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Ribosomal Proteins. VII[1]

Two-Dimensional Polyacrylamide Gel Electrophoresis for Fingerprinting of Ribosomal Proteins

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The analytical separation of complex protein mixtures as they exist in the multiple protein components of ribosomes is an excellent test of the limits of resolution of the particular method. The one-dimensional acrylamide gel electrophoresis has, indeed, brought about considerable progress. It has enabled differentiated separation not only according to charge, but also according to size and shape of the protein molecules. This method, however, shows its insufficiency when the mixture to be examined consists of numerous proteins of similar charge and size. In one-dimensional electropherograms of such mixtures some proteins are superposing, others so closely neighboring one another as to make any distinction impossible.

To separate swiftly and reproducibly the ribosomal proteins from *Escherichia coli* we developed a suitable two-dimensional polyacrylamide gel electrophoresis. By this technique more than 50 different ribosomal proteins, regularly arranged over a wide area, can be revealed. Experiments with two-dimensional electrophoresis were carried out earlier by other authors (4, 5, 7); however, the spots are sometimes placed in a diagonal pattern disadvantageous to a precise analysis. In addition electrofocusing in the first dimension described in one paper (4) cannot be employed for strong basic proteins, e.g., ribosomal proteins.

A special apparatus was constructed which allows the simultaneous two-dimensional electrophoresis of five ribosomal protein mixtures under identical conditions. Thus a series of topical problems in ribosomal research can be examined (see "Results and Discussion").

[1] Ribosomal Proteins, VI; *Analytical Biochemistry* 30, 132 (1969).

METHODS

A. *Principle*

The proteins are placed in the middle of an acrylamide gel (8% acrylamide, pH 9.6) polymerized in a glass tube (180 × 5 mm). In this first dimension the proteins migrate certain distances toward the anode or cathode according to the strength of their charge. After the first run the gel is removed from the tube, dialyzed for a period of three hours against the starting buffer of the next run, and used as horizontal starting gel for the second dimension (gel slab 200 × 200 mm, polymerized with 18% acrylamide, pH 4.6). After the run in the second dimension is terminated, the gel slabs are taken off the chamber and the proteins are made visible by means of amido black staining.

B. *First Dimension* (1-D)

For the 1-D electrophoresis, glass tubes of 180 mm height and 5 mm inner diameter are used. The glass tubes are closed at the bottom with plastic caps and filled up to 70 mm with 1-D separation gel and then overlayered with water. After polymerizing, the water is sucked off with the aid of rolled filter paper.

On the separation gel surface thus prepared, the protein mixture is layered after being mixed with the sample gel. For this purpose lyophilized ribosomal protein is used which is well soluble in sample gel. Two different techniques exist: either one can place the sample gel with a long capillary directly on the surface or one can let it run down on the inner side of the glass tube, rinsing it with a drop of protein-free sample gel. In both cases the final results are equally good. The optimal sample gel volume amounts to 0.1 ml and should not exceed 0.15 ml. It contains up to 4 mg total ribosomal protein mixture for analytical ends, the optimal concentration being 1–2 mg. The sample gel so placed is overlayered with water and then polymerized with light (mercury-tungsten lamp from OSRAM, type HWL, 250 W). After this procedure the water is sucked off the surface by means of a filter paper.

In the next step the glass tube is filled to the brim with 1-D separation gel. After polymerizing this gel, the sample gel is imbedded with plain surfaces between two columns of separation gel. The surfaces of separation and sample gels are netted with one another.

The filled glass tubes are inserted into the circular rubber gaskets (R) of the 1-D apparatus (Fig. 1, right), the shorter section of the separation gel projecting into the cathode buffer vessel (CB). The anode (A) is set on top of the anode buffer vessel (AB) and the cathode (C) on top of the lower one. The voltage applied is 90 V; this makes 2.5 mA per

tube. The electrophoresis is operated for approximately 36 hours. After 15 hours, the 3 liter electrode buffer is replaced by a new one, as TEMED and ammonium peroxodisulfate from the gel has accumulated in the first buffer filling. Then the buffers are circulated via a mixing vessel to prevent eventual electrolyte shiftings. At the end of the run the gels are carefully loosened by injecting glycerin through an injecting needle from both ends of the tubes, and squeezed out under low pressure by the aid of a water-filled syringe. Removal of the gels is facilitated by using new or SDS cleaned and unscratched tubes.

The gels are adapted to the next buffer of the second dimension through dialysis against it (see buffer systems in *Second Dimension* below). For this purpose the gels are placed in a frame subdivided into five sections (Fig. 4, right). The gels are dialyzed against 750 ml starting buffer for 1 hour. This procedure is repeated twice.

The compositions of the gels and buffer of the first dimension are:

Separation gel (pH 9.6)

54.0 gm urea	1.11 gm boric acid
12.0 gm acrylamide	14.55 gm Tris
0.45 gm bisacrylamide	0.45 ml TEMED
1.88 gm EDTA-Na_2	and water to make 148.5 ml

The acrylamide solution is deaerated and cooled before 1.5 ml ammonium peroxodisulfate solution (7%) is added. The solution is sufficient for filling 20 glass tubes.

Sample gel (pH 9.6)

48.0 gm urea	0.115 gm boric acid
5.0 gm acrylamide	0.97 gm Tris
0.2 gm bisacrylamide	0.2 ml TEMED
0.13 gm EDTA-Na_2	water to make 99.0 ml

0.5 mg riboflavin and 5 mg ammonium peroxodisulfate, dissolved in 1 ml water, are added.

Electrode buffer (pH 9.5)

360.0 gm urea	29.1 gm Tris
3.9 gm EDTA-Na_2	made up to 1 liter with water
2.1 gm boric acid	

C. *Second Dimension* (2-D)

An apparatus has been constructed for the second dimension permitting up to five electrophoreses to be effected at the same time and under identical conditions. Diagrams of this apparatus are shown in Figures 1 (left), 2, and 3. Figure 3 (right) represents the basic construction elements of the dismantled apparatus: at the left, part (LP), one of the four middle parts (MP), and at the right, part (RP). These parts are pressed together by means of screw bolts (S).

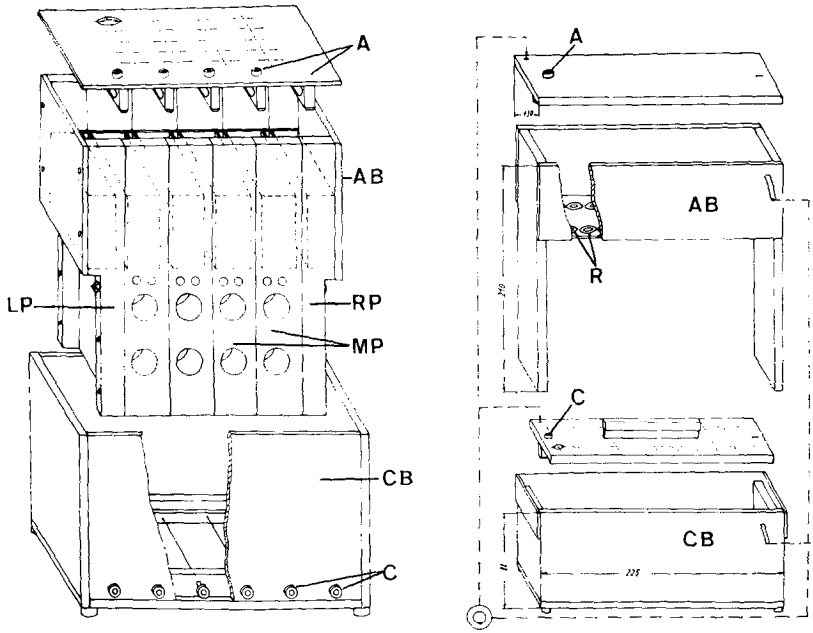

FIG. 1. *Left:* Perspective view of entire 2-D apparatus. Upper part: Plexiglas plate with the anode (A). Middle part: Chamber unit consisting of 4 middle parts (MP) and 2 side parts (LP, RP) screwed together. Above the chambers is the anode buffer container (AB). Lower part: Cathode buffer vessel (CB) with fixed cathode (C). *Right:* Perspective view of 1-D electrophoresis assembly which enables disc tubes of 180 mm length to be held in it. Upper part: Cover with the anode (A). Middle part: Anode buffer vessel (AB) with rubber gaskets (R). Lower part: Plate holding cathode (C) and cathode buffer vessel (CB). The plotted line shows direction of buffer circulation. The material used for the 1-D and 2-D electrophoreses is Plexiglas. All measurements are given in millimeters.

The apparatus may be reduced to the combination of the left and right side parts only, which hold the gel slab sandwich-like. If required, the apparatus can take up several 2-D gel slabs, five at most, provided that the appropriate number of middle parts are used.

The closing of the gel chambers at the bottom is obtained by means of acrylamide gel. A flat vessel corresponding in size to the basal surface of the 2-D apparatus is used for this purpose. This vessel is filled with 600 ml 2-D separation gel, overlayered with 30–40 ml water; then the 2-D equipment is carefully placed into the gel solution. After polymerizing, the water is sucked off the chambers with a rolled filter paper. Then the chambers are firmly sealed, prepared for the gel to be filled in and the 1-D gel to be taken up.

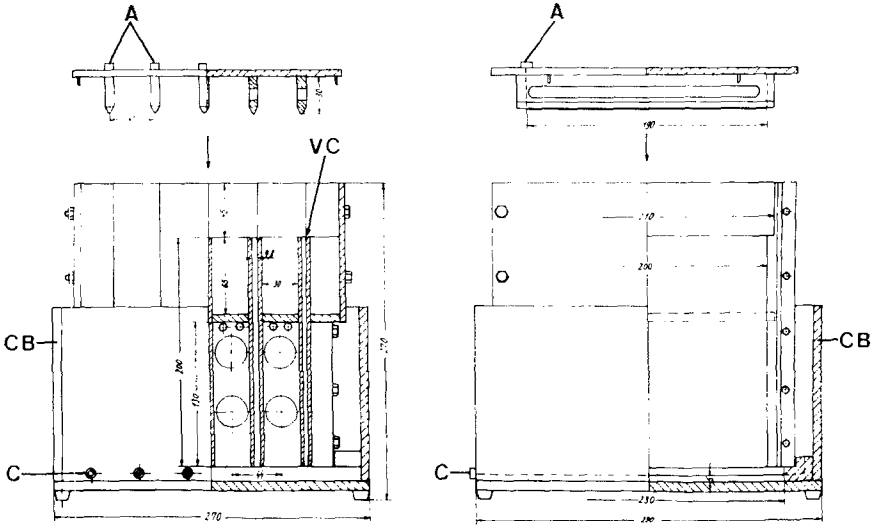

FIG. 2. 2-D electrophoresis. Left: Side view. Right: Front view. The chamber unit is placed in the cathode buffer vessel (CB) carrying the fixed cathode (C). Anode (A) is laid on top. The V-shaped crevice (VC) for the placing of the 1-D gel is visible.

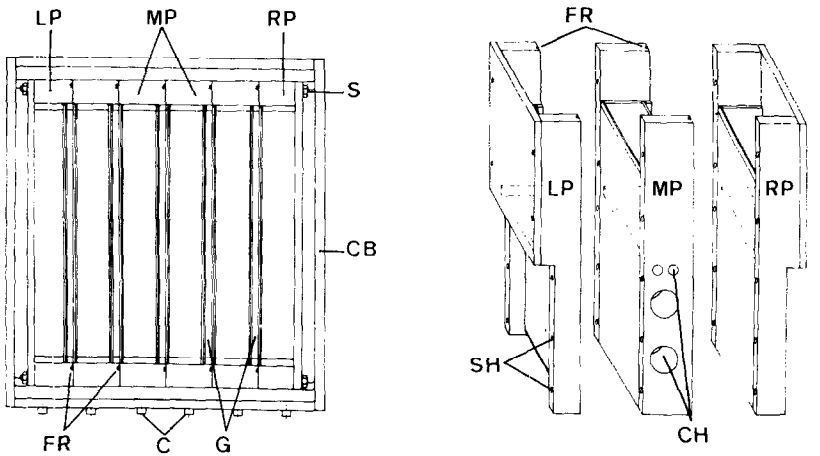

FIG. 3. Left: Top view of 2-D apparatus. The upper electrode is removed. The chamber unit consisting of left, middle, and right parts (LP, MP, RP) is placed in cathode buffer vessel (CB). The spaces for filling in the 2-D gel are visible (G). The single parts are sealed with foam-rubber gaskets (FR). The chamber parts are pressed together by aid of screws (S). Right: Perspective view of three basic construction elements (LP, MP, RP). The holes (CH) visible in the middle part assure circulation of the buffer and removal of the air. Also visible are the screw holes (SH) in all parts.

The dialyzed 1-D gel (see technique described above) is laid into the V-shaped crevice (VC, Fig. 2) at the upper end of the 2-D chambers in a horizontal position. After these preliminary steps the separation gel solution is filled in with the aid of a funnel; care is taken to avoid bubbles. The 2-D gel must rise high enough to surround the horizontal 1-D gel without covering it completely. In case air bubbles have gathered below the 1-D gel, it is lifted from one side and the bubbles are carefully removed. This procedure allows enough time for filling the five chambers with the same mixture of acrylamide and ammonium peroxodisulfate. A slight sinking of the gel level is frequently inevitable due to volume modifications of the Plexiglas chambers resulting from heat during the polymerization process. Therefore it is advisable to add some milliliters of new gel during this period. The gel solution is kept in ice water before pouring in, and the apparatus at 4°C for 2 hours to avoid a too quick polymerization of the acrylamide.

The filled apparatus is lifted from the flat vessel serving as closing device, cleaned from the superfluous gel rests, rinsed with distilled water, and put into the cathode buffer vessel (CB, Fig. 1, left), which is filled to the brim with electrode buffer. Then buffer is likewise poured into the anode buffer container (AB) of the apparatus. Altogether 11.5 liter electrophoresis buffer is used. Due to this large volume, no circulation of the buffer during the run is required. A cover plate is placed on top of the apparatus, to which the upper electrodes are fixed so that they are located directly above the chamber crevices, working as anodes (A). The cathode electrodes (C) are situated near the bottom of the lower buffer vessel (CB) between the single chambers.

The electrophoresis in the second dimension is carried out at a voltage

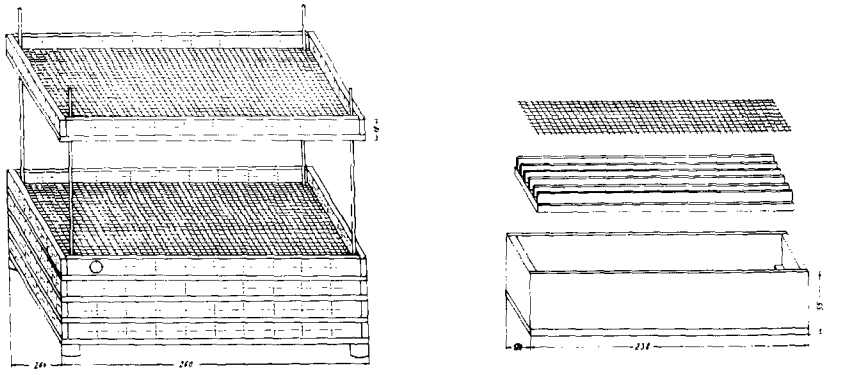

FIG. 4. Left: Frame for staining and destaining procedures. The slabs are placed on stainless-steel nets. Right: Dialysis frame for 1-D gels to be placed into the five divided sections.

of 105 V and an initial current of 480 mA. The voltage applied is kept constant for a working time of about 26 hours.

After the run, the slabs are taken out of the chambers. This is achieved by opening the screw bolt links and by a step-for-step dismantling of the apparatus.

The removed slabs are placed for 15 minutes into a solution of the following composition: 5.5 gm amido black, 50 ml glacial acetic acid, and water added up to 1 liter. Thereafter the slabs are exposed to running water for approximately 1 hour and further destained in 1% acetic acid for 40 hours. During these procedures the slabs are stored in a multi-storeyed frame, shown in Figure 4, left. The gel slabs so prepared can be analyzed and photos can be taken in transparent light. To determine more closely the protein spots, a precise measurement of the migration distances is recommended.

The following gel and buffer systems are used for the second dimension:

Separation gel (pH 4.6)

360.0 gm urea	52.3 ml glacial HAc
180.0 gm acrylamide	9.6 ml 5 *N* KOH
5.0 gm bisacrylamide	5.8 ml TEMED

and water to make 967.0 ml; for polymerizing, 33 ml of a 10% ammonium peroxodisulfate solution is added.

Starting buffer (= dialyzing buffer for the 1-D gel)

480.0 gm urea	2.4 ml 5 *N* KOH
0.74 ml glacial HAc	made up with water to 1.0 liter

Electrode buffer (for anode and cathode)

140.0 gm glycine
15.0 ml glacial HAc
made up with water to 10.0 liters

RESULTS AND DISCUSSION

The use of this technique enables us to resolve the complex protein mixture extracted from 70 S ribosomes of *E. coli* B into more than 50 single components (Fig. 5). This means that the efficiency of this method is at least twice as high as that ascertained by the 1-D electrophoresis.

The protein spots occupy specific positions on the slab; their R_f values can be measured from the starting point to both migrating directions. The coordinates of the R_f values permit an unequivocal determination of the position of the protein spots on the 2-D slab.

An alternative way for the clear determination of protein spots is provided by adding a 70 S protein mixture in small quantities. We employ this method when the ribosomal proteins isolated by preparative techniques are to be correlated with one or another of the 70 S proteins.

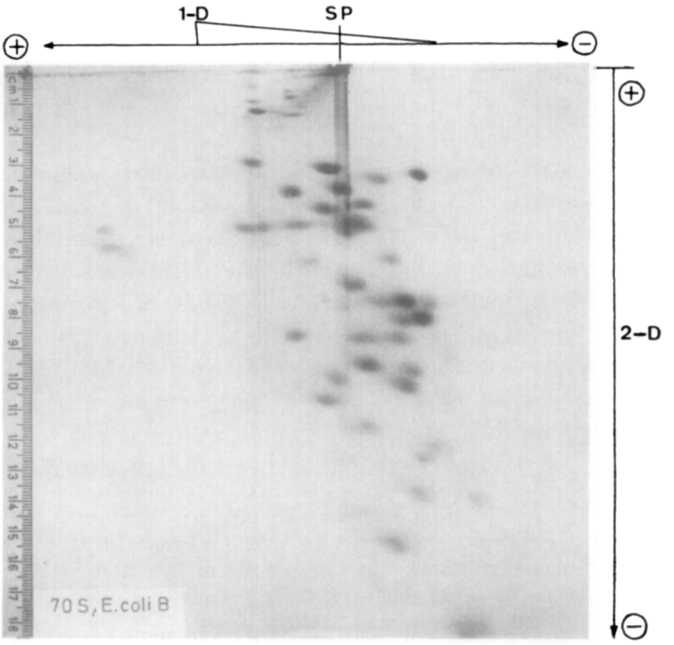

FIG. 5. 2-D electropherogram of 70 S ribosomal proteins from *E. coli* B extracted with 66% acetic acid and 3×10^{-3} *M* Mg. The ribosomes were obtained by differential centrifugation and washed with 0.5 *M* NH_4Cl. Migration directions are marked with arrows; positions of electrodes in the 1-D and 2-D steps are designated with ⊕ and ⊖. (For buffer and gel conditions see text.) SP indicates starting point. The scale on all slabs is given in centimeters.

For this purpose the quantity of the single protein is 10–20 times greater than that of a protein in the 70 S mixture. We use 80–100 μg isolated protein mixed with 8 μg $\times$ 50 (number of spots) = 400 μg of 70 S protein. Then a distinct spot is visible on the background of the 70 S proteins (Fig. 7). By comparing the spot of an isolated protein with the protein spots of 30 S or 50 S subunits the origin of this protein with regard to one of the subunits can be revealed. The protein patterns of both subunits of *E. coli* B are shown in Figure 6.

These experiments demonstrate that a single protein, either alone or in combination with others, takes the same specific position on the slab. The migration distance is not dependent on the protein concentration over a wide range.

We have compared the protein of the ribosomal subunits of the strains *E. coli* K 12 and *E. coli* B on the 2-D electrophoresis. No differences between the 50 S subunits are detected. In the 30 S subunits, however, at

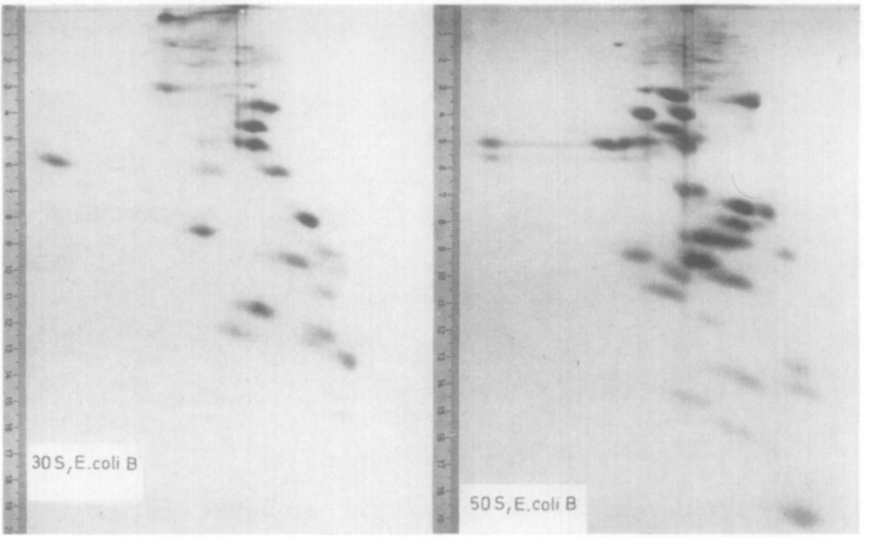

FIG. 6. Left: 30 S subunit proteins. Right: 50 S subunit proteins from *E. coli* B. Superimposing the patterns of the two subunits results in the pattern of the 70 S proteins.

least two different proteins have been found. Leboy *et al.* (3) have detected one different protein by means of 1-D acrylamide electrophoresis. Otaka *et al.* (6), in conformity with our 2-D electrophoresis results, have discovered the existence of two different proteins in the 30 S subunits by means of differentially labeled proteins, separated by CM-cellulose chromatography.

The 2-D method described opens new aspects to the exploration of ribosomal structures. At present the following studies are carried out with 2-D method:

(1) Comparing patterns and numbers of ribosomal proteins from different organisms and different cell organelles.

(2) Investigating the existence of heterogeneous populations of ribosomes in bacteria in different stages of function or growth.

(3) Testing differences in ribosomal proteins between wild type and mutants with altered ribosomes.

(4) Comparing proteins which are obtained in different stages of salt splitting from ribosomal subunits and of reconstitution.

(5) Assessing the utility of methods for group fractionations and extraction conditions of ribosomal proteins.

The sensitivity of the 2-D method can be increased by using differentially labeled proteins in some of the above-mentioned studies. Further-

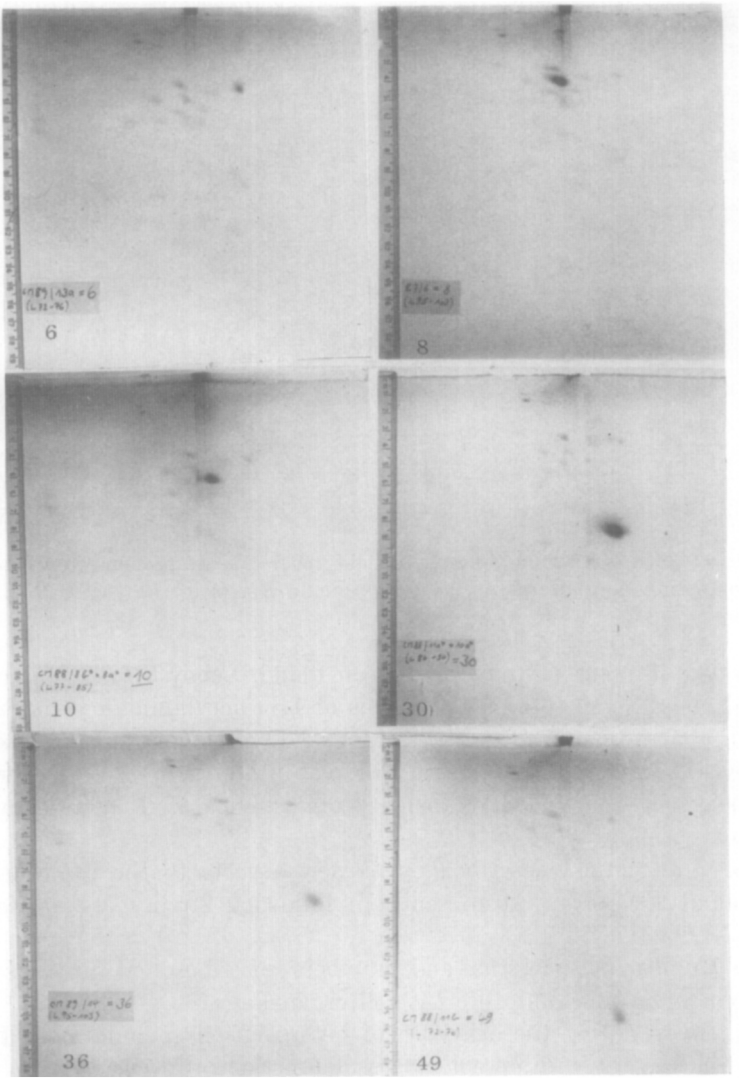

FIG. 7. Experiments to correlate isolated ribosomal proteins to one of the 70 S proteins. A dark spot of the isolated protein and a background of 70 S proteins used as reference are visible. The proteins have been designated by a provisional nomenclature. They are numbered in rows starting at the left upper corner and finishing right down.

more we are working on the modification of our present 2-D system in order to separate other complex protein mixtures. Another aim is to use the 2-D electrophoresis for the preparative separation of ribosomal proteins.

SUMMARY

A two-dimensional polyacrylamide gel system has been developed, improving the analytical separation of complex protein mixtures as obtained from ribosomes. An equipment is described in detail (Figs. 1–4) which permits a simultaneous electrophoretic separation of five protein mixtures under identical conditions.

By this method the protein mixtures of *E. coli* ribosomes can be resolved into about 50 components (Fig. 5). The use of this fingerprinting technique facilitates and accelerates considerably a number of investigations on the structure of ribosomes.

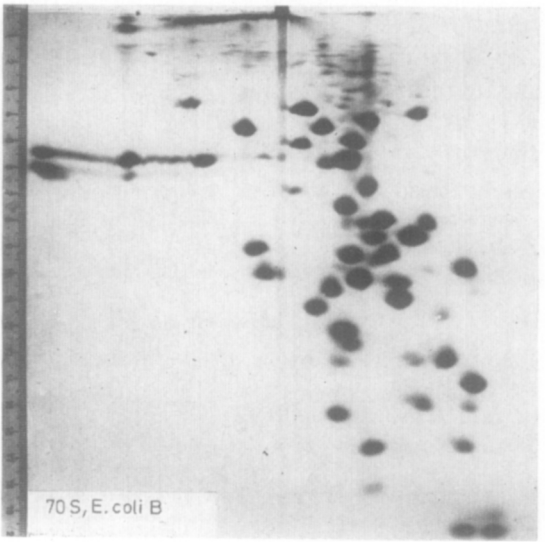

FIG. 8. 2-D electropherogram with the new system in the first dimension (pH 8.6, 4% acrylamide). Run time in the 1-D is 20 hours. The gel in the second dimension remains the same as in Figures 5 and 7. Applied is 2.0 mg protein mixture of *E. coli* B, prepared by the NH_4SO_4 precipitation method (2). The protein is extracted with 66% acetic acid and $3 \times 10^{-2} M$ Mg (1).

ADDENDUM

Since this manuscript was submitted, another gel system has also been employed for the first-dimensional step. The gel composition is as follows:

Separation gel (pH 8.6)

54.0 gm urea	4.8 gm boric acid
6.0 gm acrylamide	7.3 gm Tris
0.2 gm bisacrylamide	0.45 ml TEMED
1.2 gm EDTA-Na_2	and water to make 148.5 ml

For polymerizing 1.5 ml of a 7% ammonium peroxodisulfate solution is added.

Sample gel

48.0	gm urea	0.32 gm boric acid
4.0	gm acrylamide	0.06 ml TEMED
0.2	gm bisacrylamide	and water to make 99.0 ml
0.085	gm EDTA-Na_2	

0.5 mg riboflavin and 5 mg ammonium peroxodisulfate, dissolved in 1 ml water, are added.

Electrode buffer (pH 8.6)

360.0 gm urea	14.55 gm Tris
2.4 gm EDTA Na_2	made up to 1 liter with water
9.6 gm boric acid	

This particular system provides better resolution properties in respect to some proteins. Its result is shown in Figure 8.

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REFERENCES

1. Hardy, S. J. S., Kurland, C. G., Voynow, P., and Mora, G., *Biochemistry* **8**, 2897 (1969).
2. Kurland, C. G., *J. Mol. Biol.* **18**, 90 (1966).
3. Leboy, P. S., Cox, E. C., and Flaks, J. G., *Proc. Natl. Acad. Sci. U. S.* **52**, 1367 (1964).
4. Macko, V., and Stegemann, H., *Z. Physiol. Chem.* **350**, 917 (1969).
5. Margolis, J., and Kenrick, K. G., *Nature* **221**, 1056 (1969).
6. Otaka, E., Itoh, T., and Osawa, S., *J. Mol. Biol.* **33**, 93 (1968).
7. Raymond, S., *Ann. N. Y. Acad. Sci.* **121**, 350 (1964).