Two-Dimensional Polyacrylamide Gel Electrophoresis of Envelope Proteins of *Escherichia coli*

W. CHARLES JOHNSON, T. J. SILHAVY,[1] AND W. BOOS[1]*

Department of Biological Chemistry, Harvard Medical School, and the Biochemical Research Laboratory, Massachusetts General Hospital, Boston, Massachusetts 02114

A method of separating envelope proteins by two-dimensional polyacrylamide gel electrophoresis is described. *Escherichia coli* envelopes (inner and outer membranes) were prepared by French pressing and washed by repeated centrifugation. Membrane proteins were solubilized with guanidine thiocyanate and were dialyzed against urea prior to two-dimensional electrophoretic analysis. The slab gel apparatus and conditions were similar to the technique developed by Metz and Bogorad (1974) for the separation of ribosomal proteins. This separation occurs in 8 M urea for the first dimension and in 0.2% sodium dodecyl sulfate for the second dimension. The technique separates about 70 different membrane proteins in a highly reproducible fashion according to both intrinsic charge and molecular weight. Some examples of alterations in the membrane protein pattern are demonstrated. These alterations are caused by a mutation affecting a sugar transport system and by growth in the presence of D-fucose, inducer of the transport system. A further example of membrane protein changes introduced by growth at the nonpermissive temperature of a temperature-sensitive cell division mutant is shown. Finally, it is demonstrated that the major outer membrane component of *Escherichia coli* K-12 contains more than four proteins of similar molecular weight.

In recent years, the use of polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) for the analysis of membrane proteins has become quite popular (1, 3–5, 17). This method is advantageous because the same solvent conditions are used in the primary solubilization and subsequent electrophoretic analysis of the proteins of interest. Furthermore, it allows their molecular weight to be estimated (22). However, separation of proteins of similar molecular weight will, for the same reason, be rather poor. Indeed, despite excellent separation of many proteins in the *Escherichia coli* cell envelope, the major outer membrane protein appearing as one band by this technique has only recently been shown to consist of four proteins in some strains (18). Consequently, we looked for other methods which might combine the separating power of SDS-polyacrylamide gel electrophoresis with the additional capability of separating proteins according to their intrinsic charge under denaturing conditions. The two-dimensional polyacrylamide gel electrophoresis introduced for the separation of ribosomal proteins by Kaltschmidt

and Wittmann (6), and later modified by Metz and Bogorad (10) as well as Subramanian (21), seemed useful for this purpose. However, the major drawback of this approach is that membrane proteins are water insoluble. Detergents such as Triton or SDS used for solubilizing membrane proteins usually interfere with electrophoretic separation in urea (the system most often chosen for the expression of charge effects) and are difficult to remove. On the other hand, the presence of urea in the protein sample does not interfere with electrophoresis in the presence of SDS. Therefore, the mandatory sequence to follow for a two-dimensional electrophoresis is 8 M urea in the first dimension for separation according to intrinsic charge, followed by SDS in the second dimension for separation according to molecular weight. Membrane proteins are not readily soluble in 6 M urea, but once they are solubilized by another solvent a large portion of the proteins remain in solution when dialyzed against 6 M urea. We adopted this method and used the chaotropic reagent guanidine thiocyanate as the primary dissolving reagent, which is as effective a membrane solvent as SDS but has the added property of easy removal by dialysis against urea (12). The present paper gives an

[1] Present address: Fachbereich Biologie, Universität Konstanz, 775 Konstanz, West Germany.

account of the two-dimensional separation of the membrane proteins so treated. In particular, we applied this method to search for the still unknown but supposedly membrane-bound components of the periplasmic galactose-binding, protein-mediated β-methylgalactoside transport system (2) of *E. coli.*

(A preliminary account of this paper was given at the Mosbach Symposium, Germany, 1974.)

MATERIALS AND METHODS

Bacterial strains and genetic methods. The strains employed in this study are listed in Table 1 along with their origins. Details of the construction of these strains have been previously described (20).

Media and growth conditions. Cells were grown to stationary phase with vigorous aeration in 500-ml cultures. M188-777 and 1000 were grown in minimal media A plus 0.2% succinate and 20 μg of histidine per ml (20), and BUG-6 was grown in 0.2% D-glycerol at 35 and 42 C, respectively. D-Fucose (1 mM) was used as an inducer where noted in the text.

Membrane preparation. Cells from a 500-ml culture were harvested and washed once in 50 ml of membrane buffer [10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 8.5, containing 5 mM ethylenediaminetetraacetate, 0.2 M KCl, and 5 mM 2-mercaptoethanol (10)], resuspended in 35 ml of the same buffer, and broken by two passages through a French pressure cell at 9,000 lb/in² at 4 C. Whole cells were then removed by centrifugation at 1,000 × *g* for 10 min. The supernatant was then centrifuged at 30,000 × *g* for 1 h to pellet the membrane fraction. The membranes were resuspended in 15 ml of membrane buffer and separated into 0.5-ml aliquots and solubilized.

The outer membrane fraction was prepared as described by Schnaitman (18). The alcohol precipitate was resuspended in membrane buffer and further processed as described below.

Membrane solubilization. (i) Guanidine thiocyanate. Solid guanidine thiocyanate was added to one aliquot of washed membranes to a final concentration of 6 M. This produced an almost clear, yellow solution which was stirred at room temperature for 0.5 h and then dialyzed overnight against one change of 100 volumes of 6 M urea containing 0.1 M Tris-hydrochloride (pH 8.5) and 5 mM 2-mercaptoethanol. The dialysate was centrifuged at 12,000 × *g* for 1 h, the supernatant was carefully removed, and protein concentration was determined by the method of Lowry et al. (9). This solution was then ready for two-dimensional electrophoretic analysis. The pellet was saved for further use (see below).

(ii) SDS solubilization. Two fractions were solubilized by SDS: an aliquot of washed membranes and the insoluble material remaining after guanidine thiocyanate solubilization and urea dialysis. To the suspension of washed membranes, 20% (wt/vol) SDS containing 0.625 M Tris-hydrochloride (pH 6.8) and 0.05 M 2-mercaptoethanol was added to a final concentration of 2% SDS and 1 to 2 mg of protein per ml. The urea-insoluble pellet was suspended in 1.0 ml of a solution of 2% SDS containing 0.0625 M Tris-hydrochloride (pH 6.8) and 5 mM 2-mercaptoethanol. In addition, the urea-soluble material was prepared for one-dimensional SDS electrophoresis by adding 20% SDS (wt/vol) containing 0.625 M Tris-hydrochloride (pH 6.8) and 0.05 M 2-mercaptoethanol to a final concentration of 2% SDS and 1 to 2 mg of protein per ml. All suspensions were then placed in a boiling water bath for 10 min and dialyzed against 100 volumes of sample buffer (2% SDS [wt/vol] containing 0.0625 M Tris-hydrochloride [pH 6.8], 10% glycerol, and 5 mM 2-mercaptoethanol) (7) overnight at room temperature. The samples were then centrifuged at 12,000 × *g* for 1 h. The supernatant was removed, and the protein concentration was determined. The samples were then subjected to one-dimensional SDS electrophoretic analysis. The remaining SDS-insoluble material was clear in both the whole membrane fraction and the urea-soluble fraction. However, the pellet from the urea-insoluble material retained the original caramel color exhibited after the urea dialysis.

Electrophoresis. (i) One-dimensional SDS. The method employed was that described by Laemmli (7). A 4% stacking gel was used over an 8% separating gel.

TABLE 1. *Bacterial strains*[a]

Strain	Parent	Isolation procedure	Genotype	Origin	Reference
W3092cy⁻			F⁻ galK lacY	Wu	22
LA002	W3092cy⁻	EMS, penicillin selection	F⁻ galK lacY his		
LA021	LA002	NTG	F⁻ galK lacY his mglABC	Silhavy	20
M188-444			F⁻ malA glpD strA ptsF his galE lacY	Silhavy	20
M188-777	M188-444	P1 transduction, W3092cy⁻ donor	F⁻ malA glpD strA his galE lacY	Silhavy	20
M188-1000	M188-444	P1 transduction, LA021 donor	F⁻ malA glpD strA his mglABC galE lacY lac⁻ gal⁻	Silhavy	20
BUG-6			lac⁻ gal⁻	Clark	16, 19

[a] All strains are *E. coli* K-12; EMS, ethyl methane sulfonate; NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

(ii) Two-dimensional SDS. The method used in these studies was first described by Metz and Bogorad (10) except that 15-cm gel lengths were used (Subramanian, personal communication). The first dimensional gel (run in 5-mm [inner diameter] tubes) contained 8 M urea and was buffered with 0.1 M Tris-hydrochloride (pH 8.5). The electrode buffer was 25 mM Tris-glycine (pH 9.2). Usually 300 μg of protein in 50- to 150-μl aliquots was applied to each gel and electrophoresed with 2 mA/gel at 350 V, dropping to 1 mA/gel and 400 V at the end of the run for 4.5 h at 18 C.

The second dimension was run as described by Metz and Bogorad (10), except that the length of the apparatus was 5 cm longer. The first dimensional gels were removed from the tubes and cemented on top of the slab gels with a 4% acrylamide-cementing gel. The second dimension was run at 32 V (25 mA/gel decreasing to 4 mA/gel) for 17 h at 18 C. The gels were stained for 8 h at 37 C with a 0.1% solution of Coomassie brilliant blue containing 50% methanol and 7.5% acetic acid and destained with one change of 15% methanol containing 7.5% acetic acid.

Molecular weight markers were polymerized in the cementing gel along with the first dimensional gel. Markers used were bovine serum albumin, ovalbumin, and chymotrypsinogen purchased from Worthington Biochemicals, and hemerythrin was provided by A. R. Subramanian.

Assays. Galactose-binding protein was detected by Ouchterlony immunodiffusion (8). Transport of galactose by the β-methylgalactoside transport system was determined as previously described (15). β-Galactosidase activity was determined by the hydrolysis of O-nitrophenyl-β-D-galactopyranoside in toluenized cells or without toluene in cell-free suspension (13).

RESULTS

Preparation of membranes. Membranes were prepared in three steps: (i) harvesting and washing of the cells; (ii) breakage by French pressure cell at a pressure of 9,000 lb/in^2; and (iii) three to four washings of the membranes by centrifugation at 30,000 × g for 30 min.

The reproducibility of membrane preparations using this method, as determined by subsequent two-dimensional electrophoresis, was compared with that of membrane preparations obtained by sonic oscillation of the cells, followed by repeated washing and centrifugation at 100,000 × g or more for several hours, and was found to be superior. Membrane preparations obtained by French pressing contained apparently fewer cytoplasmic constituents than those obtained with sonic oscillation. Membranes of cells fully induced for β-galactosidase and prepared by the former method contained no detectable enzyme activity, whereas membranes obtained by the latter technique still exhibited traces of enzymatic activity even after eight washings.

Solubilization of the membrane preparation. Preliminary experiments performed to explore the optimal conditions for a two-dimensional separation of membrane proteins based on charge and molecular weight made three facts obvious. (i) The presence of detergents such as 1% Triton X-100 or SDS (agents commonly used for membrane solubilization) is undesirable when membrane proteins are electrophoresed in gels containing 8 M urea (12); such agents are difficult and time-consuming to remove by dialysis against urea, due to micell formation of the detergent. (ii) The additional presence of small amounts of urea in the protein sample does not affect electrophoresis in gels containing SDS (12, 17). (iii) Membranes cannot be solubilized significantly by 6 M urea (12), but once solubilized, 6 M urea is able to keep 70 to 80% of the proteins in solution. These facts determined the sequence of the electrophoretic separations consisting of initial electrophoresis in 8 M urea and a subsequent run in the presence of SDS. Moreover, the difficulty of removal of detergents by dialysis suggested the use of a chaotropic agent. Consequently, we used 6 M guanidine thiocyanate, an agent introduced by Moldow et al. (12), to initially solubilize the isolated membranes. Under the conditions used, this agent will solubilize 60 to 90% of the membrane preparation when protein is determined according to Lowry et al. (9). Guanidine thiocyanate had to be removed by dialysis against 6 M urea prior to electrophoresis in the first dimension, since effective electrophoresis in such a high salt concentration is not feasible. The precipitation of 20 to 30% of the detectable proteins during this dialysis is the major drawback experienced in the two-dimensional analysis of membrane proteins. Table 2 shows the loss of protein content during the solubilization of the membrane proteins as determined by the Folin reagent. However, the

TABLE 2. *Membrane yield and percentage recovery*

Sample	Amt (mg)	% Recovery[a]
Whole membranes		
M188-777 + D-fucose	10.80	100
M188-777	8.00	100
Urea pellet		
M188-777 + D-fucose	2.41	22
M188-777	2.64	33
Urea soluble		
M188-777 + D-fucose	4.11	38
M188-777	5.41	68

[a] Total recovery for M188-777 plus D-fucose was 60%; for M188-777 it was 101%.

work of Moldow et al. (12) has indicated that dialysis against urea apparently does not precipitate a particular protein, but that the precipitated protein in the urea dialysate is a representative sample of the proteins remaining in the sample. By analysis with one-dimensional polyacrylamide gel electrophoresis in the presence of SDS of all three fractions mentioned in Table 2, we came to the same conclusion. Therefore, it seemed justified to use the proteins finally solubilized in 6 M urea as the major representatives of the proteins found in the cell envelope, even though the selective loss of some proteins cannot be excluded.

Two-dimensional polyacrylamide gel electrophoresis. The three figures show stained (Coomassie brilliant blue) gel slabs obtained by two-dimensional analysis of envelope proteins of typical *E. coli* strains. The electrophoresis technique is essentially that described by Metz and Bogorad in their analysis of ribosomal proteins. Separation of the proteins in the first dimension (from left to right) in 8 M urea occurs principally according to the intrinsic charge of the denatured proteins at pH 8.5, whereas in the second dimension (top to bottom) the separation due to 0.2% SDS occurs mainly according to their molecular weight (22). Numerous proteins of the same molecular weight that would not be separated by the classical one-dimensional SDS-gel electrophoresis can now easily be detected individually. This is particularly apparent for the major protein of the outer membrane of *E. coli* and other gram-negative bacteria, which appears to be one protein band of approximately 42,000 daltons on the usual SDS-gel electrophoresis (17) but has been shown recently to consist of at least four different components (18).

Application of two-dimensional polyacrylamide gel electrophoresis. It has recently been shown by Schnaitman (18) that the major outer membrane component of *E. coli* can be separated into four distinct proteins of similar molecular weight. The proteins of the K-12 strain M188-777 solubilized from the outer membrane and prepared according to Schnaitman's procedure are shown in Fig. 1. As can be seen, the major protein components consist of more than four protein spots of very similar molecular weight. This clearly demonstrates the advantage of using a two-dimensional electrophoretic technique where the separating feature is electrical charge in one dimension and molecular weight in the second dimension. The number of the major outer membrane proteins varies

with different strains and growth conditions and might be as high as 18 (see Fig. 3A, B).

Membrane alterations can be detected in a particular strain after alteration of the growth conditions and by introducing a mutation affecting the β-methylgalactoside transport system. Figures 2A and 2B illustrate the protein pattern of membranes prepared from *E. coli* K-12 strain M188-777 grown in both the presence and absence of D-fucose. This sugar is known to induce the cytoplasmic enzymes of the galactose operon as well as the β-methylgalactoside transport system. As can be seen by comparing Fig. 2A with Fig. 2B, D-fucose causes the increase or new appearance of four to five components (numbered). Moreover, the protein pattern of strain M188-1000, an *mglABC* (14) mutant isogenic with the strain shown in Fig. 2A and 2B and grown in the presence of D-fucose, similarly fails to exhibit or show a reduced amount of the proteins seen to be inducible by fucose in the wild-type strain M188-777 (Fig. 2C). Thus, it seems likely that some of the proteins appearing in the wild-type strain after induction with D-fucose represent the hitherto unknown components of the β-methylgalactoside transport system. To correlate the particular spots with components of this transport system and to avoid the complication caused by the D-fucose-dependent induction of unrelated proteins, it will be necessary to use a constitutive (*mglR*) strain and introduce deletions or polar mutations into the known structural genes of *mglA*, *B*, and *C* (14). Only then will it be possible to coordinate the protein pattern obtained to the yet unknown transport components.

Figures 3A and 3B show the protein pattern obtained from the envelope of strain BUG-6, a temperature-sensitive mutant in cell division (septum formation) (16). The cells were grown at the permissive (Fig. 3A) and the nonpermissive (Fig. 3B) temperature. Several interesting features can be noted. (i) The envelope contains about 18 protein spots which migrate to a molecular weight position similar to that of the major outer membrane protein (see Fig. 1). (ii) One of the spots (designated 1) found to be inducible by D-fucose in strains M188-777 appears to be temperature sensitive. It had previously been shown (19) that the synthesis of the β-methylgalactoside transport system and one of its components, the galactose-binding protein in this strain, is temperature sensitive. (iii) In contrast to the large changes occurring in the protein composition of the "periplasmic compo-
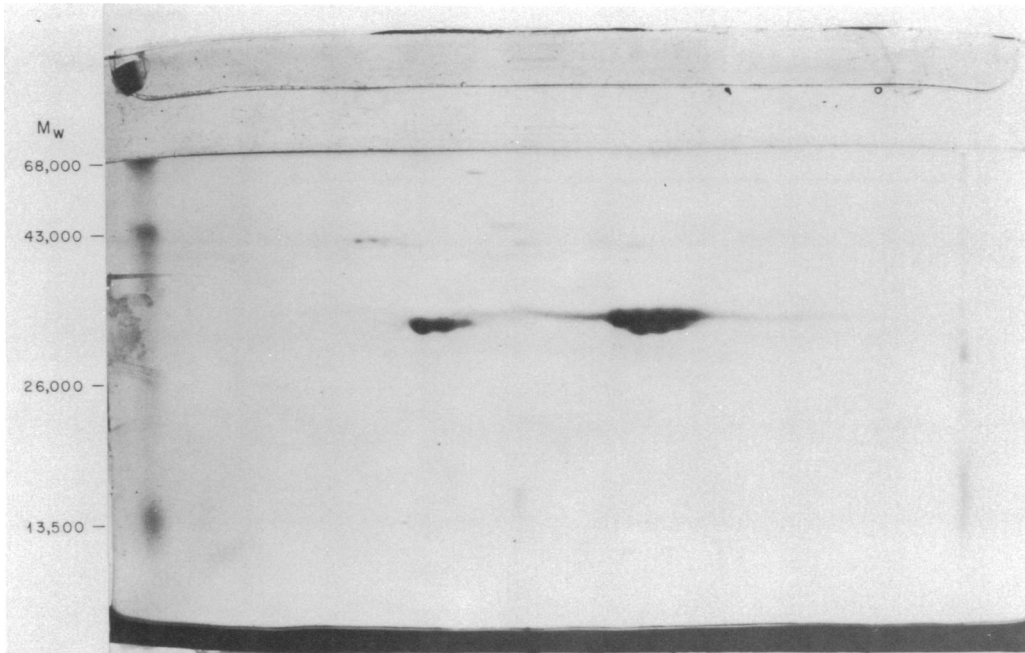
FIG. 1. *Two-dimensional polyacrylamide gel electrophoresis of outer membrane proteins. First dimension was (left to right) separation in 8 M urea, pH 8.5, for 4.5 h at 350 to 400 V; second dimension (top to bottom) was separation in 0.2% SDS, pH 6.48, for 17 h at 32 V. The separating slab gel contained 10% acrylamide, 0.5% bisacrylamide, and the cementing gel contained 4% acrylamide. The marker proteins, molecular weight indicated on the left, are (from the top) bovine serum albumin, ovalbumin, chymotrypsinogen, and hemerythrin. The gel slab is stained with Coomassie blue. The outer membranes of strain M188-777 were prepared according to Schnaitman (18) and were applied in 6 M urea (see ref. 10).*

nents" upon temperature shift (19), the envelope fraction of this strain changes less dramatically. Several spots of lower molecular weight are present at the nonpermissive temperature, but not at the permissive temperature; only a few spots appear to be temperature sensitive.

DISCUSSION

The rationale in using the present electrophoretic system was to try to separate the membrane proteins with respect to more than one parameter sequentially, thus at least in part reducing the probability of multifold spots. Two obvious parameters were intrinsic charge in one dimension and molecular weight in the other.

(i) The procedure for membrane preparation had to be simple and fast to enable the analysis of a great number of mutant strains. The method of choice was French pressing and washing by centrifugation at relatively low centrifugal forces of $30,000 \times g$, thus avoiding time-consuming ultracentrifugation. This

might result in a low yield of membranes, particularly of inner cytoplasmic membrane. However, sonication followed by repeated ultracentrifugation at $200,000 \times g$ gave qualitatively similar results. More washings were required to remove cytoplasmic constituents as measured by β-galactosidase activity. (ii) Detergents such as SDS or Triton X-100 commonly used for solubilization of membrane proteins greatly interfere with separation according to charge in a milieu of 8 M urea and are difficult to remove. Therefore, the membranes had to be solubilized in such a fashion that the dissolving agent could easily be removed by dialysis against urea, the medium for separation in the first dimension.

With this concept in mind, we employed the basic procedure of Moldow et al. (12), using the chaotropic agent guanidine thiocyanate to initially solubilize the membranes. The reagent was removed by dialysis against 6 M urea, which left the majority of the proteins in solution and ready for electrophoresis in 8 M urea. The method of Metz and Bogorad (10) established that the urea gels could then be used for
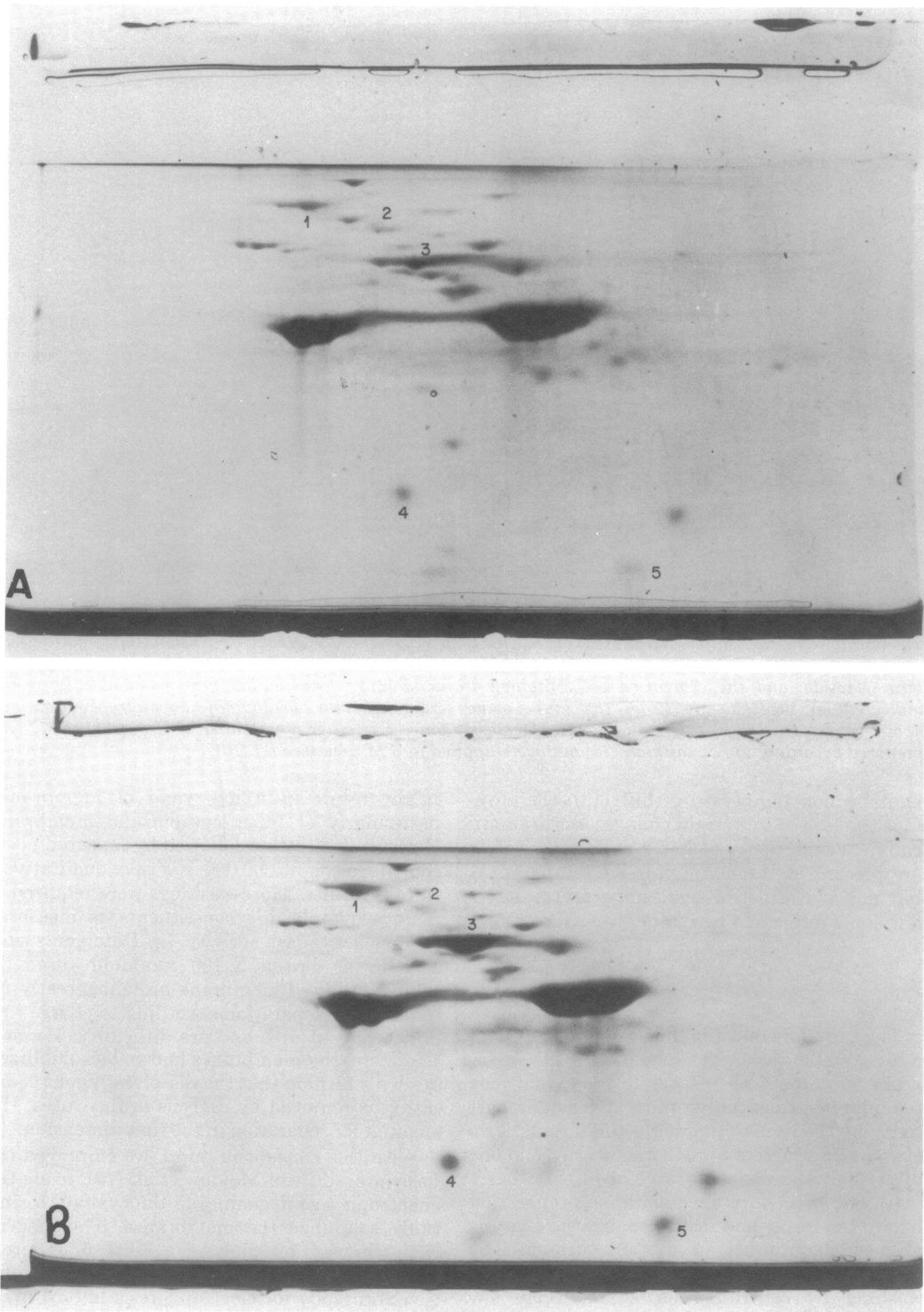
FIG. 2. *Two-dimensional polyacrylamide gel electrophoresis of total envelope proteins. (A) Strain M188-777 grown in the absence of D-fucose; (B) strain M188-777 grown in the presence of D-fucose, inducer of the β-methylgalactoside transport system; (C) strain M188-1000 defective in mglABC but otherwise isogenic with M188-777. The mutant M188-1000 was grown in the presence of D-fucose. Details as in legend to Fig. 1.*
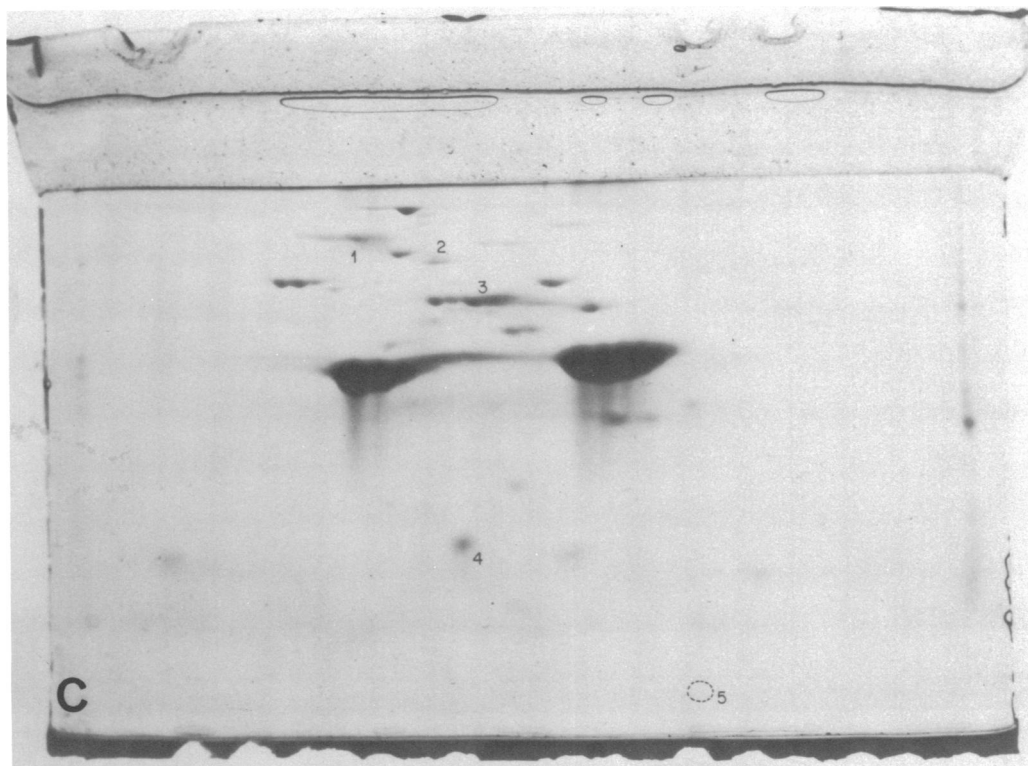
FIG. 2C

SDS-slab gel electrophoresis with no interference by the remaining urea and resulting in separation according to molecular weight.

The actual electrophoresis technique presented no problems with respect to good migration in both dimensions. The major drawback in the procedure was protein loss during dialysis against urea prior to electrophoresis. However, analysis of the proteins contained in the several steps of the solubilization procedure by conventional SDS electrophoresis shows no selective loss. The possibility of thiocarbamylation by the thiocyanate anion of primary or secondary amines present in the membrane protein during solubilization apparently presents little problem. Moldow et al. (12) found 1 nmol of [^{14}C]-labeled thiocyanate bound per 590 nmol of amino acid, or 0.17%. It is doubtful that this small amount would be stained. We therefore believe that the method as presented here is accurate and gives a better resolution of membrane protein than conventional tube urea or SDS electrophoresis alone.

The advantageous use of this two-dimensional polyacrylamide gel electrophoresis was demonstrated by the following examples. (i) Analysis of outer membrane proteins revealed more major components of similar molecular weight than had been reported before (18). (ii) The analysis of the total envelope protein of strains induced and uninduced for a particular sugar transport system showed alteration of four to five proteins, which might possibly contain the hitherto unknown components of the transport machinery. Based on genetic data, the existence of at least three components had been postulated (Boris Rotman [personal communication] has further evidence that the *mglA* and *C* gene products are necessary for translocation of substrate) (14). The electrophoretic analysis of the envelope proteins of clearly defined polar mutants or deletions will answer this question. (iii) The analysis of the envelope proteins of a strain, temperature sensitive in cell division, revealed some alterations in the protein pattern. One of the spots missing when grown at the nonpermissive temperature was found to be fucose inducible in the wild type. This correlates with a previous observation that this mutant does not synthesize an in-
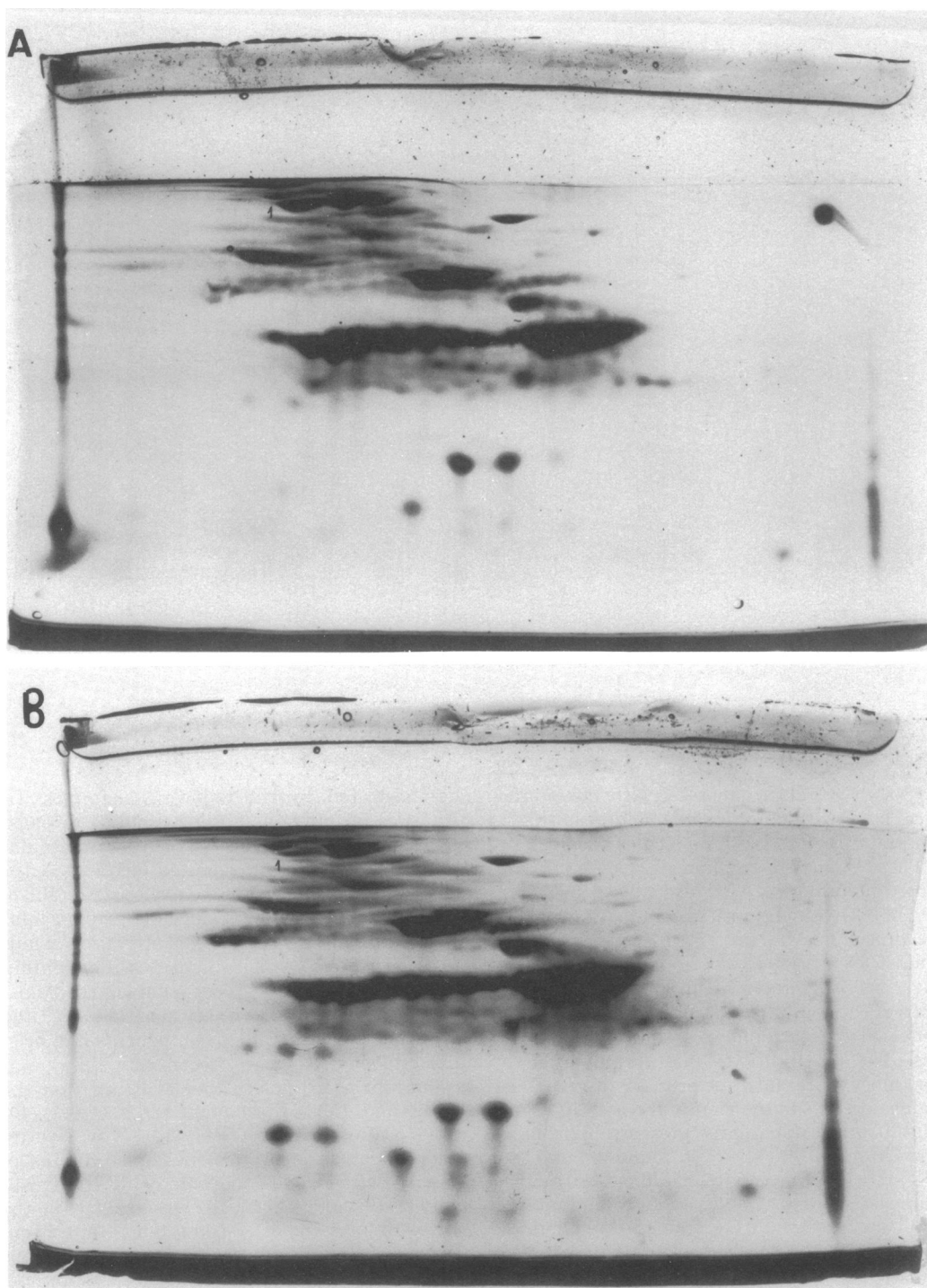
FIG. 3. *Two-dimensional polyacrylamide gel electrophoresis of total envelope proteins of BUG-6, temperature sensitive in cell division. Cells were grown at 35 (A) and 42 C (B). Details as in legend to Fig. 1.*

tact β-methylgalactoside transport system and galactose-binding protein when grown at the nonpermissive temperature.

LITERATURE CITED

1. Ames, Giovanna Ferro-Luzzi. 1974. Resolution of bacterial proteins by polyacrylamide gel electrophoresis on slabs. Membrane, soluble, and periplasmic fractions. J. Biol. Chem. **249**:634–644.
2. Boos, W. 1974. Pro and contra carrier proteins; sugar transport via the periplasmic galactose-binding protein. Curr. Top. Membr. Transp. **5**:51–136.
3. Bragg, P. D., and C. Hou. 1972. Organization of proteins in the native and reformed outer membrane of *E. coli*. Biochim. Biophys. Acta **274**:478–488.
4. Fairbanks, G., T. L. Steck, and D. F. H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry **10**:2606–2617.
5. Inouye, M., and M.-L. Yee. 1973. Homogeneity of envelope proteins of *Escherichia coli* separated by gel electrophoresis in sodium dodecyl sulfate. J. Bacteriol. **113**:304–312.
6. Kaltschmidt, E., and H. G. Wittmann. 1970. Ribosomal proteins. VII. Two-dimensional polyacrylamide gel electrophoresis for fingerprinting of ribosomal proteins. Anal. Biochem. **36**:401–412.
7. Laemmli, V. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
8. Lengeler, J., K. O. Herman, H. J. Unsöld, and W. Boos. 1971. The regulation of the β-methylgalactoside transport system and of the galactose-binding protein of *Escherichia coli* K12. Eur. J. Biochem. **19**:457–470.
9. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
10. Metz, L. J., and L. Bogorad. 1974. Two-dimensional polyacrylamide gel electrophoresis: an improved method for ribosomal proteins. Anal. Biochem. **57**:200–210.
11. Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
12. Moldow, C., J. Robertson, and L. Rothfield. 1972. Purification of bacterial membrane proteins. The use of guanidinium thiocyanate and urea. J. Membr. Biol. **10**:137–152.
13. Neu, H. C., and L. A. Heppel. 1965. The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts. J. Biol. Chem. **240**:3685–3692.
14. Ordal, G. W., and J. Adler. 1974. Isolation and complementation of mutants in galactose taxis and transport. J. Bacteriol. **117**:509–516.
15. Parnes, J. R., and W. Boos. 1973. Unidirectional transport activity of the β-methylgalactoside transport system. J. Biol. Chem. **248**:4436–4445.
16. Reeve, J. N., D. J. Groves, and D. J. Clark. 1970. Regulation of cell division in *Escherichia coli*: characterization of temperature-sensitive division mutants. J. Bacteriol. **104**:1052–1064.
17. Schnaitman, C. A. 1970. Protein composition of the cell wall and cytoplasmic membrane of *Escherichia coli*. J. Bacteriol. **104**:890–901.
18. Schnaitman, C. A. 1974. Outer membrane proteins of *Escherichia coli*. III. Evidence that the major protein of *Escherichia coli* 0111 outer membrane consists of four distinct polypeptide species. J. Bacteriol. **118**:442–453.
19. Shen, B. H. P., and W. Boos. 1973. Regulation of the β-methylgalactoside transport system and the galactose-binding protein by the cell cycle of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **70**:1481–1485.
20. Silhavy, T. J., and W. Boos. 1974. Selection procedure for mutants in the β-methylgalactoside transport system of *Escherichia coli* utilizing the compound 2R-glyceryl-β-D-galactopyranoside. J. Bacteriol. **120**:424–432.
21. Subramanian, A. R. 1974. Sensitive separation procedure for *Escherichia coli* ribosomal proteins and the resolution of high-molecular-weight components. Eur. J. Biochem. **45**:541–546.
22. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**:4406–4412.
23. Wu, H. C. P., W. Boos, and H. M. Kalckar. 1969. Role of the galactose transport system in the retention of intracellular galactose in *Escherichia coli*. J. Mol. Biol. **41**:109–120.