Resolution of Bacterial Proteins by Polyacrylamide Gel Electrophoresis on Slabs

MEMBRANE, SOLUBLE, AND PERIPLASMIC FRACTIONS*

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SUMMARY

The application of a method of electrophoretic protein analysis to the investigation of the total protein composition of Salmonella typhimurium is described. It involves electrophoresis of many samples simultaneously on a thin slab of acrylamide gel, in the presence of sodium dodecyl sulfate, with the discontinuous buffer system of Laemmli ((1970) Nature 227, 680-685). Up to about 25 samples can be run simultaneously, thus improving both efficiency and ability to compare patterns.

The sensitivity of the method for detection of changes both in soluble proteins and the insoluble membrane proteins of the cell is shown. Membrane preparations from the wild type strain and many different mutant strains have been analyzed. The major membrane proteins observed and their relationship to proteins described in the literature are discussed. The marked changes detected in a variety of mutant strains and under a variety of growth conditions are also discussed, either as to their specific meaning or as evidence of the effectiveness of the method. Solubilization artifacts concerning the proteins of the outer cell membrane are presented. The membrane fractions from two Escherichia coli strains have also been analyzed.

To identify the components of the histidine transport system in Salmonella typhimurium, a careful analysis of the proteins of both the bacterial membrane and the soluble fraction of the cell was undertaken. Several reports have appeared on methods of analysis of cell membranes (for example, References 1 to 7), mostly based on acrylamide gel electrophoresis with sodium dodecyl sulfate, in glass tubes, with or without radioactive double labeling techniques, and with or without preliminary separation of the membrane into fractions by a variety of procedures. None of the methods available was sufficiently reproducible and sensitive for the scope of the project in this laboratory. The electrophoresis technique finally adopted utilizes a thin slab of acrylamide gel in which multiple samples (up to 25) are run simultaneously, giving patterns of perfect correspondence. The buffer system is the discontinuous sodium dodecyl sulfate system of Laemmli (8), which separates protein subunits on the basis of molecular weight. The apparatus has been described by Reid and Bielesky and modified by Studier (9-11). The method is a combination of techniques already in use in other laboratories. However, to my knowledge, this powerful and convenient method has been used extensively only by workers in the field of phage protein biosynthesis and in vitro protein synthesis, and it is through them that I have become acquainted with it. (See, for example, References 10 to 13 and acknowledgments.) The powerful resolution, sensitivity, and reproducibility obtainable indicate that it would be a method of choice for many research problems. Therefore, the purpose of this paper is to describe the technique, giving all of the useful references and details, and to present its application to the study of bacterial proteins, particularly membranes.

MATERIALS AND METHODS

Preparation of Cell Fractions—Table I lists all of the strains used in this work. Cells are grown with vigorous aeration, in minimal medium (17) usually containing 0.4% glucose as a carbon source and with the necessary additions for auxotrophic strains, or in nutrient broth (Difco Bacto Nutrient Broth: 0.8%; NaCl: 0.5%). One hundred milliliters of a fully grown culture are centrifuged, the bacterial pellet is resuspended in 0.1 ml of Tris-HCl, 0.0625 M, pH 6.8, and sonicated (80 watts) for 2 min in 20-s intervals, with 40 s of cooling in between. Exponentially growing cells, or cells grown in nutrient broth, need only 80 s of sonication. The crude sonicate is centrifuged for 20 min at 6,000 × g. The supernatant is removed (for minimal medium cells, care is taken not to disturb a loose pellet floating above the tightly packed pellet in the bottom of the tube) and then centrifuged at 111,000 × g (Rmax) for 35 min. The pellet from this centrifugation (defined as the membrane fraction) is resuspended in 0.1 ml of Tris-HCl, 0.0625 M, pH 6.8; the supernatant constitutes the soluble fraction. Both fractions can be stored frozen at −20° before preparation of the samples for electropho

* This work was supported by United States Public Health Service Grant AM12121.
samples were boiled for 2 min, unless specified differently in the
preparation was also as described by Laemmli (8), except that
devised by Osborn and her collaborators (6), with the only modi-
fication that a 20-min incubation at 37°C was necessary after di-
membrane fractions were separated according to the procedure
Heppel (18).

The discontinuous sodium dodecyl sulfate buffer system of
was used. Separating gels containing acrylamide
immunolysin (Bio-Rad) and 0.8 g of
N,N’-bis-methyleneacrylamide (Bio-Rad) in 100 ml of water.
The stacking gel usually contained 5% acrylamide and was pre-
from the same stock solution. The final concentration of
accompanying as I used the apparatus. To avoid duplication
will not describe the basic apparatus, but I will refer to Plate I
for description of the changes.

I routinely use thin gels (0.8 mm) because they are easier to
dry (both stained and unstained) and to stain and utilize less material. The Lucite spacers, therefore, are 0.8 mm thick; they
must have perfectly matching thicknesses, and should make
perfect contact with each other to avoid leaking of the gel solution
from the sandwich. Before forming the sandwich, the outer edge of the plates can be lined with a very thin layer of vacuum grease (Lubriseal, Arthur H. Thomas Co.; do not use silicone grease, for easy washing), rather than sealing the edges of the formed sandwich with hot 1.5% agar. Spacers can also be cut from rubber sheets, which may give a better seal because of the compressibility of the rubber. Most materials tried for
making spacers turned out to be of very irregular thickness, thus
causing leaks. However, careful measuring of the material cor-
rects the problem.

A clamp stand has been designed to improve the stability of
the sandwich during preparation of the gel. The bottom edge of
the sandwich is clamped in a stand made from a block of wood
on which a very wide (about 12 cm) and strong clamp (like those
used in elpholids) has been fastened in a vertical position. The
lateral edges of the sandwich are aligned with vertical metal L
bars which are fastened onto the clamp stand. Large spring
paper clamps hold both the metal bars and the edges of the sand-
wich. This arrangement allows the sandwich to stand firmly
upright and eliminates accidental knocking over.

After the gel mixture is poured in the space between the two
plates, it is overlayered with buffer (of the same composition as
that in the gel mixture) by allowing the buffer to descend rapidly
in a corner of the sandwich. The interphase is deformed at
first, but it finally returns to its original position because of the
much higher density of the acrylamide solutions. No Lucite top
blank is used. After polymerization, the gel surface is rinsed
promptly with the same buffer and drained well. It is important
to rinse the surface of the separating gel promptly upon poly-
erization, otherwise it becomes irregular because residual acryla-
mide, dissolved in the overlayering buffer, finally polymerizes
too, in an irregular fashion.

A Lucite “comb” of the same thickness as the spacer strips
and with a variable number of teeth (I use combs with either 13
or 20 teeth) is inserted in the free edge of the sandwich. The
interphase is deformed at
the teeth of the comb. The teeth of
the comb should fit snugly so that no gap is formed between the
plate surface and the surface of the tooth—it would inter-
fere with the application of the samples.

The sandwich with the polymerized gels is then mounted on the
electrophoresis apparatus. A seal is formed between the notched
face of the top vessel and the notched face of the sandwich by
smearing grease on the face of the vessel, pressing the sandwich
onto it, and clamping it tightly. Air bubbles can be removed
from the bottom edge of the sandwich with a syringe with a bent
needle. With accurate greasing and clamping, no leakage
occurs from the top vessel into the bottom one. The wells are

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filled with electrode buffer, and the samples are underlayered with a Hamilton syringe (50 μl) on the needle of which is inserted a short piece of discardable polyethylene tubing (P.E. 50); this eliminates washing of the syringe. The tip of the tubing can be squashed to fit in between glass plates when the clearance is too narrow. Samples of up to 30 μl can be applied; however, better separations are usually obtained with smaller samples (10 μl or less). About 20 μg of protein per sample give optimal resolution. Electrophoresis is run routinely at room temperature, at constant current (12.5 ma); the voltage is 50 volts at the beginning and 130 volts at the end of the run, which lasts about 4 hours. More recently I have used higher current (up to 30 ma, with the voltage going from 100 to 230 volts and the run lasting about 1.5 hours), which has given excellent results; no distortion of bands occurred and sharpness of smaller molecular weight bands was improved (e.g. Fig. 9B). It might be possible to raise the current even more, thus shortening the length of the run.

Staining and Drying—The gel slab is removed from the sandwich at the end of the run by prying the plates apart gently and, if necessary, loosening the gel slab off the glass by holding the plates under running water. The gel slab is then placed into a dish containing the first of the four stain steps recommended by Fairbanks et al (7). Staining is achieved by placing the dish on a very slow platform shaker, or with a magnetic stirring bar held in a corner of the dish by an inverted plastic beaker, with cracks in its edges to allow for circulation of the liquid. The slab is sequentially stained as recommended (7). Each step takes about 1 hour, because of the thinness of the slab, although any of the steps can be prolonged up to 24 hours to match one's schedule. The slab re Paste onto a Lucite frame with a nylon stocking stretched on it, during the changes of staining solutions; this greatly decreases the chances of damaging the slab during handling.

When destaining is completed, the slab is dried on a piece of filter paper as described by Maizel (20); this takes about 30 min. The dry gel sticks to the filter paper and it can be handled as a piece of paper, it must not be allowed to get wet, as it will pick up water immediately. The dried gel can be stored in a notebook; if the separation involved labeled materials, it can be autoradiographed (Fig. 2) and later sliced for quantitative counting. If a gel is torn during handling, it can be pieced together before drying; such a gel appears after drying almost as good as an intact one (see, for example, the lower left corner of Fig. 4).

Long Gels—The resolution can be improved greatly if the separation is performed on much longer gels on a slightly modified apparatus. The glass plates are the same as described (11) except that their length is 35 cm and they are made of slightly thicker glass. The distance between the two electrode vessels of the electrophoresis apparatus is increased to match the length of the plates. The electrophoresis is run in the cold room at 20 ma. From the middle of the run onwards, the amperage drops (when the maximum voltage of the power supply has been reached) to 11 ma at the end of the run. The voltage goes from 165 to 390 volts. Under these conditions the run takes 8 hours. A sample of such a run is in Fig. 3. The samples at the edges of the slab are slightly deformed in the long gels and are not used for accurate analysis.

Radioactive Proteins: Quantitative Estimation—Separations involving labeled proteins can be easily quantitated as follows. A vertical strip including the separation of interest is cut out of the stained and dried gel, and it is then cut transversely in very thin slices with fine surgical scissors; each little slice (which can be recorded as to its distance from the origin and as carrying any particular band) is dropped in a scintillation vial containing 50 μl of water. The vials are kept inclined to make sure that the slice is properly soaked in the water. After at least 10 min of exposure to water, the slices are sufficiently wet; 10 ml of the scintillation mixture, which contains 145 ml of NCS-solubilizer (Nuclear Chicago), 3.73 liter of toluene, 14 g of 2,5-diphenyloxazole (POPOP), and 0.21 g of 1,4-bis(2-5-phenyloxazolyl)benzene (POPOP) are added to the vials. The vials are tightly capped, well mixed, and incubated overnight at 37° (or for shorter times at higher temperatures). The NCS-solubilizer causes the swelling of the wet gel slices and diffusion of the proteins into the toluene. The patterns obtained with this method of slicing and counting are very reproducible and detailed. It is possible to subdivide a sample in a very large number of slices in a relatively short time and with a great accuracy and reproducibility from experiment to experiment; alternatively one can keep the number of slices to a minimum, at least in the areas of least interest.

Molecular Weight Standards—The following proteins were used as molecular weight standards (molecular weight, reference, and source of enzyme in parenthesis): β-galactosidase (130,000; 21; Worthington), phosphorylase a (94,000; 21; Worthington), ornithine decarboxylase (88,000; donated by Dr. B. M. Guirard), bovine serum albumin (68,000; 21; Sigma), catalase (60,000; 21; Sigma), glutamic dehydrogenase (33,000; 21; Boehringer, Mannheim), ovalbumin (43,000; 21; Sigma), histidine-binding protein J (25,500; 22; this laboratory). A mixture was made containing approximately 50 μg of each of the above proteins in 1 ml of the sample buffer described by Laemmli (8). The mixture was boiled for 2 min, and 10 μl were used as a molecular weight standard. The mixture was stored at −12° and warmed up gently before each run. The KF of each protein was plotted against a logarithmic scale of the molecular weights. A linear relationship was obtained between approximately 100,000 and 30,000. Beyond either of these values the relationship was not linear anymore; the extent of deviation depended on the gel concentration used for the separation.

Identification of Bands—The KF of the proteins of interest was measured and their molecular weights were estimated from the plot of the molecular weight standards. Each protein band is referred to by the estimated molecular weight multiplied by 10−3 and followed by the letter K. The molecular weight (×10−3) of the standard proteins is indicated in each figure.

RESULTS

Three Classes of Bacterial Proteins

It is possible to analyze all of the proteins of the cell in a single electrophoretic separation of whole bacteria. The pattern obtained is shown in later figures (e.g. Fig. 9). However, it is often necessary to subdivide the proteins into functional classes: membrane and soluble (supernatant) fraction. The soluble fraction can be further subdivided, if necessary, in periplasmic proteins (18) and internal soluble proteins. Each of these classes of proteins shows a characteristic pattern, as can be seen in Samples 1 to 5 in Fig. 1. Each sample contains roughly similar amounts of protein; however, this is not representative of the relative abundance of each fraction in the cell, which will be discussed later.

2 Pointed out to me by L. M. Gold.

2 B. M. Guirard, personal communication.
this paper, however I call attention to an obvious change of a
tions of six different mutant strains were analyzed. The nature
Fig. 2 is given as an example of an autoradiogram of a gel. It
and, in addition, the slicing for quantitation is also much simpler.
was a single slab on which the membranes and the soluble frac-
dried gels are obtained as simply as with paper chromatograms
labeled proteins. Radioautograms of the whole stained and
sumably present in the soluble fraction when the routine method
repression of the 96K protein.
The radioautogram shown looks exactly like the stained gel, and comparison of the two can quite easily be made. Additional
information is obtained by slicing and counting separations ob-
tained with single- or double-labeled samples. Experiments
with double-labeled strains lacking the histidine-binding protein
J confirmed the accuracy, sensitivity, and simplicity of the
quantitation method. However, in my experience, simple visual
inspection of the obtained gels (or radioautograms) is approxi-
amately as sensitive for the qualitative detection of alterations.
The recovery of the amount of 14C applied to gels is approximately
80% for membrane proteins and over 90% for soluble proteins.
These recoveries indicate good solubilization and entering of the
proteins into the gel, especially considering that no correction
for possible quenching by the gel slices during counting was

Studies with Soluble Cell Fraction: Sensitivity and Reproducibility

The sensitivity and reproducibility of the system can be esti-
ated in separations obtained with the supernatant fraction
(Figs. 3 and 4). About 90 bands (in a long gel; 50 in a short gel)
can be separated clearly and recognized unequivocally in com-
pletely independent bacterial preparations and electrophoretic
runs. Fig. 3 shows the results obtained with the supernatant
fraction from several isogenic strains carrying single mutations
in genes involved in histidine transport. Almost every band
can be seen unchanged by scanning the gel horizontally from one
sample to the next. One obvious change is visible in Sample 1
at position 25K. This sample is prepared from strain TA1857,
which is wild type for the histidine permease gene cluster, and
therefore has a low level of the histidine transport component:
the J protein (a histidine-binding protein of molecular weight
25,000 (23)). The difference in level of the J protein is clearly
visible, even though this protein represents less than 0.3% of
the total cell protein. The lack of the P protein is not detectable
among the soluble proteins of the other three strains, which con-
tain mutations in the histP gene.

A change visible in the supernatants in Fig. 4 (also seen in
Fig. 2) is the lack of a 96K protein in strains grown in the pres-
ence of lysine plus methionine. The 96K protein is one of the
major soluble proteins of the cell. The effect of the separate
addition of lysine or methionine to the minimal medium is also
shown in Fig. 4; the purpose of this experiment was to determine
which amino acid was responsible for the 96K protein repression.
It is clear that methionine, and not lysine, is responsible for the
repression of the 96K protein.

Several other minor changes, which can be detected upon close
observation of both Figs. 3 and 4, are extremely reproducible
and give additional confidence in the reliability of the method.
They will not be discussed here.

Incidentally, I wish to point out the presence of a double band
of very high molecular weight clearly visible at the top of the
patterns in separations involving either the soluble fraction
(Figs. 3, 4, 6, and 9B) or whole cells (Figs. 8 and 9A). This
doubt is presumably corresponds to the β and β' subunits of
RNA polymerase, which have molecular weights, respectively,
of 155,000 and 150,000. A sample of pure RNA polymerase
(kindly supplied by M. Chamberlin) was run and its β and β'
subunits ran in the same position as this high molecular weight
doublet.
Studies with Membrane Fraction

The simplest and crudest membrane preparation was adopted for the investigation of the method and for quick scanning of a variety of strains and growth conditions. The cells were routinely disrupted by sonic oscillation, followed by separation into soluble proteins and insoluble ones (i.e., membranes) by centrifugation (see "Materials and Methods"). Membranes prepared by some other methods have been investigated also (see later sections) in an effort to obtain purer membrane fractions and to correlate some of the protein bands with membrane components investigated in other laboratories.

Fig. 3 shows the separation obtained with the membranes from several strains. Again the reproducibility of the separation can be estimated. About 60 bands (in a long gel; 35 bands in a short gel; e.g., Fig. 9B) can be seen, and, again, almost every band can be seen unchanged by scanning the gel across from one sample to the next. Membrane preparations from different days give quite comparable patterns. A very characteristic set of several major bands is seen at the region between 33K and 36K. These bands4 are among the major protein components of the membrane of S. typhimurium, and are characteristic of cell membranes grown under a variety of different conditions (see Fig. 6). The localization of these proteins in the outer cell membrane has been established (23a) after separation of the inner and outer cell membrane fractions according to the procedure described by Osborn et al. (6). These proteins are also subject to a very strong dependence upon temperature for solubilization (see next section), which is shown to be useful for a possible purification scheme for these proteins (23a).

Another set of major protein bands at 80K and 81K is also clearly visible in Fig. 3; however, these proteins vary greatly with different growth conditions (see later section). The only significant change visible in the strains in Fig. 3 occurs in Sample 8 where a protein (50K) is decreased and a heavier band appears at 57K. The proteins present in the 50K and 57K bands have been identified as flagellin, the constituent of bacterial flagelle (24). The 50K protein is the flagellin from Phase 1 flagella and the 57K protein is the flagellin from Phase 2 flagella; this suggests that the flagellins from flagella of the two phases differ in their molecular weight.5

Solubility and Resolution of Membrane Proteins

All of the membrane proteins are solubilized and are able to enter the gel upon heating in sample buffer at 100° for 2 min, which is the treatment recommended by Laemmli (8). I have found that treatment at a lower temperature (37° for 30 min) solubilizes all of the proteins except the major bands at 33K and 36K. These bands are among the major protein components of the membrane of S. typhimurium, and are characteristic of cell membranes grown under a variety of different conditions (see Fig. 6). The localization of these proteins in the outer cell membrane has been established (23a) after separation of the inner and outer cell membrane fractions according to the procedure described by Osborn et al. (6). These proteins are also subject to a very strong dependence upon temperature for solubilization (see next section), which is shown to be useful for a possible purification scheme for these proteins (23a).

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Another set of major protein bands at 80K and 81K is also clearly visible in Fig. 3; however, these proteins vary greatly with different growth conditions (see later section). The only significant change visible in the strains in Fig. 3 occurs in Sample 8 where a protein (50K) is decreased and a heavier band appears at 57K. The proteins present in the 50K and 57K bands have been identified as flagellin, the constituent of bacterial flagelle (24). The 50K protein is the flagellin from Phase 1 flagella and the 57K protein is the flagellin from Phase 2 flagella; this suggests that the flagellins from flagella of the two phases differ in their molecular weight.

The number of resolved bands is three in low resolution runs and six in high resolution runs.
Fig. 3. Electrophoresis of the supernatant and membrane fractions from isogenic strains. All strains were grown to saturation in minimal medium. The sample marked MW contains the molecular weight standards. Each sample contains about 20 μg of protein. Samples 1 and 5 are from TA1857; Samples 2 and 6 are from TA1861; Samples 3 and 7 are from TA1863; Samples 4 and 8 are from TA1879. The arrow on the left points to position 25K, which corresponds to the band of the histidine-binding protein J (19). Sample 1 (strain TA1857) has a low level (i.e. wild type level) of the J protein. The two bottom arrows on the right point to the 33K and 36K bands. The top arrow on the right points to the absence of the 50K protein (phase 1 flagellin) in Sample 8; in this sample a heavier band at 57K can be seen (see text for discussion). Separation is on 10% acrylamide, on a long gel (see “Materials and Methods”).

Fig. 4. Effect of methionine in repressing a 96K protein. Cells (TA1859) were grown in minimal medium containing the indicated amino acid additions (10^{-3} M). Cells were harvested in the exponential stage and the supernatant and membrane fractions prepared as usual. Electrophoresis was on 8% acrylamide, on approximately 20 μg of protein per sample. The arrow points to the 96K protein. This protein is less clearly separated in cells grown to the stationary phase (Fig. 3).

Studies with Periplasmic Fraction

The periplasmic fraction (shock fluid) from several strains carrying mutations in the histidine permease genes is shown in Fig. 5. The only significant difference is in a 25K band which is the histidine-binding protein J (the 25K standard is the J protein). The J protein is elevated in the dhuA strain in Sample 2 and missing in the hisJ mutant strain in Sample 3 (14); these two strains are compared to the wild type strain which has wild type level of the transport protein J. The hisP gene codes for a different component of histidine transport (14); therefore in Sample 4 (a dhuA strain containing a hisP mutation) the level of the J protein is still as elevated as in Sample 2.

The resolution of the membrane proteins is always worse than the sharp separations observed with the supernatant fraction. In an attempt to obtain sharper separations, a lipid extraction was performed on a membrane fraction. A previous report (25) had demonstrated that after lipid extraction the mobility of some proteins on sodium dodecyl sulfate gel electrophoresis was altered. However, a lipid extraction with chloroform-methanol (2:1, v/v) was without noticeable effect on the mobility of membrane proteins and did not give sharper bands.

Various temperatures of solubilization has been studied (23a). In contrast to membrane proteins, no effect of temperature of solubilization on the supernatant fraction of the cell has been observed.

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the membrane fraction. However, by the use of appropriate components undergoing the changes either in the soluble or in the membrane fraction, many proteins are shown in Samples 6 to 8. A variety of changes is also clearly visible in these separations, but will not be discussed. The effect of the addition of methionine to the minimal medium has been a large increase in a protein at 77K. Cells grown on a poor carbon and nitrogen source (Sample 4) have a large increase in a band at 100k; anaerobically grown cells (Sample 9) have a large increase in a protein with a slightly different mobility. The presence and abundance of the proteins in the 33K to 36K region is dependent upon the nature of the lipopolysaccharide in S. typhimurium (23a); therefore it is possible that the major differences in that region observed between the Salmonella and the Escherichia strains are related to the different lipopolysaccharide composition of these bacteria (27). These major E. coli protein bands are also subject to the same temperature dependence for solubilization as the Salmonella outer membrane proteins.

It is also obvious from Fig. 7 that no major protein is found at 48K in either of the E. coli strains analyzed. The presence of such a major protein and its anomalous response to solubilization procedures has been reported by several investigators (1, 3, 6, 28, 29). However, Fig. 7 shows that different E. coli strains vary in the composition of the major membrane proteins. Because of this and because of the difference in growth media used to grow the cells and in the solubilization buffers, no attempt has been made to correlate the results obtained with two different E. coli strains in this study with those reported for other strains. Many other differences among the three strains are quite obvious. Among them, the Salmonella 50K protein (flagellin) is clearly absent or greatly decreased in the E. coli strains. Many minor bands show differences; they have not been studied further.

The supernatant fractions also show many differences which have not been analyzed further.

**Alternative Methods of Membrane Preparation**

**Membrane Vesicles—Kaback** has developed a method of preparation of purified cell membranes which are active in a variety of transport processes (19). The method, which is completely different from the sonication procedure used throughout this paper, involves spheroplast formation (by means of lysozyme) and osmotic shock of the spheroplasts, followed by extensive washing. The protein composition of membrane vesicles has been compared with that of membranes prepared by sonication (Fig. 1). The total number of protein bands and the general pattern in membranes prepared by the two methods are strikingly similar. A few small differences are observed: there is the loss of a band at 50K and of the bands at 81K and 86K. A difference in growth medium might account for some of the differences between the membrane preparations; this question has not been explored further.

**Membranes Prepared According to Osborn et al.** (6)—The constitution of the total membrane preparation (6) see “Materials and Methods”), after brief sonication and before the sucrose gradient centrifugation, is shown in Fig. 1. The pattern for the

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**Fig. 5.** Electrophoresis of the periplasmic proteins from a variety of histidine permease mutants. Cells were fully grown in minimal glucose medium and shocked as described under “Materials and Methods.” The shock fluids were concentrated by vacuum dialysis about 10-fold and stored frozen. The 25,000 molecular weight standard is the J protein. It is this protein which is present in different amounts in the shocked strains: low in the wild type (Sample 1, TA831), high in the dinA strains (Sample 2, TA1014; Sample 4, TA1195), missing in the hisJ mutant (Sample 3, TA1050). Concentration of acrylamide was 9%.

**Preservation of the major E. coli protein bands are also subject to the same temperature dependence for solubilization as the Salmonella outer membrane proteins.**

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mutant strains and a study of known enzymatic changes occurring upon changes in the growth medium, they presumably could be fully identified.

**Escherichia coli Proteins: Membranes and Supernatants**

Two different strains of E. coli were used for the preparation of membranes by the routine method: ML208-225 and K12 (HfrH, thi-). Fig. 7 shows that membranes from either strain are different from those from S. typhimurium. Particularly notable is the difference in the major proteins of the outer membrane: 33K to 36K. The E. coli strains both differ from S. typhimurium and from each other. They both lack the 36K protein and both have a new major protein at 29K; the K-12 strain also lacks the 33K protein and might have a 35K protein with a slightly different mobility. The presence and abundance of the proteins in the 33K to 36K region is dependent upon the nature of the lipopolysaccharide in S. typhimurium (23a); therefore it is possible that the major differences in that region observed between the Salmonella and the Escherichia strains are related to the different lipopolysaccharide composition of these bacteria (27). These major E. coli protein bands are also subject to the same temperature dependence for solubilization as the Salmonella outer membrane proteins.

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The vesicles were kindly prepared by H. R. Kaback.
MEMBRANES | SUPERNATANTS

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**Fig. 6 (left).** Effect of growth conditions on protein composition of membrane and soluble fractions. All samples were strain TA1859. Samples 1 and 6, fully grown culture in minimal medium with glucose as carbon source; Samples 2 and 6, fully grown culture in minimal medium with glucose as carbon source, in a tightly capped bottle filled to the brim; Samples 3 and 7, fully grown culture in nutrient broth; Samples 4 and 8, fully grown culture in a nitrogen and carbon-free medium (26) containing 0.8% sodium citrate as carbon source and 5 x 10^{-3} M L-proline as nitrogen source. Acrylamide concentration was 9%. The white streak in Sample 6 is due to damage inflicted on the photograph in the course of printing.

**Fig. 7 (right).** Comparison of *Escherichia coli* with *Salmonella typhimurium*. All strains were fully grown in nutrient broth, ML308-225 and K-12 are *E. coli* strains. Acrylamide concentration was 9%.

**Fig. 8** shows a set of 12 otherwise isogenic strains in which the presence of the two different flagellins (phases (24)) can easily be followed. Other examples of the usefulness of scanning whole cell separations can be seen in Fig. 9. The effect of a lipopolysaccharide defect on the major outer cell membrane proteins, because of a deep rough (rfa) mutation in TA1978 (16), is shown (23a). The loss of some of the 33K to 35K proteins is clearly visible in the whole cell separation (Fig. 9A) and confirmed in runs with the separated membrane fraction (Fig. 9B).

The density of the method is such that some proteins can easily be detected in a separation involving the whole cell protein. This allows a very fast scanning of many cell cultures in one experiment, because it eliminates both the cell disruption and all centrifugation steps.

As an illustration, a partial study is shown of the 50K membrane protein (flagellin) which is absent in some strains which concomitantly show an elevation of a 57K protein (Fig. 3). The 50K protein is produced in larger amounts in cells grown on nutrient broth. In such cells it is clearly visible as a band even in a whole cell separation. Fig. 8 shows a set of 12 otherwise isogenic strains in which the presence of the two different flagellins (phases (24)) can easily be followed.

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Another example concerning the level of the histidine-binding protein J is also shown in Fig. 9. In the wild type (TA1772) the level of J protein is low as compared to the dhuA-containing strains (TA1014 and TA1195), while the lack of the J protein in hisJ mutants (TA1650 and TA1771) is visualized easily in whole cells.

The β and β' subunits of RNA polymerase (155K and 150K) can also be detected in whole cells (discussed under “Studies with Soluble Cell Fraction: Sensitivity and Reproducibility”).

**DISCUSSION**

The purpose of this report is to demonstrate the power of this particular method of protein analysis for the identification and study of membrane proteins in addition to other cell fractions. While both acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and the use of a slab apparatus have been exploited for several years (20), no combination of apparatus, buffer system, and other details is as powerful and convenient as the
Fig. 8. Electrophoresis of whole cells showing the phase of flagella (50K or 57K proteins). The bacteria were fully grown in nutrient broth and the samples prepared as described under "Materials and Methods." Samples 1 to 12 are, in order: TA1615, TA1619, TA1620, TA1625, TA1631, TA1635, TA1850, TA1705, TA1708, TA1711, TA1713, TA1720. The arrow points to the 50K protein, which is decreased in Samples 8 to 12 where a heavier band appears instead at 57K. Sample 1 also has decreased level of 50K, but it contains an extra large amount of the 57K band. Concentration of acrylamide was 9%.

Fig. 9. Electrophoresis of whole cells carrying mutations in histidine transport or in the lipopolysaccharide (deep rough): composition of the membrane of the deep rough strain. The samples labeled wild type are prepared from strain hisD3052, which is the parent of the deep rough strain, TA1978. The other strains carry mutations in the histidine permease genes (see Table I and text). A, cultures were fully grown in minimal glucose (TA1772, TA1014, TA1650, TA1771, and TA1195) or in nutrient broth (hisD3052 and TA1978). Acrylamide concentration was 9%. B, membranes and supernatants from the parent of the deep rough strains (hisD3052; sample labeled wild type) and the deep rough strain (TA1978) grown in minimal medium with glucose as carbon source, 2 × 10^{-4} M L-histidine and 5 × 10^{-6} M biotin, were prepared by sonication as described under "Materials and Methods." Concentration of acrylamide was 9%. This gel was run more rapidly: 1.5 hours (see "Materials and Methods").
one presented in this report. The full power of this technique has already been demonstrated in elegant studies involving soluble phage proteins (see, for example, References 10 to 13). However, the method has not been applied to the study of cell membranes, which has depended up to now upon the cumbersome electrophoresis in tubes (for example References 1, 4, 5, 6, 29, 30) which does not allow efficient comparison between samples or handling of numerous samples. Moreover, it is extremely hard to present the results so that other laboratories can compare their separations with those appearing in the literature. A common method of presentation of the results has been through the use of labeled protein and the slicing and counting of the gels. This is an extremely laborious, expensive, and relatively inaccurate way of presenting the results of an electrophoretic separation in a tube.

In this paper I present electrophoretograms obtained with whole bacterial cells and with three main fractions in which cells can be subdivided: the periplasmic proteins, the soluble proteins, and the membrane proteins, accounting, respectively, for about 10, 70, and 15% of the cell protein. Several alterations occurring in mutant strains or upon change of the growth medium are shown to be detected quite sensitively in several of the separations. The specific meaning of most of the alterations is not discussed in detail here; rather, some of the advantages of the method will be pointed out, and only the patterns obtained with the membranes will be analyzed in more detail.

No quantitation of minor individual bands has been attempted. However, it is possible to judge the sensitivity of the method from the examination of separations performed on 20 μg of protein. Taking into consideration that at least 40 bands of varying intensity are clearly visible in each pattern, one can safely assume that about 0.2 μg of protein (i.e. about 1% of total) is visible in a single band.

The detection of any particular protein can be greatly improved by using gel concentrations which are lower (for high molecular weight proteins) or higher (for low molecular weight proteins), thereby spreading out the area of the separation of most interest. For very small proteins (below 20,000 molecular weight) it is necessary to use gels of very high acrylamide concentration (up to 22%). However, the drying of these high concentration gels presents problems (20). The method published by Swank and Munkres (31) for the separation of oligopeptides should be quite suitable for that purpose; it makes use of the addition of urea to moderate concentrations of acrylamide (about 12%), with the result of producing gels with effectively smaller holes.

The composition of the membranes of S. typhimurium has been studied in detail. The membrane fraction, prepared in three different ways, gives remarkably similar patterns (Fig. 1). Among the most characteristic and abundant protein bands are several proteins of molecular weights between 33,000 and 36,000, which are typical of all S. typhimurium strains which I tested, and which are unaffected by the conditions of growth. These proteins are components of the outer cell membrane and are strongly dependent upon temperature for solubilization (23a). It has not been established whether these bands correspond to completely different proteins or whether they are the same protein attached to different non-protein components which affect its mobility. Osborn et al. (6) have reported the presence of two major proteins, A and B, in the outer cell membrane of S. typhimurium which respond with an altered mobility to variation in the temperature of solubilization. Even though the 33K to 36K proteins described here are not solubilized at all at 37° under my conditions, rather than have changed electrophoretic mobility, as in the case of the A and B proteins (6), it is likely that they are the same components. The difference in behavior might be due to any of the many variables involved (exact strain genotype, medium of growth, medium of solubilization, etc.). In both cases the components are major outer membrane components and have similar molecular weights. Moreover, the finding that there are multiple major proteins, as opposed to a single major protein, is in agreement with a recent report on a purification procedure for the outer cell membrane proteins (32).

The 34K and 36K proteins are greatly decreased in lipopolysaccharide mutants of the deep rough (rfa) class (Fig. 9) (23a). The meaning of this alteration is under current study. It might be related to the alteration in an outer membrane protein described recently by Wu (33) in a lipopolysaccharide mutant of E. coli. No information is available on the function of any of the outer membrane proteins. An investigation of the properties of mutants defective in these proteins as a consequence of a deep rough mutation might be helpful in this respect.

As already discussed in the text, it is not possible to compare the composition of S. typhimurium membrane with that published for E. coli (1, 3, 29) because the reported molecular weight for the major outer membrane component from E. coli is different from that shown in this paper for either S. typhimurium or E. coli. A comparison of appropriate strains by the slab method would be the most direct and unequivocal approach.

A report has recently appeared (34) in which the presence of a small protein, first discovered covalently attached to the peptidoglycan (35), could be detected in the unbound form in the E. coli envelope. The large amount (34) of the unbound form of this protein and its small molecular weight should have allowed its detection among the smaller proteins of S. typhimurium. However, no heavy band of molecular weight 7,000 was visible in separations of the membrane on a 22% acrylamide gel (data not shown). It is of course possible that S. typhimurium has such protein in smaller amounts, or not at all, or not in the free form, or finally it might have a similar protein of different molecular weight.

Other membrane protein changes have been detected in a variety of mutant strains involving functions of the cell envelope. Only some examples have been chosen to illustrate the power of the method.

Similarly, numerous changes have been observed in the soluble and periplasmic fractions. Only the case of analysis of a known protein, the periplasmic transport protein J which binds histidine, will be discussed. The J protein, of 25,000 molecular weight, is about 0.3% of the total cell protein in strains carrying a promoter mutation, dhuA (14, 22). Mutants lacking this protein can easily be recognized even in separations involving the whole cell protein. Of course, it becomes increasingly easier to detect the difference between strains with and without the J protein as the cells get fractionated. Thus, the protein represents a large percentage of the total periplasmic proteins (about 5%) and can be seen as a major band (Fig. 5). The mobility of J proteins with altered properties and the search for fragments in mutants defective in the protein is in progress.

The advantage of being able to analyze the protein of whole cells is obvious. The manipulations are minimal and within a few hours the electrophoresis is completed. The idea has been utilized for the scanning of numerous colonies arising as transductants in a genetic experiment involving the mapping of the mutation affecting the phase of the flagellin (i.e. the 50K variable protein, Figs. 3 and 8). The segregation of the mutation re-
sponsible for the altered mobility could easily be followed.5 Examples of other proteins easily recognized in whole cells are the major outer membrane proteins, the J protein, and the β and β' subunits of RNA polymerase.

In sodium dodecyl sulfate-acrylamide gel separations, a change in the position of a band is due to a change in molecular weight of that protein (20); mutations which alter the molecular weight of any particular protein will be detected. Thus missense mutations in the position of a band is due to a change in molecular weight of subunits of RNA polymerase.

In the case of the major outer membrane proteins, the J protein, and the β and β' subunits of RNA polymerase, it might be possible to identify the fragment which is still being synthesized by analyzing the oligopeptide composition of the cell. This principle has been brilliantly exploited in the case of phage T7 by Studier and his colleagues (11) who actually produced me to this technique; B. I. Kirk and E. Spudich for their help in some of the electrophoresis runs; L. Gold for suggesting some of the refinements so useful in the proper handling of the gels; and W. Studier for making available the drawings of the apparatus and numerous details on its operation.


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