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Two-Dimensional Gel Electrophoresis of Membrane Proteins†

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ABSTRACT: A high-resolution method for two-dimensional separation of membrane proteins is described. It involves a nondiscriminating solubilization of a membrane preparation with sodium dodecyl sulfate, followed by electrophoresis in the first dimension according to charge (by isoelectric focusing). The electrophoresis in the second dimension is in the presence of sodium dodecyl sulfate, thus separating proteins on the basis of molecular weight. Electrophoresis in the first dimension is either on a thin slab gel, or on a small-diameter tube; electrophoresis in the second dimension is on a thin slab gel. Up to 100 μg of protein can be analyzed. The two-dimensional system is a modification of the one recently described by O'Farrell (1975). About 150 different proteins can be visualized in *Escherichia coli* or *Salmonella typhimurium* cell envelopes; examples of differences between mutant and wild-type strains are presented. The method is applicable also to membrane preparations from other sources: a two-dimensional separation of plasma membrane proteins from HeLa cells is presented.

One of the main problems encountered in the study of membrane proteins is the lack of an adequate assay for their presence and of good methods for their separation. Recently several one-dimensional electrophoretic methods, mainly on acrylamide gels in the presence of sodium dodecyl sulfate (Ames, 1974), have been applied successfully to the analysis of membrane proteins (for example, Ames, 1974; Fairbanks et al., 1971; Neville, 1971; Schnaitman, 1973). By use of this powerful method about 30–60 protein bands can be identified and separated in *Escherichia coli* and *Salmonella typhimurium* membranes (Ames, 1974).

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Among the studies performed with sodium dodecyl sulfate acrylamide gel electrophoresis have been several involving analysis of the proteins of the outer cell membrane in both wild-type and mutant strains (Ames et al., 1974; Koplow and Goldfine, 1974), the identification of a succinic dehydrogenase subunit (Spencer and Guest, 1974), of nitrate reductase components (MacGregor and Schnaitman, 1971), of proteins involved in the conjugation process (Skurray et al, 1974), of proteins involved in colicin tolerance (Chai and Foulds, 1974) and many others. However, in all of these cases the proteins involved are major membrane proteins. Minor components, or even relatively abundant ones, cannot be distinguished if their molecular weight is the same as that of one of the major components: thus the resolution obtained by one-dimensional dodecyl sulfate[1] gel electrophoresis is not adequate in many cases.

In this paper we present a two-dimensional separation of membrane proteins which has a much greater power of resolution. At the basis of a two-dimensional separation is the spreading of proteins in each of two dimensions, each according to a completely different principle. Thus, while one dimension can be electrophoresis in the presence of dodecyl sulfate, which separates proteins on the basis of their molecular weight, the separation in the other dimension could be on the basis of protein charge. Using these two parameters, O'Farrell (1975) developed a high-resolution, two-dimensional separation to be performed on radioactive whole cell protein. We adapted his elegant method to use with bacterial envelopes, which in *E. coli* and *S. typhimurium* constitute about 10–15% of the whole cell protein (Ames, 1974). Our method involves solubilization with dodecyl sulfate prior to the two-dimensional separation. We assessed the power of resolution of membrane proteins by analysis of a variety of both unlabeled and labeled membrane preparations, from wild-type and mutant strains, from *E. coli* and *S. typhimurium*. We also applied the method successfully both to a plasma membrane preparation from HeLa cells and to an Influenza virus preparation.

Experimental Procedure

Materials. Acrylamide was from either Bio-Rad or Eastman Kodak. *N,N'*-Methylenebisacrylamide was from Bio-Rad. *N,N,N',N'*-Tetramethylethylenediamine (TEMED)[1] was from Eastman-Kodak. Ammonium persulfate was from Mallinckrodt. Nonidet P-40 (NP-40) was from Particle Data Laboratories. Urea was "Ultra Pure grade" from Schwarz/Mann. Sodium dodecyl sulfate was from Gallard-Schlesinger. The proteins used as molecular weight standards are, from top to bottom of dodecyl sulfate gels: β-galactosidase (130 000); phosphorylase *a* (94 000); bovine serum albumin (68 000); catalase (60 000); glutamic dehydrogenase (53 000); ovalbumin (43 000); horse liver alcohol dehydrogenase (37 000); beef heart lactic dehydrogenase (36 000[2]); histidine-binding protein J (25 500). The numbers in parentheses are the respective subunit molecular weights. The source of these proteins and references to their molecular weights are as described in the work of Ames (1974). All other chemicals were from standard commercial sources. *E. coli* JF404 (K12, HfrH *thyA*) and its derivative JF404-2a (HfrH *thyA tolG2*) were obtained from J. Foulds and grown as described in the paper of Chai and Foulds (1974). *E. coli* WGAS (K12, W3110 *trpA*) and its derivative defective in succinic dehydrogenase, WGAS *sdh9*, were obtained from J. R. Guest and grown as described in the paper of Spencer and Guest (1974). *S. typhimurium* TA1857 (strain LT2, wild-type) was grown in a minimal medium (Vogel and Bonner, 1956) with glucose (0.4%) as a carbon source. HeLa cell plasma membranes and an Influenza virus type A preparation were kindly supplied by E. E. Penhoet.

Cell Envelope Preparation. Cells are harvested during late exponential growth and resuspended in one-hundredth the original volume of 1 mM Tris-HCl (pH 7.8) containing 1 mM $MgCl_2$. All operations are conducted at 0–4°. The cells are disrupted with a French pressure cell at 8000 psi. The crude extract is centrifuged at 6000*g* for 20 min and the supernatant is centrifuged again at 41 000*g* for 60 min. The pellet is washed twice by resuspending it in the same buffer and centrifuging. The final, washed pellet (cell envelope) is resuspended in the same buffer at a protein concentration of 25–30 mg/ml, and stored at −20°. Preparations are used immediately or at most within 2 weeks. Longer storage causes the appearance of artifactual spots.

The effect of RNase and DNase pretreatment on the sharpness of focusing in the neutral pH range was determined by adding RNase (20 μg in 10μl of 0.1 M Tris-HCl (pH 6.8)) to an envelope preparation (3 mg of protein in 100 μl), followed by sonication and DNase treatment as described by O'Farrell (1975). The reaction mixture was centrifuged at 40 000*g* for 60 min and washed once with 10 volumes of 1 mM Tris-HCl (pH 7.8)–1 mM $MgCl_2$. The final pellet was resuspended to a final volume of 100 μl of the same buffer and solubilized as usual.

Solubilization. A volume of cell envelope preparation containing about 3 mg of protein is placed in a heavy wall glass centrifuge tube (5 ml capacity); to it are added: 0.5 M Tris-HCl (pH 6.8), 20 μl; 10% dodecyl sulfate (w/v), 40 μl; 0.1 M $MgCl_2$, 1 μl; water to 200 μl. The tube is capped with Parafilm, incubated at 70° for 30 min, then centrifuged at 40 000*g* in the Sorvall SE12 rotor for 60 min. The supernatant, containing the solubilized protein, is removed with a micropipet and kept at 0° until ready to use on the same day. Under these conditions the ratio (w/w) of the dodecyl sulfate/protein is 1.3. In some experiments the amount of dodecyl sulfate was altered to give a ratio of 2.6 or 0.77. When mercaptoethanol was added during solubilization, its concentration was 10% (v/v). We have not stored the solubilized protein, though it may be possible to do so.

Sample Preparation. The freshly solubilized envelope protein is diluted with two volumes of sample dilution buffer which contains: 9.5 M urea, 2% ampholines (comprising 0.4% pH 3.5–10, 0.8% pH 5–6, 0.8% pH 6–8), 5% mercaptoethanol (v/v), and 8% NP-40. The final composition of the sample thus prepared is somewhat different from that described by O'Farrell (1975). In some cases the final urea concentration was raised by adding 15 mg of solid urea to 20 μl of solubilized protein, and allowing it to dissolve at room temperature, prior to dilution with the sample dilution buffer. It is important to use a ratio of the final percentages of NP-40 to dodecyl sulfate of 8, otherwise there will be streaking in the neutral area of the gel. Once the sample has been prepared, it is run the same day. We have not tested whether it can be stored frozen.

[1] Abbreviations used are: TEMED, *N,N,N',N'*-tetramethylethylenediamine; NP-40, Nonidet P-40; IF, isoelectric focusing. Throughout this paper dodecyl sulfate refers to the sodium salt.

[2] The molecular weight of beef heart lactic dehydrogenase has been reported to be 36 000 (Weber and Osborn, 1969). However, it consistently runs with an apparent molecular weight of 30 500. This material was purchased from Sigma.

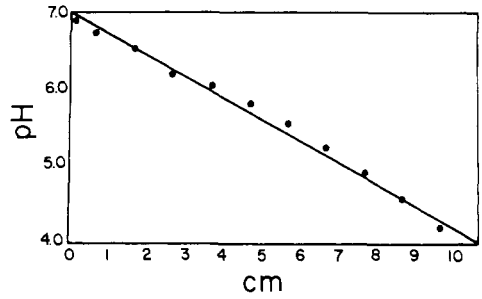

FIGURE 1. pH gradient for the isoelectric-focusing gel (first dimension) used in Figure 2. The zero point on the abscissas is the bottom of the well, i.e., the origin of the electrophoresis.

Isoelectric Focusing. The first dimension (isoelectric focusing) is as described by O'Farrell (1975) with the following modifications. The total concentration of ampholines is 2% and it comprises either of the following pH ranges: (a) 4-6, 6-8, and 3.5-10 in the ratio of 2:2:1; (b) 4-6, 6-8, and 7-9 in the ratio of 1:1:1. Polymerization is achieved by riboflavin and exposure to light, thus obviating the addition of ammonium persulfate. Riboflavin is added from a stock solution (0.14 mg/ml) containing also TEMED (1%): 0.4 ml is added to 15 ml of final acrylamide gel mixture. The gel is made in a 1-mm thick slab apparatus assembled as described (Ames, 1974) and containing a comb with 13 teeth, each 6 mm wide. Alternatively, the above acrylamide gel mixture can be used for filling small glass tubes which have been prepared as described by O'Farrell (1975) and overlayered with 8 M urea. Polymerization is started by exposing the slab (or the tubes) to a Luxo fluorescent 15-W light (placed about 5 cm away) for 30 min. At this time the apparatus is removed from the clamp stand (Ames, 1974) and exposed to light for 30 more min, to allow polymerization of any gel areas which might have been screened from the light.

After polymerization, the comb is removed, each well or tube top surface is emptied of any remaining liquid with a syringe and fine needle, and without any further cleaning of the wells, the slab is mounted on the electrophoresis apparatus as described (Ames, 1974). The tubes are mounted in a standard cylindrical gel apparatus as described (O'Farrell, 1975). Without any prerunning procedure, the prepared samples are placed into the wells by pipetting 25 μl (or more, if necessary) with a Hamilton syringe. Ten microliters of a sample overlay solution (O'Farrell, 1975) is added to each of the wells (or the tubes), which are then filled to the top with the cathode electrode solution (0.02 M NaOH, degassed by boiling, and stored under vacuum). The cathode reservoir is filled with the cathode electrode solution, and the anode reservoir is filled with the anode electrode solution (0.01 M H_3PO_4). The electrophoresis is performed at 300 V for 18-19 h, followed by 400 V for 1.5 h.

Samples which will undergo a separation in the second dimension are placed in the central area of the slab with empty wells on either side.

At the end of the run, the slab is disassembled as described (Ames, 1974), taking care to allow the gel slab to stick in position to either one of the glass plates. The plate, with the gel slab, is placed on a template which indicates the position of the wells and the vertical edges of each run. The strips to be run in the second dimension are cut out of the gel by pressing down with the edge of a large stainless steel spatula. For easy cutting, the edge of the spatula should be quite straight, and no sawing motion is performed. The strip is picked up by inserting a spatula underneath the whole length of its vertical edge and transferred to a dodecyl sulfate slab gel for the second dimension.

The pH gradient is obtained from the center strip corresponding to a well where no sample was placed. The strip is cut transversally in 1-cm pieces, each of which is then placed in a test tube containing 1 ml of water. After standing about 18 h the pH values are determined. A representative pH gradient thus obtained is shown in Figure 1.

Those portions of the slab which will be stained are placed in 10% trichloroacetic acid for 18 h. They are then stained in Coomassie Blue (1 mg/ml) in ethanol-acetic acid-water (9:2:9) for 2 h. Destaining is in three changes (1 h each) of ethanol-acetic acid-water (25:8:65). The gel is finally equilibrated with 5% acetic acid prior to drying.

The cylindrical gels are removed from the glass tubes, equilibrated, and stored frozen as described (O'Farrell, 1975).

Sodium Dodecyl Sulfate Gel. A 10% dodecyl sulfate acrylamide gel slab, about 2 mm thick, is prepared as described (Ames, 1974), except that the stacking gel is 2 cm high, has no wells, its top surface is flat, and has been overlayered with a buffer of the same composition as that in the stacking gel mixture (stacking buffer). This leaves about 1 cm of space between the top of the stacking gel and the edge of the notch in the glass plate: this is the space which will be occupied by the isoelectric focusing strip. After the stacking gel has polymerized the overlayered stacking buffer is removed and the isoelectric focusing strip is allowed to slide in place with the aid of a small amount of the same buffer (enough to cover the strip). This buffer is removed with a syringe and replaced once with fresh buffer. To keep the strip in place, the buffer is thoroughly removed, and warm (45°) 0.5% agarose (Seakem, MCI Biomedical) dissolved in the stacking buffer is added with a Pasteur pipet. The whole operation, from the cutting of the isoelectric-focusing gel to the adding of the agarose, takes no more than 15 min: longer times may cause diffusion of the protein bands.

If necessary, for increased sensitivity of autoradiography, the thickness of the dodecyl sulfate slab gel can be reduced, by using Lucite spacers of appropriate thickness for both dimensions. Lucite sheets are available in a variety of thicknesses, and because of their irregularity it is possible to obtain spacers of a wide variety of thickness. We had good results with a first dimension gel 0.75 mm thick and a second dimension gel 1 mm thick: i.e., a 30% increase in thickness between the first and the second gel is adequate for comfortable manipulations.

If desired, it is possible to make a well in the agarose to use for a molecular weight standard. A small Lucite strip is inserted between the two plates, at one end of the slab, prior to addition of the agarose. After the agarose has solidified, the Lucite strip for the molecular weight is removed; the slab is mounted as usual on the electrophoresis apparatus. After the molecular weight sample has been applied the electrophoresis is started at 25 mA (initial voltage: 60 V) for 1 h; then it is raised to 50 mA, and the run continued until the dye in the molecular weight standard reaches the bottom. If the second dimension slab is thinner than 2 mm, the amperage should be decreased appropriately (15 mA initial amperage, then raised to 30 mA, for a 1 mm thick slab).

Equilibrated cylindrical gels (either stored frozen or not) are applied to a second dimension dodecyl sulfate acrylam-

ide gel slab, 1 mm thick, prepared as described above, except that the stacking gel is about 2.5 cm high and its flat top surface resides immediately below the level of the notch in the glass plate. The cylindrical gel (2 mm diameter) is placed on the inclined slab gel apparatus, where it rests on the edge of the glass notch. Hot agarose-containing solution O (O'Farrell, 1975) is pipetted below and on top of the cylindrical gel, being careful not to trap air bubbles below it and thus sealing it in place (P. H. O'Farrell, personal communication). The electrophoresis is then run as usual.

At the end of the run the gel is stained and dried as described (Ames, 1974). An occasional gel needs further destaining with ethanol–acetic acid–water (25:8:65) for 1 hr prior to drying. If the gel is 2 mm thick, the temperature of the water bath used for drying the gel is kept at about 70° and drying takes about 1 h. This avoids cracking of the gel during drying. If the separation involved labeled protein, the dry gel can be autoradiographed.

Following the convention used by O'Farrell (1975), each slab gel has been photographed with metric rulers along the vertical and horizontal sides. The origin of the ruler indicates the origin of the electrophoresis, which is by isoelectric focusing (IF) from left to right, and by dodecyl sulfate electrophoresis from top to bottom. Individual spots are referred to by coordinates, read on the two rulers: the horizontal coordinate precedes the vertical one.

Results and Discussion

The Two-Dimensional System

Solubilization. In developing this method of membrane protein analysis we aimed at obtaining an extensive and reproducible solubilization immediately followed by an electrophoretic separation in a first dimension, which takes advantage of intrinsic protein charge (by isoelectric focusing). An important reason for separating proteins by isoelectric focusing as the first dimension, is that much larger amounts of protein can be applied to an isoelectric-focusing gel without running into overloading problems (as compared to what can be applied to a dodecyl sulfate gel); thus unlabeled membrane proteins can be analyzed effectively also in cases where it is not feasible to obtain or to process labeled material. The isoelectric-focusing gel can then be exposed to separation in the second dimension according to molecular weight (dodecyl sulfate gel electrophoresis).

In the O'Farrell method (1975) the proteins in whole cells are denatured and solubilized by treatment with urea, the nonionic detergent NP-40,[3] and mercaptoethanol; the solubilized sample is then applied to the isoelectric-focusing gel, which also contains both urea and NP-40. In our hands, this solubilization procedure, when applied to whole cells (with freezing and thawing, but without sonication), does not fully solubilize all membrane proteins. In fact, if a radioactive sample of *S. typhimurium* whole cells is solubilized according to this procedure and then centrifuged (40 000*g* for 60 min), a pellet is obtained which contains about 15–20% of the total radioactivity. About 90% of the label in this pellet can be solubilized with dodecyl sulfate (at 100°) and then separated by two-dimensional electrophoresis. Several proteins, which were absent from the supernatant, are present in this solubilized pellet, such as the group of very abundant outer membrane proteins which focus at pH 4.8. This is in agreement with the known insolubility of many membrane proteins in nonionic detergents, or even in anionic detergents without extensive heating (for example, Ames et al., 1974; Rosenbusch, 1974). Therefore, we felt that it was necessary to adopt a different, more effective solubilization procedure, specific for use with the membrane fraction.

Table I: Solubilization of Membrane Proteins.

	Percentage of Initial Envelope Protein.[a]						
	70° 5 min	70° 30 min	100° 3 min	70° 30 min	100° 3 min	70° 30 min	100° 3 min
SDS/protein	1.3	1.3	1.3	1.3 + 10% mercaptoethanol		2.6	2.6
Soluble	71	71	86	79	87	85	92
Insoluble	12	13	9	7	9	13	5

[a] Envelopes from *S. typhimurium* (TA1857) were prepared and solubilized as described in Experimental Procedures. With a ratio of dodecyl sulfate/protein (w/w) of 1.3 the concentrations of dodecyl sulfate and protein were 20 and 15.1 mg/ml, respectively. With a ratio of 2.6 the concentrations of dodecyl sulfate and protein were 20 and 7.7 mg/ml, respectively. Protein concentration was assayed as described (Lowry et al., 1951): dodecyl sulfate, up to 0.5 mg/ml, did not interfere with the assay. When mercaptoethanol was present, the protein was precipitated with a 30-fold excess of cold acetone prior to assaying as usual.

Several reports (Rüchel et al., 1974; Weber and Kuter, 1971) had suggested that proteins that had been denatured with dodecyl sulfate could be separated from the dodecyl sulfate electrophoretically and would then migrate according to their native charge. O'Farrell (1975) suggested that if dodecyl sulfate was added to an isoelectric-focusing gel containing NP-40, it would combine with the NP-40 to form micelles, which upon electrophoresis, migrate to the front (i.e., to the acid pH region, in an isoelectric focusing separation). Therefore, we developed a dodecyl sulfate solubilization procedure which could be combined with an isoelectric-focusing separation. Membrane proteins were solubilized with dodecyl sulfate with heating: this detergent is known to be an efficient and fairly nondiscriminating solubilizing agent, especially at higher temperatures (for a review on detergent action: Helenius and Simons (1975)).

Table I shows the results obtained on *S. typhimurium* membranes, with various concentrations of dodecyl sulfate and protein, and with treatments at various temperatures. It shows that optimal solubilization is achieved with heating at 100° for 3 min. Heating at 70° for 30 min was almost as efficient and was chosen as the standard condition for solubilization: it employs a ratio (w/w) of dodecyl sulfate/protein of 1.3. Increasing the ratio to 2.6 improved solubilization slightly; however, in order to achieve this ratio without increasing the absolute concentration of dodecyl sulfate it is necessary to lower the protein concentration. Therefore, a higher dodecyl sulfate.protein ratio was not used in any of the experiments presented, because we were aiming at applying as much protein as possible and an increase in dodecyl sulfate interferes with isoelectric focusing. Decreasing the dodecyl sulfate/protein ratio to a value of 0.77 decreased the extent of solubilization only at 100° and very slightly (data not presented). The presence of mercaptoethanol during solubilization also improved very slightly the

[3] NP-40 is a nonionic detergent, closely related to Triton X-100. Both are alkyl (octyl) phenol ethoxylates: NP-40 contains 7 to 9 relative mol of ethoxy linkages while Triton X-100 contains 9 to 10 ethoxy linkages (G. B. Cook, Shell Chemical Co., personal communication).

solubilization, though it was not added routinely. If mercaptoethanol is present during solubilization the preparation should be electrophoresed immediately, otherwise precipitation occurs upon freezing. Mercaptoethanol is always present in the sample at the time it is applied to the gel.

The average value obtained with *E. coli* membranes, under standard conditions, from eight different experiments, was 78% solubilization and it agreed with the values obtained for *S. typhimurium*. The remaining insoluble protein (approximately 10–20%) can be accounted for in both organisms by the peptidoglycan-bound lipoprotein (which, in the bound form, may represent about 10% of the cell envelope proteins (Braun et al., 1974)) and by one or more major proteins which are fully solubilized only upon heating at 100°.[4]

Attempts at using a solubilization in guanidinium thiocynate, followed by removal of the chaotropic agent by dialysis against urea (Moldow et al., 1972), were unsatisfactory for two reasons. One is that large amounts of protein are lost by precipitation during dialysis against urea, especially in *S. typhimurium* (for *E. coli* see also Johnson et al., 1975). The second, and more important reason, is that, after this treatment, many proteins run as multiple spots in the isoelectric-focusing dimension: this is apparently an artifact due to the appearance of charge heterogeneity (O'Farrell, 1975; Herbert et al., 1973). It may be due to carbamylation by degradation products of urea, to which the protein solutions are exposed for long periods of time during dialysis, or to thiocarbamylation by exposure to the 6 M thiocyanate during solubilization. Even small amounts of derivatization (Moldow et al., 1972) would be put readily in evidence by the isoelectric focusing, because of its high sensitivity to charge differences.

Isoelectric Focusing. We would like to refer the reader to the excellent paper by O'Farrell (1975) for a full discussion on the properties of the two-dimensional separation and on the importance of a variety of parameters. We will discuss presently only the factors involved in the specific handling of membranes and in the modifications we introduced.

When membrane preparations are solubilized in dodecyl sulfate as described above and then applied to an isoelectric-focusing gel (as described in Experimental Procedure) a separation is obtained that is very reproducible (see discussion of individual figures). We have not ascertained whether all proteins are completely freed of dodecyl sulfate during the separation. However, either they all are or, if any retain bound dodecyl sulfate, it must be a fixed and reproducible amount, which does not give erratic results or poorly shaped spots. (See also section on Membrane Proteins from Mammalian Cells and an Envelope Virus.) The pH gradient is linear (Figure 1), ranging from about 4.2 to about 7.0 (or 7.5 if the gel contains ampholines in the pH 7–9 range; see Experimental Procedure). When dodecyl sulfate is present the basic end of the gradient reaches a lower pH value than in its absence. There are very few proteins visible in the very acidic region,[5] while some proteins which have isoelectric points above 7.5 are probably lost. If those proteins are of interest the basic range could be extended by adding a portion of more basic ampholines: we have not explored this possibility.

Quantitation of the amount of protein which enters the isoelectric-focusing gel was obtained by focusing ^{14}C-labeled envelope proteins, then cutting the stained and dry gel and counting each slice as previously described (Ames, 1974). Approximately 80% of the applied label could be accounted for in the gel. Part of the missing 20% of the label could have been lost because it focuses at pH higher than 7.5.

Most of our isoelectric focusing was performed on slab gels approximately 11–12 cm long. Trials with longer slab gels were quite satisfactory, and those should be used whenever an improved resolution is desired. In most of our separations there was no need for spreading out the proteins any further.

The amount of protein which can be applied is quite large: we have applied up to 100 μg of protein and obtained very good resolutions. The large amount of protein is necessary in order to visualize a large number of proteins, because in membranes a few proteins make up a large percentage of the total (possibly because they are structural proteins). In separations involving large amounts of protein, the major spots will often show a small amount of trailing, which does not interfere greatly with the resolution.

At the neutral end of the gel the protein bands do not focus as sharply as in the other regions of the gel. This results in horizontal streaking of spots in the final two-dimensional gel. This problem may be due to the presence of nucleic acids in the sample, which apparently interfere with proper focusing in that area (O'Farrell, 1975). It is also possible that the solubility of membrane proteins is incomplete at pH near neutrality. Streaking is considerably reduced by treatment of the sample with RNase and DNase (O'Farrell, 1975), or by increasing the amount of urea present in the sample (see Experimental Procedure). Treating the membrane preparations with chloroform and methanol prior to solubilization (thus extracting the lipid components of membranes) did not remove the problem of streaking.

Sodium Dodecyl Sulfate Gel Electrophoresis. After isoelectric focusing is completed a strip is cut out of the slab gel and applied horizontally to the top of a dodecyl sulfate slab gel, as described in Experimental Procedure. The strip does not undergo any equilibration (except for what occurs as part of the handling and transferring the strip to the second dimension gel, as described in Experimental Procedure). The resolution in the second dimension is quite good under these conditions, with no obvious sign of vertical streaking. We were not able to store frozen isoelectric-focusing strips (as described by O'Farrell [1975] for cylindri-

[4] One-dimensional dodecyl sulfate gels were run on both the solubilized and insoluble portions of envelopes from both organisms (after heating both portions at 100°). In *E. coli* the major component of the insoluble portion is a single protein with apparent molecular weight of 32 000. Presumably this corresponds to the protein characterized in *E. coli* B by Rosenbusch (1974) and which constitutes about 15% of the envelope proteins. Evidently *E. coli* K12 has a protein of similar behavior. A minor band corresponding in molecular weight to another major membrane protein (apparent molecular weight 29 000) is also present in the insoluble fraction in qualitatively larger amounts than expected from a contaminant; therefore, also this protein may be not fully soluble at 70°. In *S. typhimurium* there are several outer membrane proteins which are only partially soluble at 70° (Ames et al., 1974); these may be related in structure and function to the Rosenbusch protein from *E. coli*. The bound form of murein-liproprotein is insoluble even after boiling in sodium dodecyl sulfate (Braun and Rehn, 1969) and presumably does not enter the gel.

[5] Essentially no protein appears at, or below, pH 4.8 in *E. coli*. However, a group of major membrane proteins from *S. typhimurium*, which are partly solubilized by our procedure, focus at pH 4.8: these may be related to the Rosenbusch protein in *E. coli* (Rosenbusch, 1974; see also footnote 4).

cal gels), because it always resulted in diffusion of protein bands with consequent loss of resolution. This may be due to the extreme thinness of the slab gel. Therefore, cylindrical gels were used for isoelectric focusing, whenever storing was necessary. The results were excellent.

The thickness of the dodecyl sulfate slab gel is usually twice that of the isoelectric-focusing gel, so that the gel strip from the first dimension can be inserted with ease between the glass plates for the second dimension, without damaging it and without delaying the transfer process. The double thickness of the dodecyl sulfate gel does not affect sensitivity of detection when the Coomassie Blue stain is used. However, as expected, the sensitivity of ^{14}C-detection by autoradiography is considerably decreased in thicker gels. Therefore, if very high sensitivity in the detection of radioactivity is needed, either more labeled material (or of higher specific activity) should be used, or the thickness of the dodecyl sulfate gel can be considerably reduced (see Experimental Procedure).

The concentration of acrylamide in the dodecyl sulfate gel is usually 10%. Increasing the concentration or using a gradient does not reveal any more proteins of low molecular weight (which would have run at the front in a 10% gel). With membrane proteins, the distribution of spots in a 10% gel is adequate for most purposes. Gradient gels can be prepared for special cases as described by O'Farrell (1975).

Analysis of Membrane Proteins

We applied the two-dimensional method to the analysis of bacterial cell envelopes from *E. coli* and *S. typhimurium* with equal success. Because of the good solubilization characteristics of dodecyl sulfate, it presumably can be applied to membrane fractions from other organisms, as shown in the section on Membrane Proteins from Mammalian Cells and an Envelope Virus. Below we discuss the important features of the method as applied to the envelope fraction from *E. coli*.

E. coli Membrane Proteins. Figure 2 is a photograph of two two-dimensional separations performed on isolated membranes from a K12 (HfrH) strain and from a *tolG* mutant derived from it (Chai and Foulds, 1974). It can be used as an illustration to discuss some of the characteristics of the method.

(i) The resolving power of the separation is much higher than any previously published method. About 150 spots are visible in each separation in Figure 2: this is three to four times more than the best one-dimensional separation has yielded up to now. This number is also in agreement with the total number of proteins visible in *E. coli:* 1100 (O'Farrell, 1975); membrane proteins account for about 10–15% of total cell protein (i.e., 100–150 proteins if the protein is uniformly distributed). In autoradiograms of separations involving labeled envelopes, about 200 proteins are visible. In the neutral region of the gels there are a number of spots which have streaked in the isoelectric focusing dimension. This problem, which may be due to either poor solubility of those proteins in that area, or to the presence of nucleic acids (or both), has been discussed above and can be eliminated if necessary. The streaking does not interfere excessively with the identification of most proteins.

No obvious problems with charge heterogeneity arise during the separation. The occurrence of artifactual charge heterogeneity would appear as multiple spots which separate in the isoelectric focusing direction, but run with the same apparent molecular weight in the dodecyl sulfate dimension. This problem has been amply discussed by O'Farrell (1975), who also pointed out the protective effect exerted by the amino groups of the ampholines in the presence of urea. A recently published two-dimensional method (Johnson et al., 1975) has patterns vastly different from ours: we feel that those patterns may include numerous spots due to artifactual charge heterogeneity.

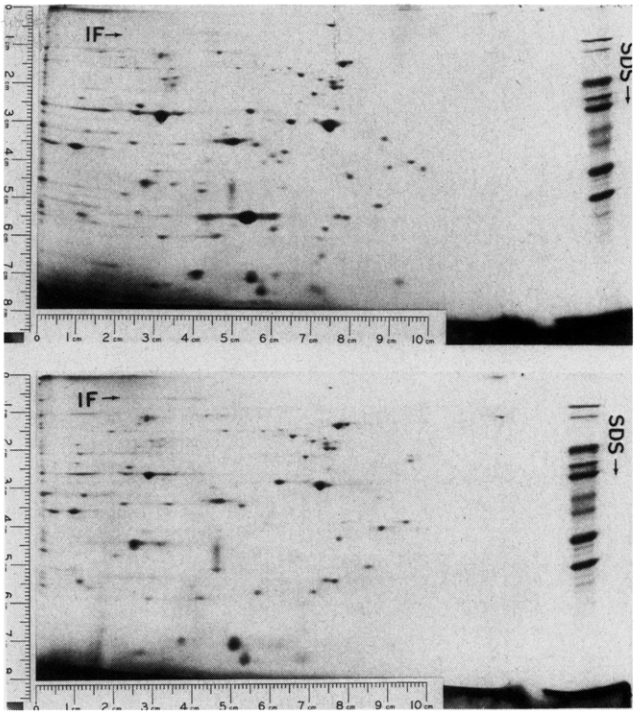

FIGURE 2: Photograph of two-dimensional slab gels of *E. coli* cell envelopes from JF404 (parent strain, top) and JF404-2a (*tolG* mutant, bottom). For growth of bacteria and envelope preparation see Experimental Procedure. The ampholine composition was pH 4–6; pH 6–8; pH 3.5–10, in the ratio of 2:2:1. In these gels the pH values of the isoelectric focusing gel were: 6.9 at the origin; 5.7 in the center; 4.2 at the front. The gradient was linear (see Figure 1). The front of the isoelectric focusing gel is at 12.6 cm from the origin; but there are very few proteins past pH 4.8. The gels stretch a little during staining and drying; therefore appropriate corrections should be made when comparing a dry gel with the pH gradient obtained with the wet gel. The gels have been stained with Coomassie Blue. Each separation contained about 60 μg of protein. The dark area at the front of the dodecyl sulfate gel is due to the ampholines, which run in that position and stain intensely with Coomassie Blue. The vertical series of bands at the extreme right is the molecular weight standard, which is electrophoresed in the dodecyl sulfate gel dimension.

(ii) The reproducibility of the separations is very good. The two gels are qualitatively identical except for a few spots, which are missing in the mutant and will be discussed below. We have compared the pattern obtained for the parent strain in Figure 2 with the pattern obtained (Figure 3) from a different *E. coli* strain (WGAS, which was obtained from J. R. Guest, and is K12, W3110, F^-, with a few mutations and grown under slightly different conditions). We found that about 90% of the spots visible in the parent strain in Figure 2 could be tracked down in equivalent relative positions in this second *E. coli* strain. The few differences are probably due to the different conditions of growth and to the different genetic backgrounds.

(iii) The sensitivity of the method depends on the maximum amount of protein which can be added. According to O'Farrell (1975), Coomassie Blue detects 0.01 μg of protein. We feel that in a two-dimensional gel the sensitivity is

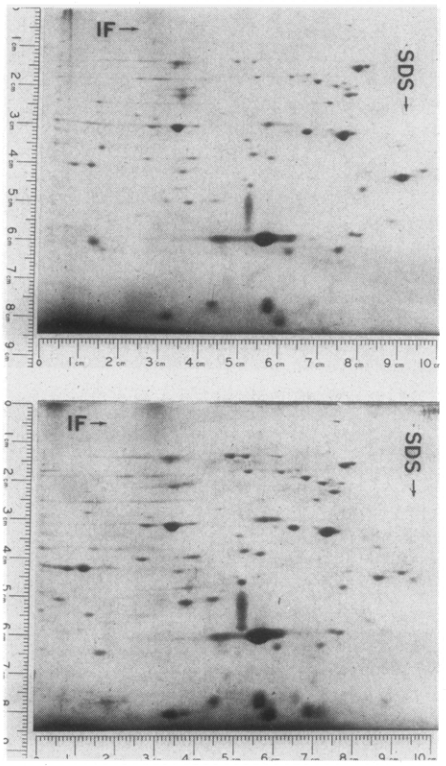

FIGURE 3: Photograph of two-dimensional slab gels of *E. coli* cell envelopes from WGAS (parent strain, top) and WGAS *sdh9* (succinic dehydrogenase mutant, bottom). For details see legend to Figure 2. This photograph covers only the area of the gel containing protein. The right-hand portion, containing the molecular weight standard, is not included.

somewhat decreased, though we have not established this with certainty. If the sensitivity is 0.1 μg, it should be possible to detect a protein accounting for 0.1% of the total protein, if 100 μg had been applied to the gel. Of course, if labeled preparations are available, the sensitivity can be greatly increased (see O'Farrell, 1975, for a full discussion on this subject). A comparison between the two separations in Figure 2 reveals some qualitative differences in several of the spots. Because of the high sensitivity of the Coomassie Blue stain it is possible to determine that a certain spot is still present, though in much smaller amounts than in the parent. This allowed us to single out in the parent three spots (coordinates 5.4 × 5.6; 5.4 × 2.7; 4.2 × 4.8) which are completely lacking in the *tolG* mutant, and two which are greatly decreased (coordinates 5 × 3.4; 4.1 × 7.0). One of the spots completely absent in the mutant (coordinates 5.4 × 5.6) is among the most abundant membrane proteins and its absence is easily noticeable even in a one-dimensional dodecyl sulfate gel separation (Chai and Foulds, 1974). It corresponds to the outer membrane protein (band G) which has been linked previously (Chai and Foulds, 1974) to the *tolG* mutation.[6] The loss or decrease of the other four spots had not been detected previously. All should be taken into consideration as the possible product of gene *tolG*. This is a good illustration of the power of this two-dimensional separation in uncovering differences among minor proteins, which would not be detectable by a one-dimensional separation.

[6] This protein has a different mobility depending on what temperature it has been exposed to previously. If it has been heated at 70° (as in Figure 2) it runs with an apparent molecular weight of 22 000; if it has been heated at 100° it runs with an apparent molecular weight of 29 000. This protein may correspond to protein B of Reithmeyer and Bragg (1974) and to other heat-modifiable proteins studied in other laboratories (Schnaitman, 1973; Koplow and Goldfine, 1974).

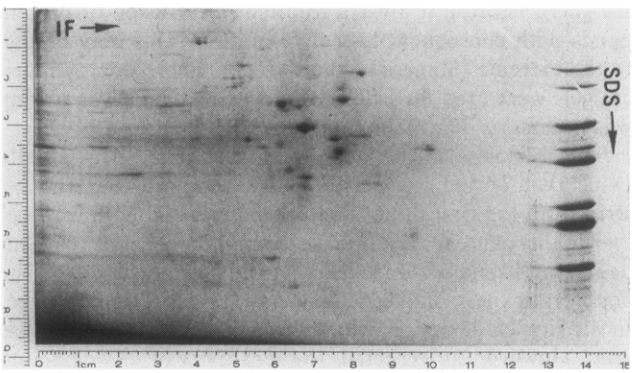

FIGURE 4: Photograph of a two-dimensional slab gel of plasma membranes from HeLa cells. The crude plasma membranes were isolated by E. E. Penhoet from cells grown in suspension, as described (Kiehn and Holland, 1970). They were collected from the first sucrose gradient centrifugation and washed twice in 0.05 M Tris-HCl (pH 7.4) containing 0.0025 M $MgCl_2$. The two-dimensional separation was performed twice (on the same membrane fraction) giving essentially the same pattern. About 35 μg of protein was applied to the IF gel. Other details are in legend to Figure 2.

(iv) The improved resolution can easily settle a question of possible identity between proteins, which has been suggested on the basis of molecular weight alone. For example, in the previous one-dimensional separation of membranes from *tolG* and its parent (Chai and Foulds, 1974) a protein band was still visible in the *tolG* cytoplasmic membranes in the same position as band G (though none was visible in the outer membrane, where band G is normally located). The possibility was raised of its being a residual amount of band G which was located in the cytoplasmic membrane. With our improved resolution this problem can be settled. In Figure 2 there are several proteins with the same molecular weight as band G (29 000, after heating at 100°;[6] vertical coordinate 4.5), but with different isoelectric properties. They can easily account for the band still visible in *tolG* membranes in one-dimensional separations.

Figure 3 shows that in a succinic dehydrogenase mutant (WGAS *sdh9,* bottom) a spot which has an apparent molecular weight of 68 000 is missing (coordinates 3.6 × 2.3) when compared with the parent. This presumably is one of the succinic dehydrogenase subunits, as described by Spencer and Guest (1974). Several other differences are visible: the mutant has some new spots (possibly a consequence of the *sdh9* mutation, if the strains were fully isogenic in the rest of the chromosome), the most obvious one being a small molecular weight protein (coordinates: 6.9 × 8.1). A comparison of the two *E. coli* parent strains used in this work (Figures 2 and 3, respectively) indicates that there are many similarities (as expected from related *E. coli* K12 strains), and, as already mentioned above, it further illustrates the reproducibility of the system and its usefulness in characterizing a full complement of membrane proteins.

Membrane Proteins from Mammalian Cells and an Envelope Virus. The validity of the method for its use in nonbacterial systems was tested on a plasma membrane preparation from HeLa cells as shown in Figure 4: more than 50 proteins can be seen clearly.

Envelopes from Influenza virus type A were also analyzed by this method (data not shown). The pattern obtained for the envelope proteins was identical with that ob-

tained with a preparation that had never been exposed to dodecyl sulfate (E. E. Penhoet, personal communication). This indicates that the exposure to dodecyl sulfate has not altered the value of the isoelectric point of those proteins, and supports the contention that dodecyl sulfate can be separated electrophoretically from proteins. All three major envelope proteins were detectable, and one of them showed multiple spots in the isoelectric-focusing dimension. This latter protein is a glycoprotein with a molecular weight of about 56 000 and its displays charge heterogeneity presumably because of the presence of variable amounts of carbohydrate.

Conclusions

In conclusion, we feel that the method we have presented will be a valuable tool in the analysis of membrane proteins because of its capability to solubilize and then resolve with high reproducibility and sensitivity membrane proteins. It takes advantage of the good solubilizing power of dodecyl sulfate and it should therefore be applicable to a wide variety of membrane preparations. Relatively large amounts of the solubilized protein can be analyzed by separating it initially on the basis of charge, thus taking advantage of the larger capacity of isoelectric-focusing gels. Thus it should be useful in cases where it is not possible to prepare labeled membrane of high specific activity, while they can be obtained in large amounts. The combination of large amounts of protein with the good sensitivity of Coomassie Blue staining may allow direct visualization of most proteins in a preparation. Combining a separation on the basis of charge with one on the basis of molecular weight theoretically allows resolution of several thousand proteins (O'Farrell, 1975). Therefore, it should be possible to resolve fully even membranes of very complex compositions.

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