out a small scale two-dimensional

electrophoresis run. Such a set up is diagrammed on page 72. This permits 1-D electrophoresis to be carried out in the standard size tubes. However, no pumping is required and the 1-D buffer is reduced from eight liters to one. The 1-D polyacrylamide gel is removed as described and dialyzed 1 hour against 100 ml of starting buffer in a 200 ml test tube stoppered and layered horizontally. The dialysis is repeated twice.

Electrophoresis in the second dimension is performed in the single chamber formed by combination of the right and left side pieces. A paraffin mold (see figure 16) corresponding to the base of the 2-D chamber is encased in a 1 liter glass tray. The 2-D apparatus is fitted into the paraffin mold and 50 ml of polymerizing acrylamide monomer are added to the chamber; the gel is then overlaid with 10 ml of water and allowed to polymerize. This seals the 2-D chamber and prepares it for the addition of the separation gel. The remaining steps are identical to those for a full scale run.

In addition to the small scale modification, several other electrophoretic procedures can be carried out with this apparatus. Zonal one dimensional electrophoresis can be performed using either the apparatus for the first dimension or that for the second dimension as a vertical bed one dimensional system. While this results, of course, in lower resolution, it has the advantage of allowing the simultaneous electrophoretic analysis of some 75 samples

when the second dimensional system is used. Such a large number of samples cannot be electrophoresed simultaneously by conventional 1-D electrophoresis systems.

Disc electrophoresis, as described by Ornstein (7) and Davis (8), can be substituted for zonal electrophoresis in either the first or the second dimensional system and, if desired, the polyacrylamide can be replaced by or combined with other gels such as agarose (16) or sephadex (17). Polyacrylamide substitutes or polyacrylamide composites also permit the use of this apparatus for the electrophoresis of high molecular weight species (molecular weight greater than 100,000) such as nucleic acids and lipoproteins. These species are otherwise excluded.

Lastly, inserts may be fitted into the second dimension apparatus to decrease the gel slab width. Taking precautions to prevent gel shrinking (15), the gel can then be dried to a very thin film. This permits the use of radioautography using caron 14 (18) or even low energy beta emitters such as tritium.

## Electrophoresis of Ribosomal Proteins

### Yield and purity

The ribosomes prepared as described under methods have previously been shown to be active in in vitro amino acid incorporation and are therefore fully functional subcellular particles.

In order to compare the proteins of such ribosomes isolated from different organisms, it is essential to prepare the ribosomal protein in high yield with a minimal contamination of ribosomal RNA. Several methods were tried which have been successfully applied to the isolation of ribosomal proteins from E. coli. These results are shown in Table 1. It can be seen that only the 2-chloroethanol method, which had been used previously for a strain of B. stearothermophilus(10) provided a satisfactory preparation of ribosomal protein. The yield and purity of the ribosomal protein isolated by this method from the four organisms studied here are shown in Table 2. In all cases more than 80% of the protein in the initial ribosomes was recovered and more than 99.5% of the ribosomal RNA was removed during the isolation.

Table 1

Isolation of Ribosomal Protein

| Method | Percent Recovery* | |
|---|---|---|
| | Protein | RNA |
| Acetic Acid (19) | 50.3 | 3.0 |
| Modified Acetic Acid (20) | 47.2 | 1.0 |
| Lithium Chloride (21) | 55.1 | - |
| Chloroethanol (10) | 80.4 | 0.5 |

*B. licheniformis

Table 2

Yield and Purity of Ribosomal Protein

| Organism | Percent Recovery | |
|---|---|---|
| | Protein | RNA |
| *B. licheniformis* | 80.4 | 0.5 |
| *B. pumilus* | 82.1 | 0.5 |
| *B. stearothermophilus* 10 | 86.8 | 0.5 |
| *B. stearothermophilus* 2184 | 83.4 | 0.5 |

Reproducibility

In order to assess the design qualities of the apparatus, the following experiment was performed. Four samples of the ribosomal protein from the four species of the genus Bacillus were subjected to simultaneous electrophoresis as described. Following electrophoresis and staining, the gel slab from slot one was photographed. The gel slab from each of the other slabs was then placed directly on the first slab and, after removal of air bubbles, photographed. The results are shown in figures 20 through 23.

Figure 20. Slab 1

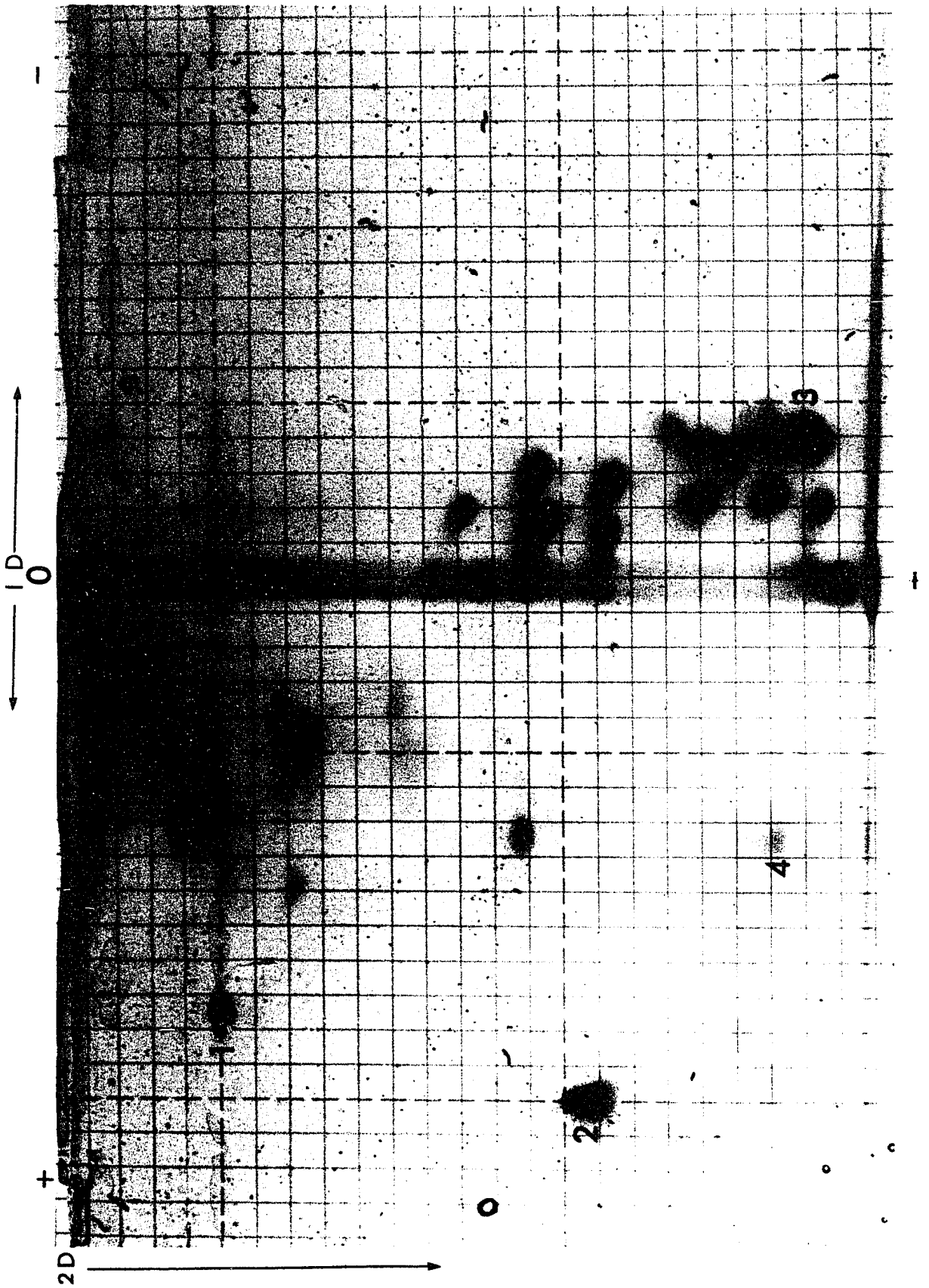


Figure 21. Slab 2 on slab 1

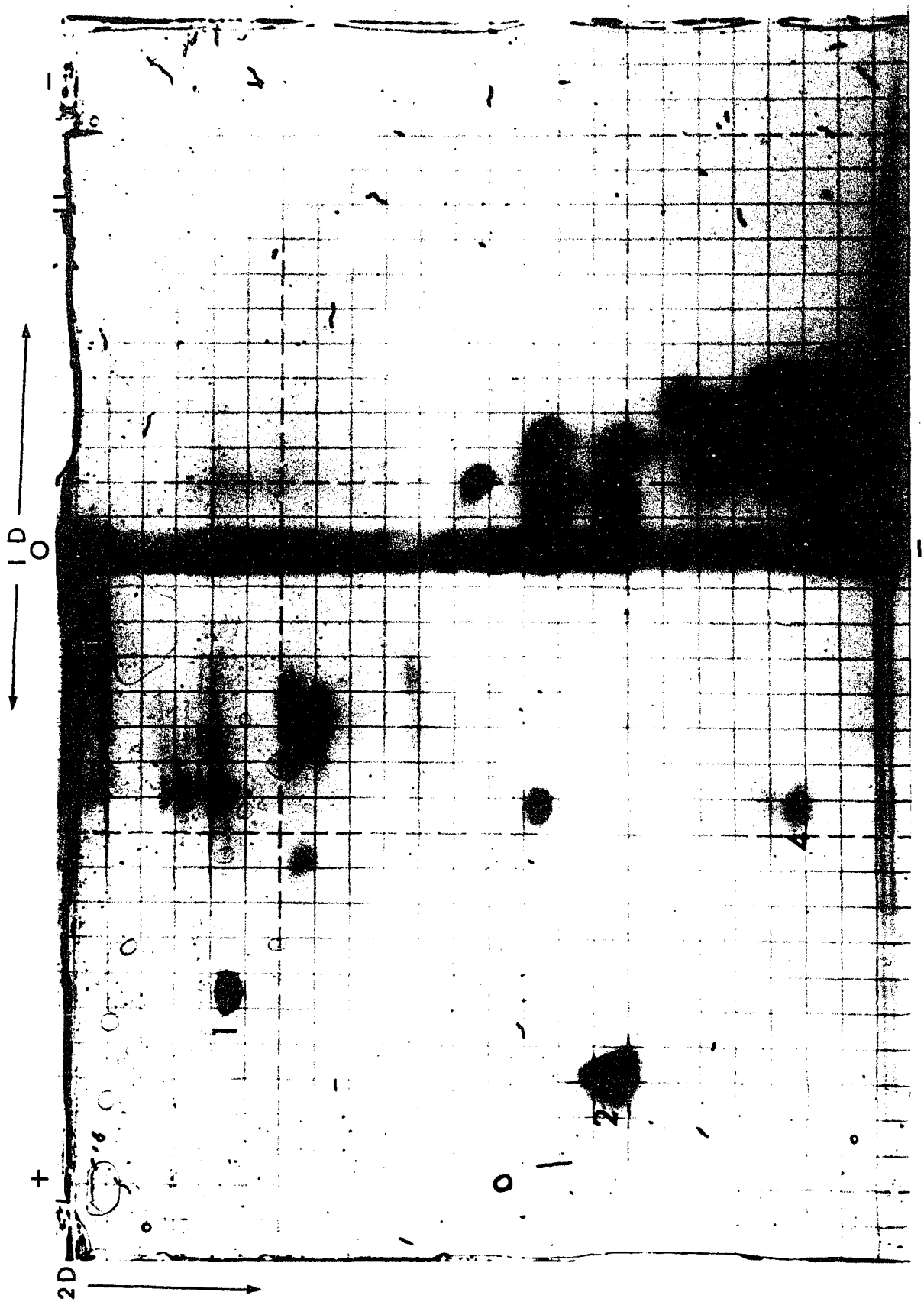


Figure 22. Slab 3 on slab 1

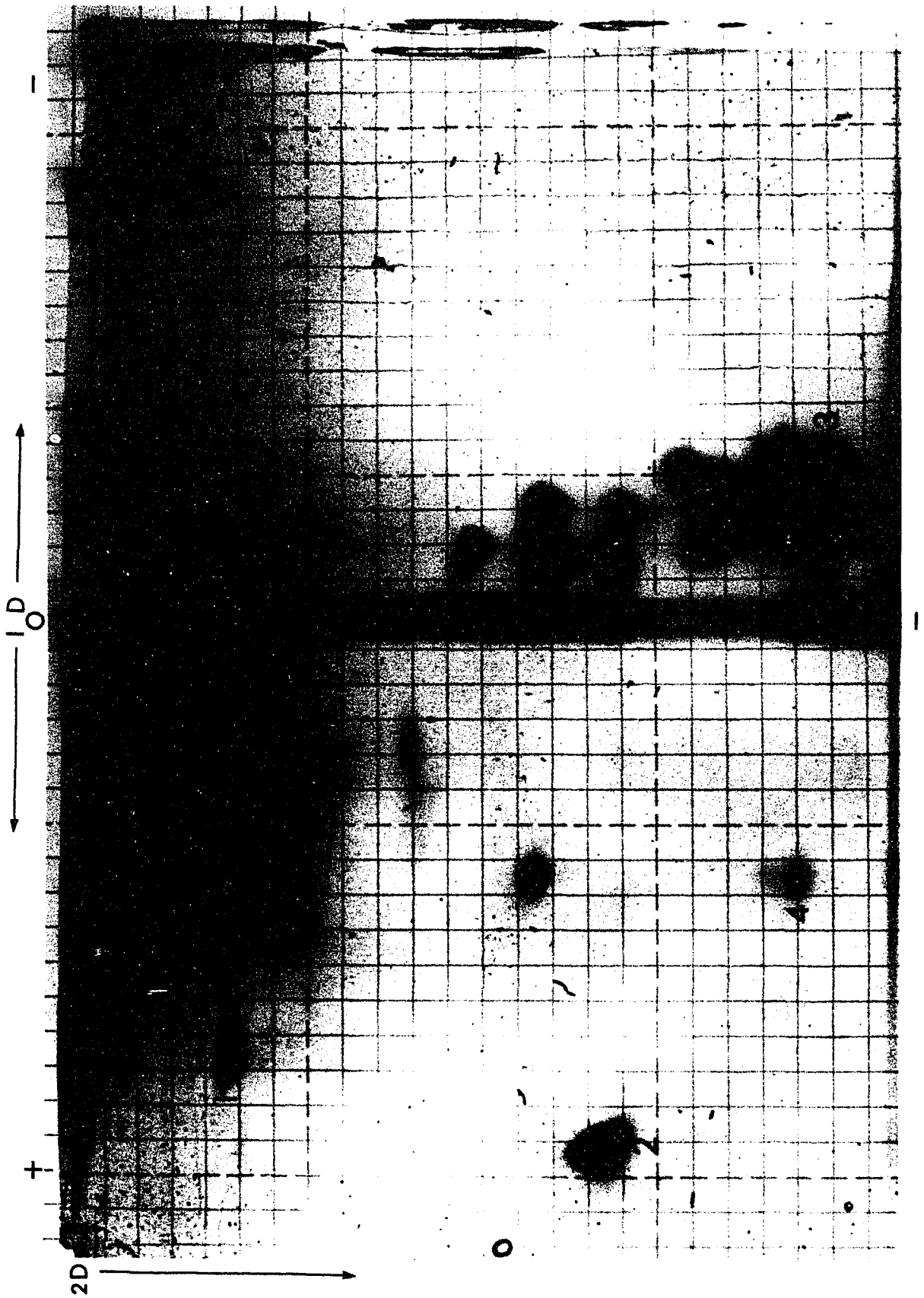
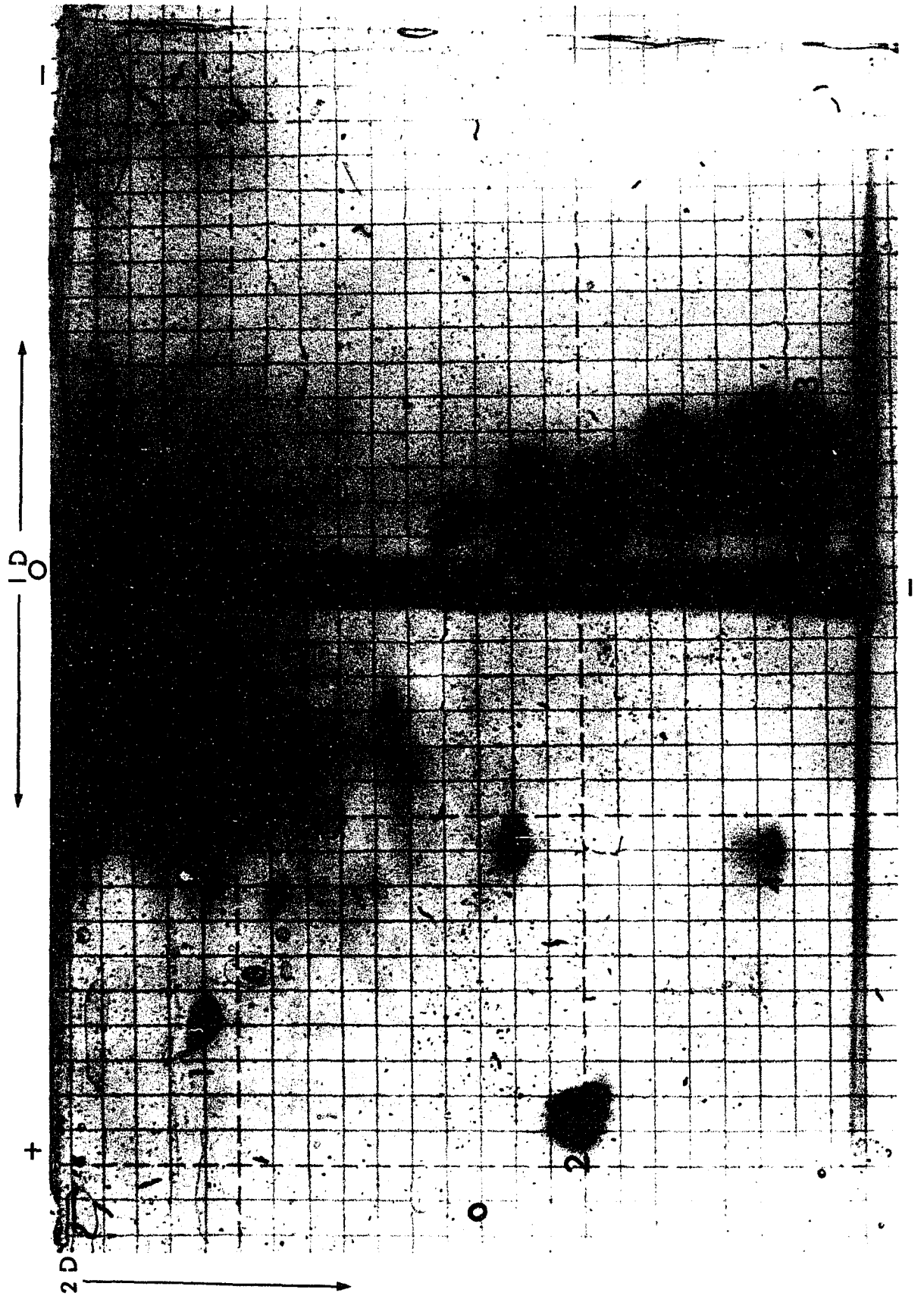


Figure 23. Slab 4 on slab 1



Inspection of these photographs shows that the patterns are nearly identical in all cases which indicates that the apparatus has indeed been properly designed and constructed.

A numerical check on the reproducibility of these patterns is presented in Table 3 which lists the actual migration distances of four proteins. The proteins chosen were those that moved the farthest, since any deviations from the migration distances of the proteins in slab 1 would be most noticeable for such proteins. It can be seen from table 3 that the reproducibility is, in fact, very satisfactory.

Table 3

Reproducibility of Electrophoretic Patterns

| Gel slab | Protein #1 | | Protein #2 | |
|---|---|---|---|---|
| | cm* | %** | cm* | %** |
| | | 1-D | | |
| 1 | -7.60 | - | -9.10 | - |
| 2 | -7.60 | 0 | -9.00 | 1.10 |
| 3 | -7.70 | 1.32 | -9.20 | 1.10 |
| 4 | -7.65 | 0.66 | -9.20 | 1.10 |
| | | 2-D | | |
| 1 | -2.60 | - | -9.00 | - |
| 2 | -2.60 | 0 | -9.10 | 1.11 |
| 3 | -2.55 | 1.92 | -8.90 | 1.11 |
| 4 | -2.65 | 1.90 | -9.10 | 1.11 |

*Migration distance: a negative sign indicates migration toward the positive electrode

**Percentage deviation of the migration distance from that measured in gel slab 1

Table 3 (continued)

| Gel Slab | Protein #3 | | Protein #4 | |
|---|---|---|---|---|
| | cm | % | cm | % |
| | | 1-D | | |
| 1 | 2.60 | - | -4.60 | - |
| 2 | 2.50 | 3.80 | -4.50 | 2.20 |
| 3 | 2.60 | 0 | -4.60 | 0 |
| 4 | 2.60 | 0 | -4.50 | 2.20 |
| | | 2-D | | |
| 1 | -12.70 | - | -12.30 | - |
| 2 | -12.60 | 0.74 | -12.30 | 0 |
| 3 | -12.60 | 0.74 | -12.10 | 1.62 |
| 4 | -12.80 | 0.74 | -12.35 | 0.41 |

Solubility of thermophilic proteins

The electrophoretic conditions established in this study for the separation of the mesophilic ribosomal proteins were not suitable for the separation of the thermophilic ribosomal proteins. It was first suspected that the polyacrylamide pore size was such as to prohibit the migration of the proteins. However, varying the gel concentration from 9% to 4% did not result in increased resolution. It became apparent that the lack of resolution was related to the low solubility of the thermophilic proteins which, by itself, is an interesting finding that may have some bearing on the problem of thermophily. The following approaches were taken in an attempt to solubilize the proteins. First, the pH of the buffer and of the gel was varied over the pH range of 9 to 5. While this was not successful, it indicated that poor resolution was not related to isoelectric precipitation.

Experiments were next performed in which the composition of the sample and/or the separation gel was altered. Since LiCl had been used to extract ribosomal proteins from other ribosomes, it was added to the gels at various concentrations. However, one dimensional electrophoresis did not lead to resolution of the sample.

Lastly, the urea concentration of the sample gel was increased from 48% to 60% which increased the solubility

of the proteins and resulted in the presence of bands migrating toward both the cathode and the anode in 1-D electrophoresis. In order to further increase the amount of protein in solution, the sample gel volume was increased from 0.1 to 0.2 ml which decreased the protein concentration at the same time. The increased volume of sample gel had little effect on the size of the spots of the two-dimensional pattern. The solubility of the proteins was further improved by layering the 1-D separating and sample gel monomer with a urea solution rather than with water as suggested by Ornstein (7), and thus eliminating the diffusion of urea out of the gels. Increasing the urea concentration in the separation gel so that it was equal to that of the sample gel (60%) did not increase resolution; therefore, the urea concentration in the separation gel was kept at 36%. Apparently only initial solubilization of the protein was necessary. The procedure worked out for the thermophilic proteins was then used for all the preparations (see Materials and Methods).

Comparison of mesophiles and thermophiles

The ribosomal proteins from the two mesophiles and the two thermophiles were subjected to two dimensional electrophoresis as described. The photographs of the patterns obtained are shown in figures 24 through 27 and the migration distances are compiled in Tables 4 and 5.

These migration distances were obtained from the individual gels and the gels were then superimposed as a direct check on the migration distances listed in tables 4 and 5.

Table 4

Migration Distances of Mesophilic Proteins

| Spot number | B. licheniformis | | B. Pumilus | |
|---|---|---|---|---|
| | 1-D | 2-D | 1-D | 2-D |
| | (cm) | | (cm) | |
| 1 | | | 4.70 | 1.80 |
| 2 | 5.10 | 1.80 | | |
| 3 | | | 4.60 | 2.05 |
| 4 | 4.70 | 2.05 | | |
| 5 | | | 2.60 | 2.50 |
| 6 | 8.60 | 2.50 | 8.60 | 2.50 |
| 7 | 5.80 | 2.60 | | |
| 8 | | | 4.70 | 2.65 |
| 9 | 2.20 | 3.00 | | |
| 10 | 2.60 | 3.20 | | |
| 11 | 3.40 | 3.20 | | |
| 12 | 5.80 | 3.30 | | |
| 13 | | | 3.40 | 3.80 |
| 14 | | | 5.80 | 3.80 |
| 15 | 3.00 | 4.10 | 3.00 | 4.10 |
| 16 | 0 | 5.10 | 0 | 5.10 |
| 17 | 0 | 5.60 | 0 | 5.00 |
| 18 | 1.40 | 6.05 | 1.40 | 6.05 |
| 19 | 0 | 6.40 | 0 | 6.40 |
| 20 | 3.70 | 7.10 | | |
| 21 | -2.20* | 7.15 | -2.20 | 7.15 |
| 22 | 0 | 7.20 | 0 | 7.20 |
| 23 | | | -1.30 | 7.20 |
| 24 | -1.50 | 7.60 | | |
| 25 | 0 | 8.40 | 0 | 8.40 |
| 26 | 9.90 | 8.40 | 9.90 | 8.40 |
| 27 | -1.40 | 8.50 | -1.40 | 8.50 |
| 28 | -2.40 | 8.50 | -2.40 | 8.50 |
| 29 | -3.00 | 9.00 | | |
| 30 | | | 3.50 | 9.00 |
| 31 | | | -2.90 | 9.70 |
| 32 | -3.10 | 9.70 | | |
| 33 | 0 | 9.70 | 0 | 9.70 |
| 34 | -1.60 | 9.80 | -1.60 | 9.80 |
| 35 | -2.80 | 10.00 | -2.80 | 10.00 |

Table 4 (continued)

Migration Distances of Mesophilic Proteins

| Spot number | B. licheniformis | | B. Pumilus | |
|---|---|---|---|---|
| | 1-D | 2-D | 1-D | 2-D |
| | (cm) | | (cm) | |
| 36 | | | 5.10 | 10.70 |
| 37 | 7.80 | 10.70 | | |
| 38 | -1.80 | 10.90 | 1.80 | 10.90 |
| 39 | -3.00 | 11.00 | 3.00 | 11.00 |
| 40 | -3.50 | 11.00 | 3.50 | 11.00 |
| 41 | -3.40 | 11.40 | -3.40 | 11.40 |
| 42 | -1.00 | 11.50 | -1.00 | 11.50 |
| 43 | 0 | 11.60 | 0 | 11.60 |
| 44 | -1.60 | 11.60 | -1.60 | 11.60 |
| 45 | 0 | 12.00 | 0 | 12.00 |
| 46 | -2.40 | 13.05 | -2.40 | 13.05 |
| 47 | -4.00 | 13.05 | -4.00 | 13.05 |
| 48 | -5.50 | 13.05 | -5.50 | 13.05 |

* "-" indicates protein migrates toward the negative electrode in 1-D electrophoresis.

Table 5

Migration Distance of Thermophilic Proteins

| Spot number | B. stearothermophilus 2184 | | B. stearothermophilus 10 | |
|---|---|---|---|---|
| | 1-D | 2-D | 1-D | 2-D |
| | (cm) | | (cm) | |
| 1 | 3.00 | 2.00 | 3.60 | 2.00 |
| 2 | 10.60 | 2.40 | 5.20 | 2.40 |
| 3 | 4.50 | 2.50 | | |
| 4 | | | 3.50 | 3.00 |
| 5 | | | 4.90 | 3.20 |
| 6 | 0 | 6.70 | | |
| 7 | 0 | 6.80 | | |
| 8 | | | 0 | 7.20 |
| 9 | 0 | 7.70 | | |
| 10 | 0 | 8.00 | 0 | 8.00 |
| 11 | | | -1.40* | 8.16 |
| 12 | 8.00 | 8.60 | | |
| 13 | | | -3.00 | 9.20 |
| 14 | 8.00 | 9.60 | | |
| 15 | 5.50 | 9.60 | | |
| 16 | 0 | 9.70 | | |
| 17 | | | 0 | 10.50 |
| 18 | 0 | 10.90 | 0 | 10.90 |
| 19 | -1.50 | 11.20 | | |
| 20 | | | 0 | 11.50 |
| 21 | | | -1.10 | 11.60 |
| 22 | 0 | 11.90 | | |
| 23 | -1.40 | 12.60 | | |
| 24 | 0 | 13.10 | 0 | 13.00 |
| 25 | -1.50 | 13.10 | | |
| 26 | -2.50 | 13.10 | | |

* "-" indicates protein migrates toward the negative electrode in 1-D electrophoresis

Figure 24. B. licheniformis

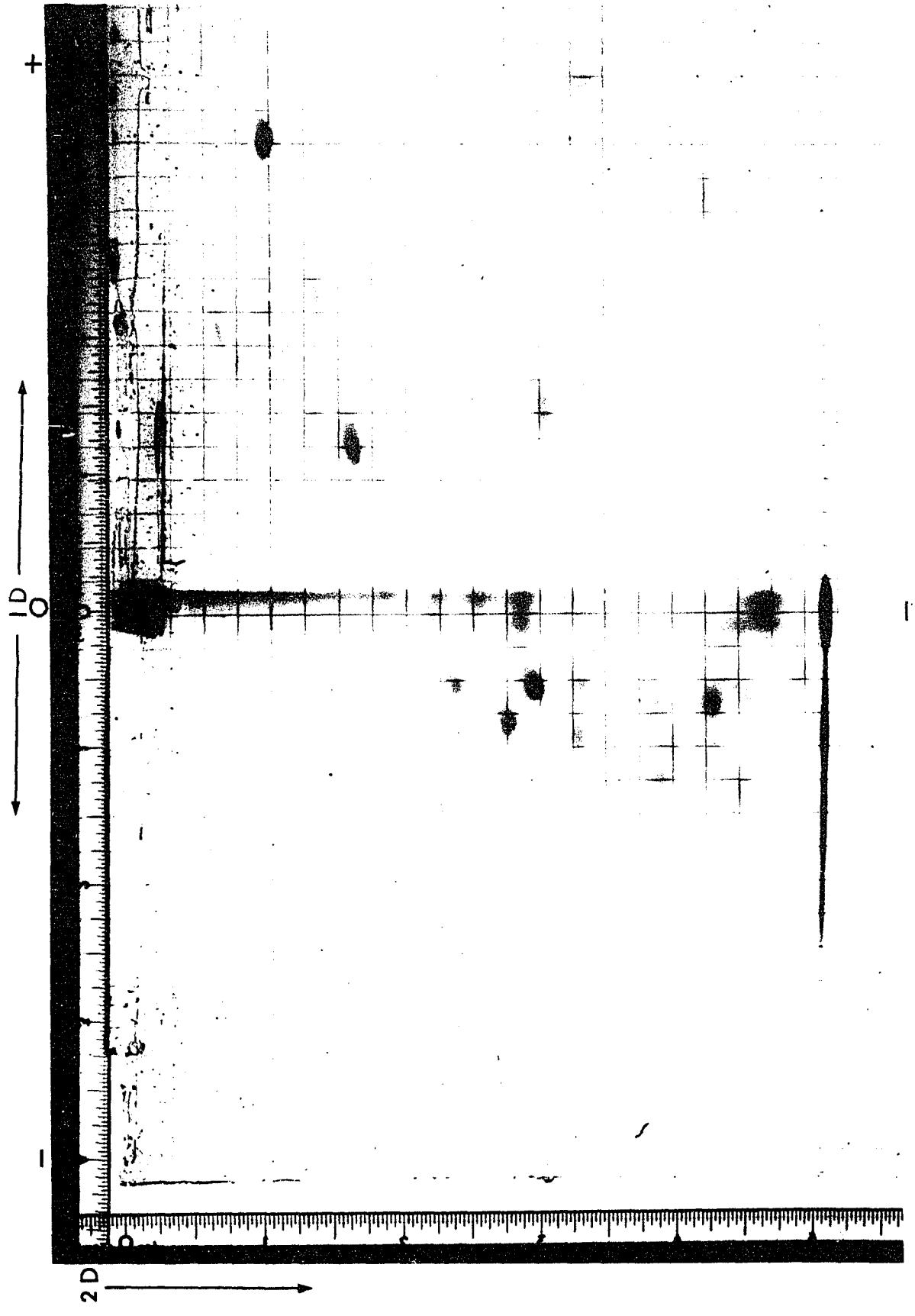


Figure 25. *B. pumilus*

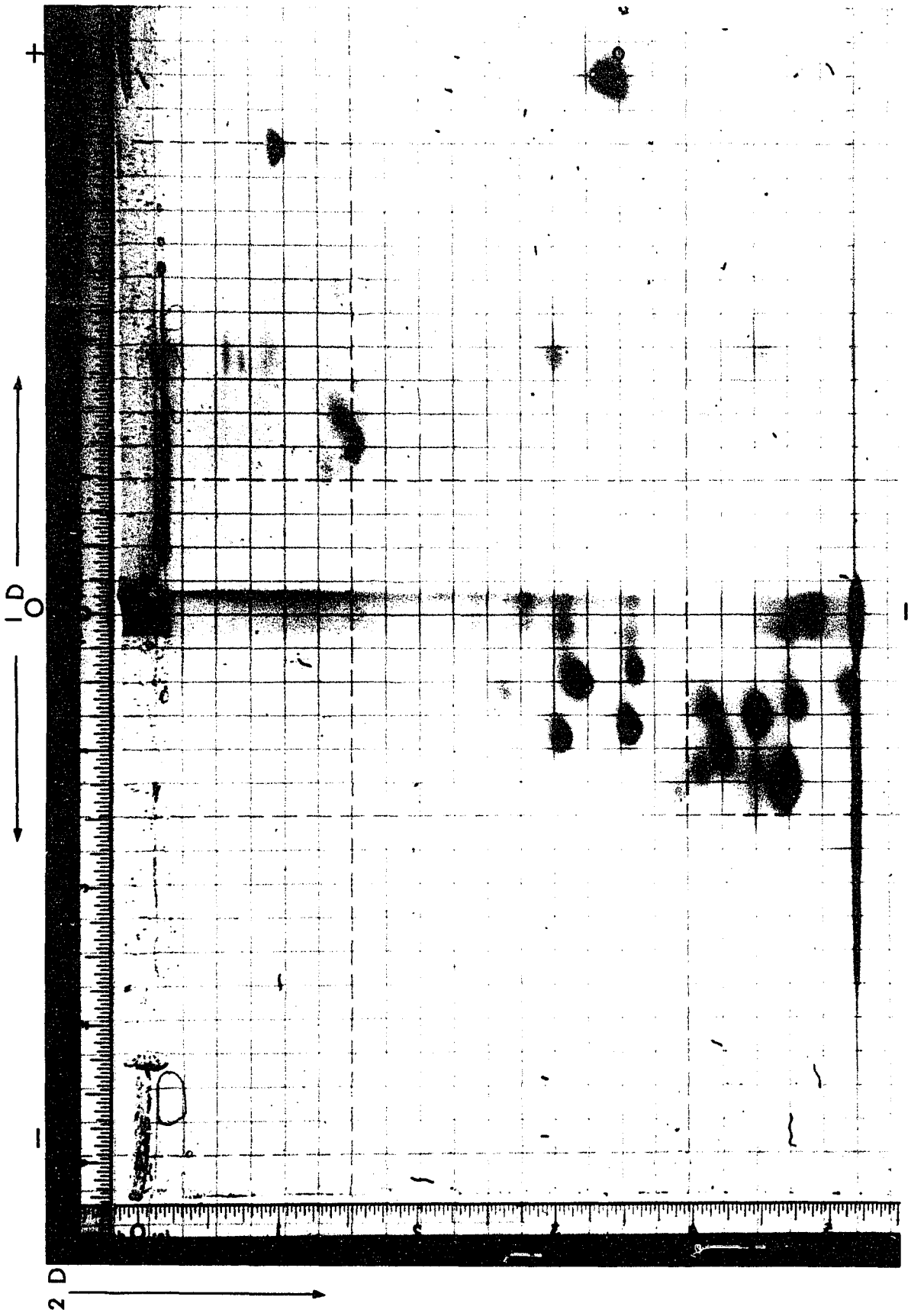


Figure 26. B. 2184

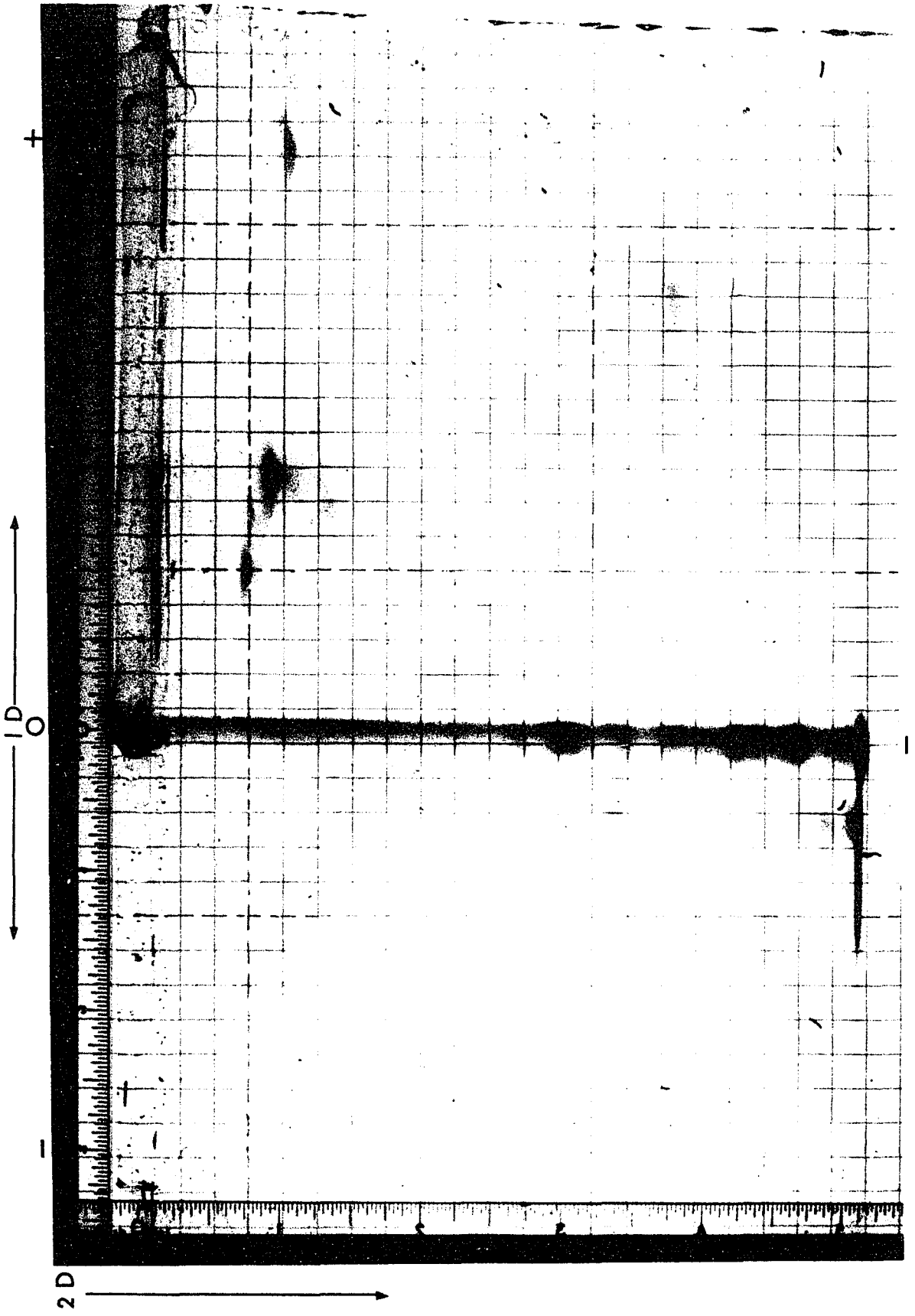
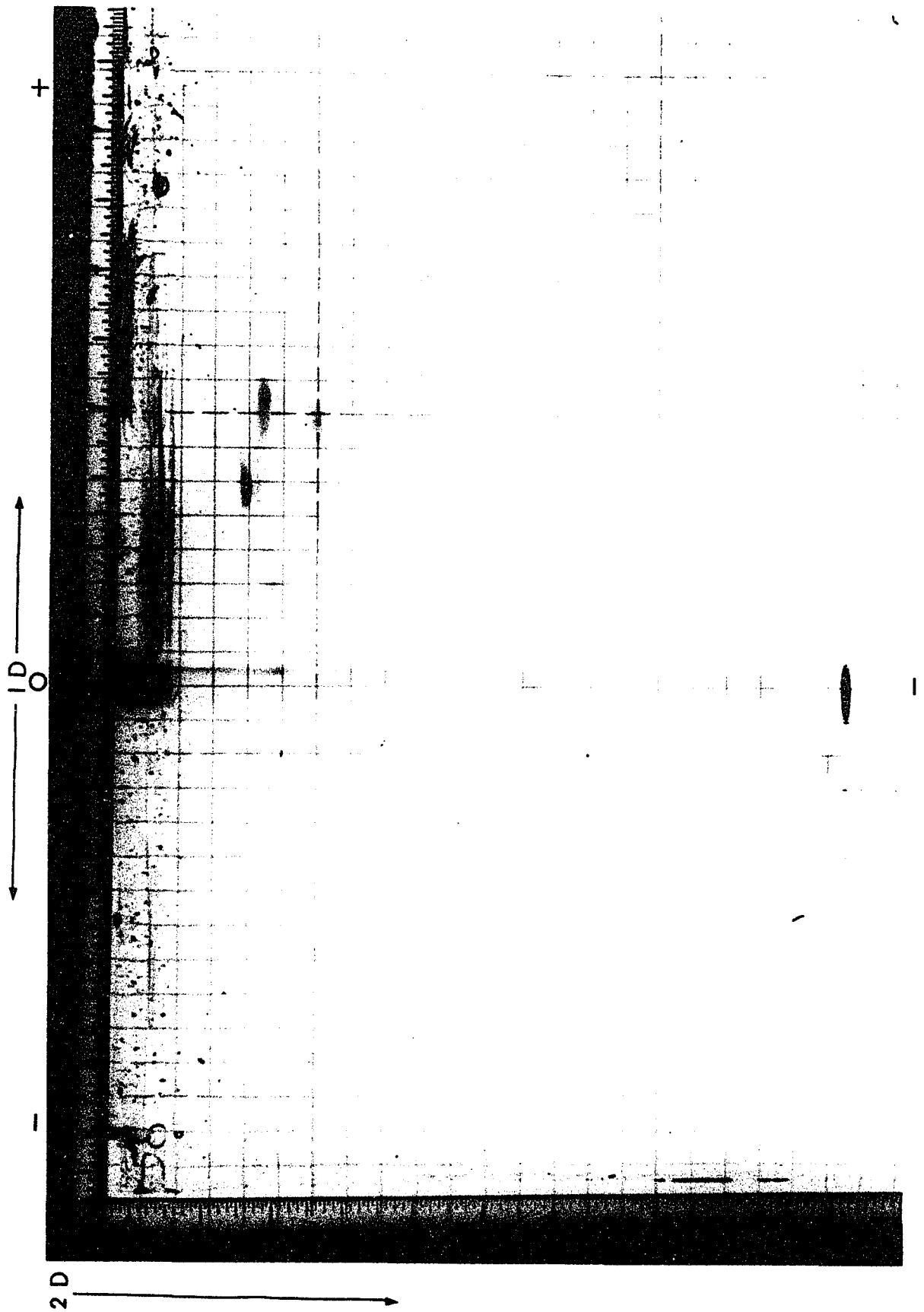


Figure 27. B. 10



It can be seen from Figures 24 & 25 and Table 4 that the resolution of the mesophilic proteins was very good, yielding 48 different proteins on the assumption that each spot represents one protein. The electrophoretic patterns for the ribosomal proteins from B. licheniformis are rather similar to those from B. pumilus though about a third of the proteins have different mobilities. The two patterns are best distinguished by spots 1,3,8,21,36 which are essentially perpendicular to the first dimension in B. licheniformis while the corresponding spots (2,4,7,21,37) in B. pumilus form a staggered pattern.

The thermophilic ribosomal proteins yield 26 different spots which means either that the resolution was less effective than that for the mesophiles or that there is a significant difference in the structure of thermophilic versus mesophilic ribosomes. The electrophoretic patterns of the ribosomal proteins from B. stearothermophilus 10 and 2184 are more unlike than the electrophoretic patterns of the two mesophilic preparations. Furthermore, both thermophilic patterns differed from the two mesophilic ones in having a much smaller number of proteins that migrated as cations in the first dimension.

The present experiments have indicated significant differences between ribosomal proteins of different species

from one genus (B. licheniformis versus B. pumilus or both of these versus B. stearothermophilus) and even between different strains of one species (B. stearothermophilus 10 versus B. stearothermophilus 2184). Hence the report (21) that the ribosomal proteins from E. coli are similar to those of B. stearothermophilus when assessed by one dimensional polyacrylamide disc electrophoresis may have to be modified when these preparations are analyzed by more sophisticated instruments such as the instrument constructed in this study.

The degree of resolution of the mesophilic proteins agrees well with that reported for the proteins from other mesophilic 70s ribosomes where some 50-60 different proteins have been identified (22). Since the recovery of the ribosomal proteins in the present experiments amounted to about 80%, it is conceivable, but unlikely, that the sampling was not entirely random and that a few additional proteins may be identified if the yield of ribosomal proteins approaches 100%. More likely, however, additional proteins will be identified if the solubilization of the ribosomal proteins is improved.

Despite improvements made, the extent of solubilization achieved for the thermophilic proteins was not satisfactory. The mild technique employed here may have to be replaced by more drastic procedures involving detergents (23) and/or organic solvents (24) which have been used to solubilize

ordinarily insoluble proteins. These agents, however, lead to micellar charge inversion and protein denaturation (26). While this is helpful for molecular weight studies (23) where size is the major separation criterion, it is not desirable for studies concerned with the native electrostatic nature of the protein. Since the interactions between the ribosomal proteins and the ribosomal RNA is suspected to be largely electrostatic in nature (25), detergents and organic solvents were avoided in this study. The electrostatic nature of the protein was taken as a major criterion for their separation in the hope of relating peculiarities in the electrophoresis patterns to the phenomenom of thermophily.

## SUMMARY

A two-dimensional polyacrylamide gel electrophoresis apparatus has been designed and constructed. The apparatus is capable of high resolution, exact comparison and simultaneous two-dimensional separation of up to five different samples.

The apparatus has been used for a comparative study of ribosomal proteins from two mesophilic species and two thermophilic strains of the genus Bacillus.

The ribosomal proteins were isolated by the 2-chloroethanol method with a yield of over 80% and with less than 0.5% contamination by ribosomal RNA.

The mesophilic organisms had similar polyacrylamide gel electrophoresis patterns, each consisting of thirty-eight resolved spots. The patterns obtained from the thermophiles were rather dissimilar and showed only 13-18 resolved spots. The lower resolution of the thermophilic proteins was due, in part, to the greater insolubility of these proteins compared to those from the mesophiles.

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VITA

I was born May 19, 1948 in Kalamazoo, Michigan and received my primary and secondary education at Comstock Public Schools. Prior to entering Western Michigan University I received a B.S. degree in chemistry at Adrian College. In 1970 I was married to Jacklynn Flynn and in 1971 and 1973 our sons were born. Currently I am a research assistant at the Harvard Medical School.