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Purification of Bacterial Membrane Proteins

The Use of Guanidinium Thiocyanate and Urea

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Summary. Guanidinium thiocyanate was shown to be effective in solubilizing over 80% of the protein of the *E. coli* cell envelope. Fractionation of the solubilized membrane polypeptides by ion exchange chromatography was achieved following removal of the guanidinium thiocyanate by dialysis against 6 M urea.

Proteins are major constituents of biological membranes and are thought to be responsible for a wide variety of membrane functions. Although some membrane proteins have been successfully extracted and purified, most integral membrane proteins have not been accessible to standard techniques of biochemical fractionation. In large part this has been due to the tendency of these proteins to aggregate in aqueous solvents.

To facilitate the study of hydrophobic proteins, Hatefi and Hanstein (1969) suggested the use of "chaotropic" agents, which promote the solubilization of many hydrophobic compounds in aqueous solvents. According to these authors, the order of effectiveness of these agents is $SCN^- > ClO_4 >$ guanidinium$^+ >$ urea $> CL^- > F^-$. We have found (Robertson, Moldow + Rothfield, 1972) that the combination of the most efficient chaotropic anion and cation in the form of guanidinium thiocyanate is an effective solubilizer of membrane proteins. Following solubilization with guanidinium thiocyanate, ion exchange chromatography in urea was used to isolate several groups of membrane proteins.

Previous studies of the membrane proteins of gram-negative bacteria by gel electrophoresis in sodium dodecyl sulfate (SDS) have revealed many

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peptide bands, with a major component of molecular weight approximately 40,000 (Schnaitman, 1970*a*). The "major band" was located in the cell wall-outer membrane fraction and accounted for more than half of the total protein of this fraction. It has been suggested that this component may be a single "structural protein" (Schnaitman, 1970*b*). The present studies confirm that components of the major band are located in the outer membrane of the *E. coli* cell envelope but indicate that it includes several polypeptides, rather than being a single species.

Materials and Methods

Materials

All chemicals used were reagent grade. Deionized water was used in all solutions. Solutions of 6 M urea (Baker) were freshly prepared, stirred with a mixed bed ion exchange resin (Mallinkrodt Amberlite MB-3) and the resin removed by filtration immediately before use. Guanidinium thiocyanate (Eastman) was recrystallized from methanol and washed with diethly ether in early experiments; it was found that this procedure has no effect on the amount of protein solubilized and the recrystallization procedure was omitted in the later large scale solubilization experiments. Guanidinium [^{14}C]thiocyanate was prepared by mixing 8 mg of sodium [^{14}C]thiocyanate (ICN-Tracerlab, 3.45 mC/nmole) with 4.9 g of guanidinium thiocyanate to obtain a final specific activity of 3.5×10^8 cpm/mmole thiocyanate. [^{14}C]urea and the magnesium salt of 1-anilino-8-napthalene sulfonate (ANS) were purchased from New England Nuclear and Eastman Organic Chemicals. *E. coli* C90 was obtained from Dr. A. Garen.

Analytical Procedures

The procedure of Lowry, Rosebrough, Farr and Randall (1951) was used to measure protein with bovine serum albumin as standard. Heptose and 2-keto-3-deoxyoctonate (KDO) were assayed as previously described (Osborn, 1963). For phospholipid determinations, the sample was extracted twice with five volumes of chloroform-methanol (3:1) at room temperature and the organic phase back extracted with 0.2 volumes of 0.05 M KCl-methanol-chloroform (47:48:3). Total phosphate was then determined in the final organic phase by the method of Ames and Dubin (1960). RNA was estimated by the method of Munro and Fleck (1966). Lipopolysaccharide concentrations were calculated from heptose measurements using the value of 0.5 μmole heptose per mg lipopolysaccharide obtained from parallel measurements of authentic lipopolysaccharide. Similar values were obtained when KDO content was used as the basis for determination of lipopolysaccharide.

Polyacrylamide Gel Electrophoresis

All gels were run at room temperature with tap water cooling. Acrylamide and N,N′-methylene bisacrylamide were punchased from Eastman Organic Chemicals. Ammonium persulfate and N, N, N′, N′-Tetramethylethylenediamine were used to catalyze polymerization. All gels were prerun for 20 min before use. Stacking gels were not used in any electrophoretic system and 10 cm × 0.29 cm^2 gels were used; in some experiments a modified E – C slab gel apparatus, with 2-mm thick gels was employed.

Method I

Gel electrophoresis was performed on 7.5% polyacrylamide gels in the presence of 2% sodium dodecyl sulfate (SDS) as described by Fairbanks, Steck and Wallach (1971). Samples (50 to 100 µg protein per gel) were heated to 100 °C for 5 min in 2% SDS, 25 mM dithiothreitol and 25 mM Tris-HCl buffer, pH 7.8, before being applied to the gels. The gels were fixed and stained with Coomassie blue (Fairbanks *et al.*, 1971).

Method II

Polyacrylamide gels 6.8 × 5 (notation of Neville, 1971) were prepared in the presence of 6 M urea and 0.1 M Tris-HCl buffer, pH 8.7. Samples (100 µg protein per gel) were applied in 0.1 ml of 50 mM Tris-HCl buffer, pH 8.7, containing 6 M urea and 35% sucrose. The running buffer was 25 mM Tris-glycine, pH 9.2. Gels were run at 3 ma per gel for at least 3 hr, stained for 2 hr with 0.25% Amido Black in 10% acetic acid and destained electrophoretically.

Method III

Electrophoresis was performed in 7.5% polyacrylamide gels containing 6 M urea and potassium acetate buffer, pH 4.5, as described by Reisfeld, Lewis and Williams (1962). Samples (75 to 100 µg protein per gel) were applied in 10 mM sodium acetate buffer, pH 4.5, containing 6 M urea, 5 mM β-mercaptoethanol and 10% sucrose. Gels were run at 3 ma per gel for 10 to 12 hr. After cooling to 4 °C, the gels were stained with Amido Black as described above.

Column Chromatography

Samples to be chromatographed were dialyzed extensively at 4 °C against the equilibrating buffer until the pH and conductivity of the sample were identical to the column eluate. Protein concentrations in the column fractions were estimated by measuring the absorbance at 280 nm and salt concentration was determined by measuring conductivity. All buffers contained 6 M urea, 5 mM β-mercaptoethanol and 5 mM EDTA; columns were equilibrated and poured in the cold, and all chromatography was done at 4 °C.

DEAE cellulose (Cellex D, BioRad) was extensively washed with 0.5 N NaOH and 0.5 N HCl and then equilibrated with 5 mM Tris-HCl buffer, pH 7.8. The urea soluble fraction was applied to a column (19.5 cm^2 × 50 cm), the column was washed with 1,500 ml of 5 mM Tris-HCl buffer, pH 7.8, and a linear gradient of 0 to 0.5 M NaCl in the same buffer (1,000 ml of each, was applied, followed by a second linear gradient of 0.5 to 1.0 M NaCl in the same buffer (500 ml of each). Finally, the column was washed with 300 ml of 5.5 M guanidinium thiocyanate. Fractions of 10 ml were collected at a flow rate of 80 ml/hr.

SP-Sephadex C-50 (Pharmacia) was equilibrated in 10 mM sodium acetate, pH 4.5, with several changes of buffer and repeated decanting of fines. Columns (12.5 cm^2 × 15 cm) were poured under an 8-cm pressure head and extensively washed with the same buffer. Carboxymethyl Sephadex C-25 (CM-Sephadex, Pharmacia) was treated in a similar fashion using 10 mM sodium phosphate, pH 6.2, as the equilibrating buffer. After applying the sample, the columns were washed with 250 ml of the starting buffer followed by a linear gradient of 0 to 0.5 M NaCl in the starting buffer (250 ml of each). The columns were finally eluted with 200 ml of 1 M NaCl. Fractions of 8 ml were collected.

Cell Envelope Preparation

E. coli C-90 was grown to late exponential growth phase in enriched medium (10 g Difco Tryptone, 5 g Difco yeast extract, 5 g NaCl and 2 g glucose per liter) with pH maintained at 7.0 by addition of 2 M Na_2CO_3. Cells were harvested by continuous-flow centrifugation, washed once with 10 mM Tris-HCl buffer, pH 7.8, and resuspended at a concentration of 1 g (wet weight) per ml in cold 10 mM Tris-HCl buffer, pH 7.8, containing 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 2 mg/ml lysozyme (Sigma) and 10 µg/ml pancreatic RNase (Worthington). The suspension was mixed thoroughly at 4 °C, frozen and thawed once and sonicated intermittently for a total of 5 min with temperature maintained below 10 °C. The sonicate was centrifuged at 4 °C for 12 hr at 40,000 $\times g$. The pellet was resuspended in 10 mM Tris-HCl buffer, pH 7.8, containing 0.2 M KCl, 5 mM EDTA and 5 mM β-mercaptoethanol and washed in this same buffer until no further protein appeared in the supernatant fluid. The pellet was suspended in 10 mM Tris-HCl buffer, pH 7.8, 5 mM EDTA, 5 mM β-mercaptoethanol, and spun at 3,000 $\times g$ for 10 min to remove whole cells. Finally, this supernatant fluid was centrifuged at 40,000 $\times g$ for 12 hr and the pellet resuspended in 10 mM Tris-HCl buffer, pH 7.8, 5 mM EDTA, 5 mM β-mercaptoethanol, at a protein concentration of 25 to 30 mg/ml. This material contained no visible intact cells when examined by phase microscopy. It banded at the same density in a sucrose gradient and had an identical SDS gel electropherogram to material isolated by isopycnic centrifugation as described below. As noted below, the SDS pattern was identical to the combination of the gel patterns of purified inner and outer membrane fractions. No material was present at the bottom of the gradient, where intact cells are normally recovered.

Membrane proteins were labeled by growing cells to late exponential growth phase in medium M 9 (Clowes & Hayes, 1968) containing 2% glucose and 4,5 [^{3}H]leucine (20 mg/liter, 13 µC/mmole). The cells were collected by centrifugation, washed twice in 10 mM Tris-HCl buffer, pH 7.8, and suspended in the same buffer at an optical density of 15 at 600 nm. This suspension was sonicated discontinuously for 5 min in Branson sonifier with temperature maintained below 10 °C, and the sonicate centrifuged at 250,000 $\times g$ for 30 min at 4 °C. The pellet was washed three times in a solution containing 0.2 M KCl, 5 mM EDTA, 10 mM Tris-HCl, pH 7.8, and resuspended in 2 volumes of 10 mM Tris-HCl, pH 7.8. This suspension was applied to a linear gradient of 35% to 60% sucrose buffered with 10 mM Tris-HCl, pH 7.8. After centrifugation at 80,000 $\times g$ for 12 hr, the band located at density 1.22 g cm^{-3} was isolated and again subjected to gradient centrifugation in an identical manner. The membrane fraction from this gradient was isolated, collected by centrifugation and resuspended in 10 mM Tris-HCl, pH 7.8 (final specific activity, 10^5 cpm per mg protein).

Isolated inner and outer membrane fractions were prepared by the procedure of Osborn, Gander, Parisi and Carson (1972). The combination of the SDS gel patterns of the purified inner and outer membrane fractions was identical to the gel patterns of the total cell envelope prepared as described above.

Solubilization of the Cell Envelope

The cell envelopes were buffered with 10 mM Tris-HCl, pH 7.8, containing 5 mM β-mercaptoethanol, and 5 mM EDTA during all steps of the solubilization procedure ("standard buffer").

The membrane suspension (30 mg protein/ml) was sonicated briefly at 4 °C to obtain a homogenous dispersion. Solid guanidinium thiocyanate was added to achieve a final concentration of 6 M. This turbid suspension became clear during 30 min of gentle agitation at room temperature and the entire mixture was centrifuged at 250,000 $\times g$ for 2 hr at 25 °C. The supernatant fluid was immediately dialyzed at 25 °C against 100 volumes

of standard buffer containing 6 M urea until the pH and conductivity of the solubilized cell membranes was equal to that of the standard buffer in 6 M urea. The dialysate was then centrifuged at 250,000 × g for 2 hr at 4 °C. The pellet obtained from this step was combined with the earlier pellet and re-extracted with guanidinium thiocyanate in a similar fashion. The second extraction usually solubilized an additional 5 to 10% of the protein of the membrane suspension.

The degree of thiocarbamylation of the solubilized polypeptides was determined by solubilizing the membranes with guanidinium [^{14}C]thiocyanate (3.5 × 10^8 cpm/mmole). Specific activity of the solubilized protein was determined by mixing 300 μg of sample with 0.3 mg of bovine serum albumin (Pentex), adding 10 volumes of cold 10% trichloracetic acid, and immediately filtering under suction through a 045-μ Millipore filter. No detectable protein remained in the supernatant fluid after the trichloracetic acid precipitation. The filters were washed twice with cold 5% trichloracetic acid, air-dried and counted in 5 ml of toluene-Bio-Solv-Liquifluor (Beckman) to a 1% level of significance in a Beckman LS 230 liquid scintillation spectrometer. Similar experiments to assess the possibility of carbamylation during the exposure to urea were done by carrying out the purification procedure in the presence of [^{14}C]urea (3 × 10^5 cpm/mmole). The amount of [^{14}C]urea that was incorporated into acid-insoluble material was measured at each stage of the protein purification as described above.

Preparation of Membrane Antisera

Urea gels (*Method II*) were stained with the magnesium salt of 1-anilino-8-napthalene sulfonate (ANS) as described by Hartman and Udenfriend (1969). Utilizing UV light to visualize the bands, the regions of the gel indicated in Fig. 6 were cut out and homogenized in 0.15 M NaCl by forcing the suspension through a fine mesh screen. It was possible to reproduceably cut out the top band from the slowly migrating group of bands (band 5 in Fig. 6), but because of the insensitivity of staining with ANS, single bands could not accurately be cut out of the intermediate and rapid regions. Therefore, all the bands in each of these regions were cut out and treated as a unit. Each of the homogenates, containing approximately 500 μg of protein, were then mixed with an equal volume of incomplete Freund's adjuvant and rabbits were inoculated in multiple subcutaneous locations on days 1 and 21. The rabbits were bled by cardiac puncture on day 60. The sera was collected, heat inactivated at 56 °C for 30 min and dialyzed against 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.4, centrifuged at 12,000 × g for 20 min, and the supernatant fluid stored at −20 °C.

Membrane protein fractions prepared by ion exchange chromatography as described in the text were dialyzed against 0.15 M NaCl, centrifuged at 12,000 × g for 30 min to remove precipitate, and then were used as antigens in precipitin reactions.

Precipitin tests were done by carefully overlaying 0.1 ml of antiserum with 0.1 ml antigen (0.3 mg protein per ml) in 6 × 50 mm tubes). The tubes were incubated at 37 °C for 1 hr, then kept at 4 °C for 36 hr. A positive reaction was the appearance of a sharp band at the antigen-antiserum interface.

Results

Solubilization of Membrane Proteins

Guanidinium thiocyanate was approximately as effective in solubilizing[1] *E. coli* membrane proteins as SDS and more effective than guanidinium

[1] Solubility was operationally defined as the failure to sediment after centrifugation at 200,000 × g for 2 hr at 4 °C.

Table 1. Solubilization of membrane proteins by different agents

Reagent	Concentration	Protein solubilized (%)
Guanidinium SCN	6 M	84
Sodium SCN	6 M	42
Guanidinium Cl	6 M	13
Cetyltrimethylammonium Br	2%	61
Sodium deoxycholate	2%	41
Triton X-100	2%	54
SDS	2%	82
Urea	6 M	<4
Thiourea	2 M	<4

4,5[^{3}H]leucine-labeled cell envelope (52,000 cpm/ml) was incubated with the agents listed above for 1 hr at room temperature with mixing. Radioactivity in the supernatant fluid after centrifugation at 250,000 × *g* for 2 hr was measured and is expressed as per cent of total radioactivity in the original labeled cell envelope.

chloride or sodium thiocyanate when solubilization was determined by the use of [^{14}C]leucine-labeled membranes (Table 1) or by the direct measurement of protein in the supernatant solution. Detergents other than SDS solubilized less protein, and urea alone was almost completely ineffective. The degree of solubilization was proportional to the concentrations of guanidinium thiocyanate and maximal solubilization was achieved with a 6-M solution of this chaotropic agent (Fig. 1). Varying the temperature between 4° and 37 °C did not affect the amount of membrane protein solubilized.

Essentially, all the polypeptides remained in solution when the guanidinium thiocyanate was removed by dialysis against 6 M urea (Fig. 2), and all subsequent fractionation steps were performed in the presence of urea.

When the solution of membrane polypeptides in guanidinium thiocyanate was dialyzed directly against 0.1 M Tris-HCl, pH 7.8, a heavy white precipitate rapidly appeared, containing 75% of the solubilized protein. Approximately 40% of the total protein remained in solution when the urea was removed from the urea-soluble fraction by dialysis against 0.15 M NaCl.

In addition to protein, the other major membrane components in these organisms are phospholipid and lipopolysaccharide. Less than 5% of the lipopolysaccharide and phospholipid of the original membrane preparation was present in the urea-soluble fraction (Fig. 2) and this small residual amount was removed by DEAE column chromatography in the next step of the fractionation procedure. There was no detectable RNA in the urea-

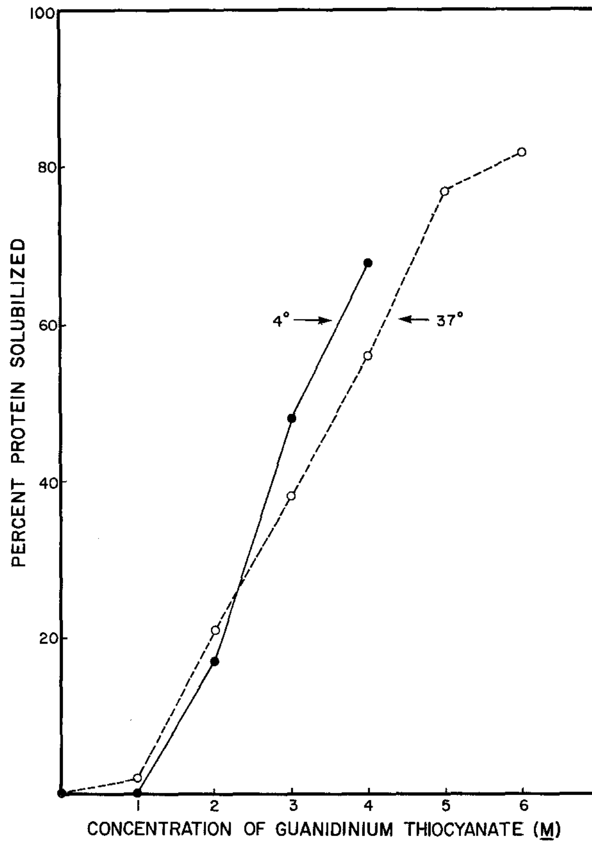


Fig. 1. Solubilization of cell envelope protein by increasing amounts of guanidinium thiocyanate at 4 and 37 °C. The percentage of protein solubilized was estimated as described in Table 1

soluble fraction. The insoluble polypeptides remaining in the residue after the extraction with guanidinium thiocyanate appeared to contain a representative sample of the major bands present in the urea-soluble fraction when both were examined by SDS gel electrophoresis.

The urea-soluble fraction contained approximately 18 polypeptide bands when examined by polyacrylamide gel electrophoresis in the presence of urea at pH 8.7 (Fig. 3). Electrophoresis in urea at pH 4.5 gave generally similar results but with poorer resolution of the bands. As indicated below, the solubilized polypeptides were then fractionated by ion exchange chromatography.

To determine if thiocarbamylation of membrane polypeptides occurred to a significant degree during solubilization, guanidinium [^{14}C] thiocyanate was used as the solubilizing agent. The urea-soluble fraction contained

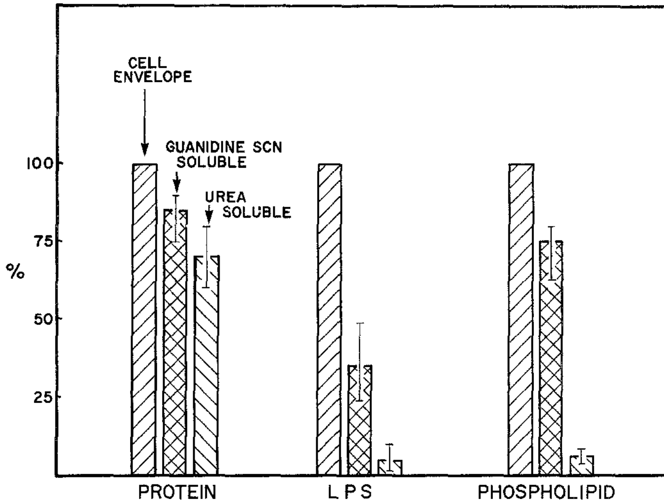


Fig. 2. Composition of solubilized fractions. Protein, lipopolysaccharide and phospholipid were determined in the supernatant fluid after centrifugation at 250,000×*g* for 2 hr as described in *Methods* and are expressed as percentage of the amounts present in the original cell envelope fraction. The urea-soluble fraction contained 42 μg of lipopolysaccharide and 34 μg of phospholipid per mg of protein

17 nmoles of $S^{14}CN$ per mg of protein, corresponding to 1 nmole of $S^{14}CN$ per 590 nmoles of amino acid. The isolated SP-Sephadex fractions contained <2 nmoles of $S^{14}CN$ per mg of polypeptide, corresponding to <1 nmole of SCN per 5,000 nmoles of amino acid. Experiments were also performed to determine whether the exposure to urea resulted in carbamylation of the solubilized polypeptides. Studies done in the presence of [^{14}C] urea revealed no detectable carbamylation at any stage of protein purification (<6.7 nmoles [^{14}C]cyanate per mg of protein).

Anion Exchange Chromatography

Five peaks were obtained when the soluble polypeptides were chromatographed on DEAE cellulose in the presence of 6 M urea (Fig. 4), accounting for 60% of the protein applied to the column. The remainder of the polypeptides could not be eluted with 1 M NaCl but were eluted by 6 M guanidinium thiocyanate (not shown in figure). This final fraction precipitated when dialyzed against 6 M urea. Gel electrophoresis in SDS or urea showed it to be highly aggregated, barely penetrating the gel, and this material was not further studied.

When each of the DEAE fractions was rechromatographed under identical conditions, a single peak containing all of the material applied to the

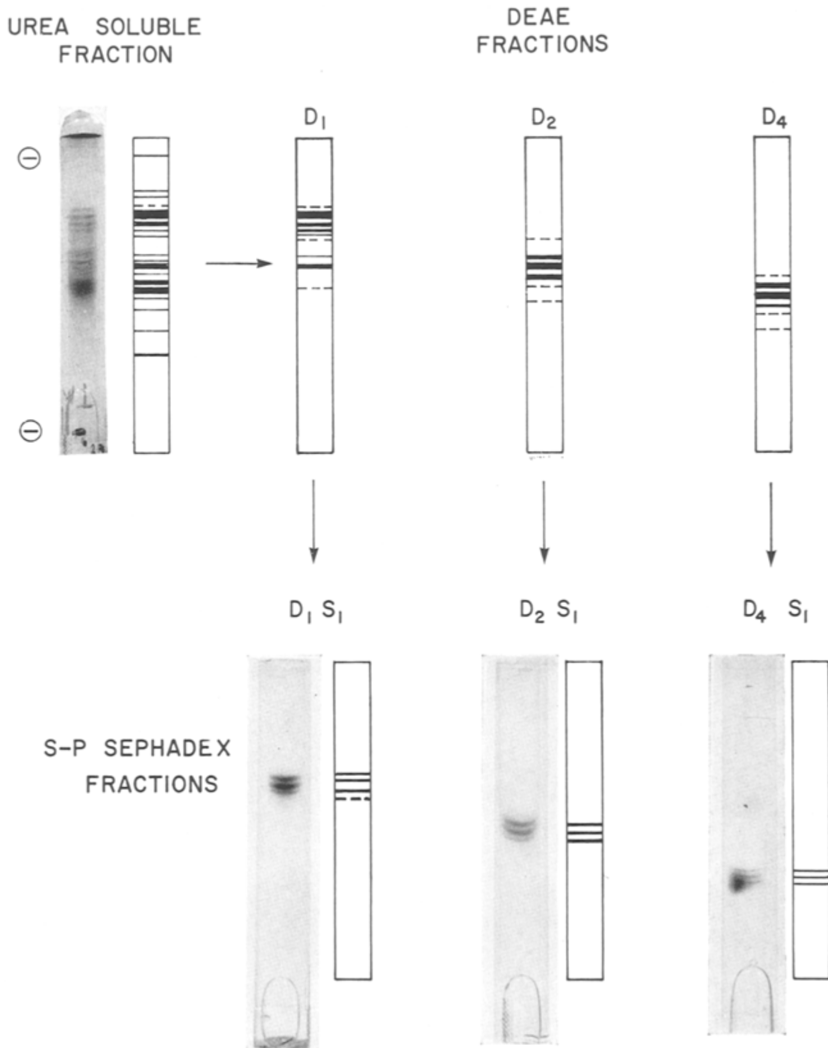


Fig. 3. Urea gel electrophoresis at different stages of isolation. Urea gel electrophoresis was performed by *Method II*. The symbols over the individual gels refer to the chromatographic peaks shown in Figs. 4 and 5. The gels were run for 3 hr

column was eluted at the same salt concentration. In each case, the gel patterns of the rechromatographed polypeptides were unchanged.

The first DEAE fraction D_1 was enriched for the polypeptides of slowest mobility (Fig. 3). Fraction D_2 contained polypeptides of intermediate mobility in the urea gel electropherograms, while D_4 corresponded to the major group of rapidly migrating polypeptides. Fraction D_3 appeared to

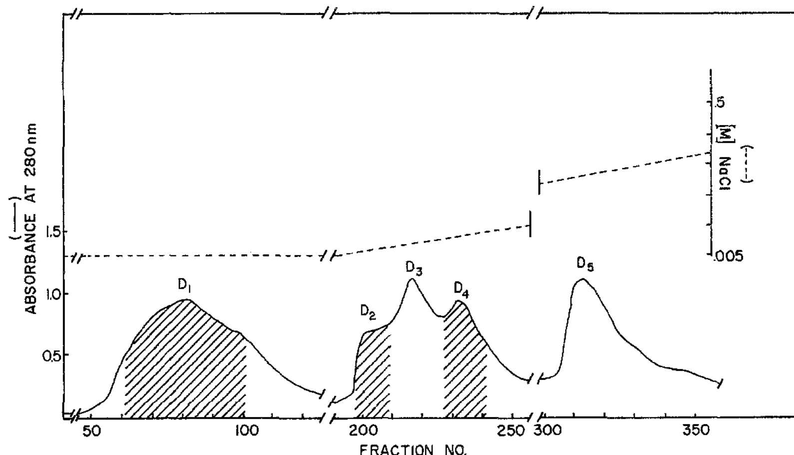


Fig. 4. DEAE cellulose chromatography of the urea-soluble fraction. 1.5 g of protein was applied to the column. The fraction eluted with 5.5 M guanidinium thiocyanate is not shown

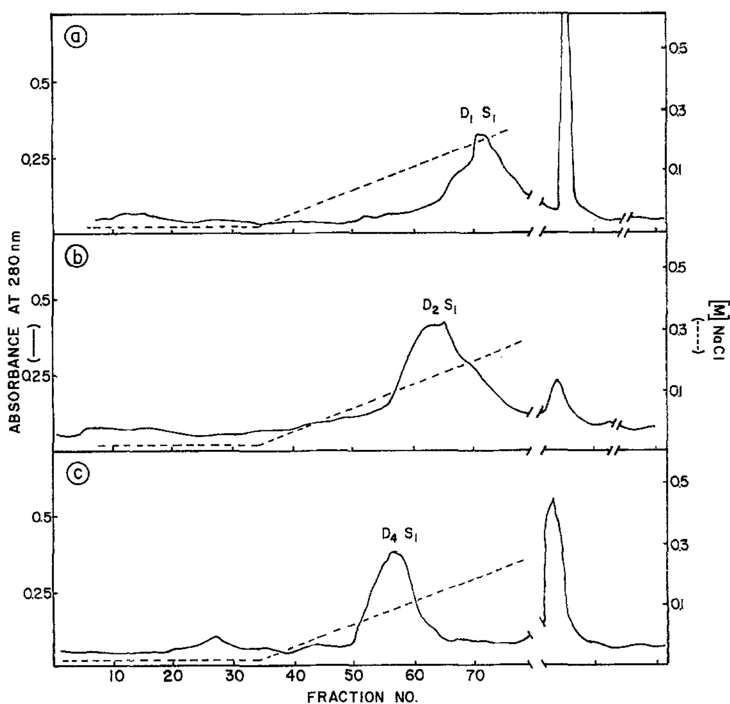


Fig. 5. Chromatography on SP-Sephadex of DEAE peaks (*see* Fig. 4). DEAE peaks (*see* Fig. 4) were concentrated by ultrafiltration in an Amicon ultrafiltration cell using a PM10 membrane filter and were then subjected to chromatography of SP-Sephadex as described in *Methods*. (*a*) D_1, 100 mg protein; (*b*) D_2, 75 mg protein, and (*c*) D_4, 75 mg protein. Following completion of the NaCl gradient, the final peaks were eluted with 0.6, 0.5, and 0.35 M, respectively

contain some polypeptides from fraction D_2 and D_4 and was not further fractionated.

Fraction D_5 failed to penetrate urea gels. SDS gel electropherograms indicated two bands that migrated closely together in the molecular weight region corresponding to the "major" band, plus at least four faintly staining bands of more rapid migration.

Cation Exchange Chromatography

Fraction D_1 was further fractionated by chromatography on SP-Sephadex to give two major peaks (Fig. 5*a*). The first peak (D_1S_1) contained the slowly migrating polypeptides (Fig. 3) which together accounted for 10% of the total protein of the original membrane suspension.

Fractions D_2 and D_4 were chromatographed on SP-Sephadex (Fig. 5*b*, *c*) in a similar fashion. Peak D_2S_1 contained the major polypeptides of intermediate migration on urea gels while D_4S_1 included the most rapidly migrating polypeptides (Fig. 3). Peaks D_2S_1 and D_4S_1 accounted for 3% and 2% of the total membrane protein, respectively.

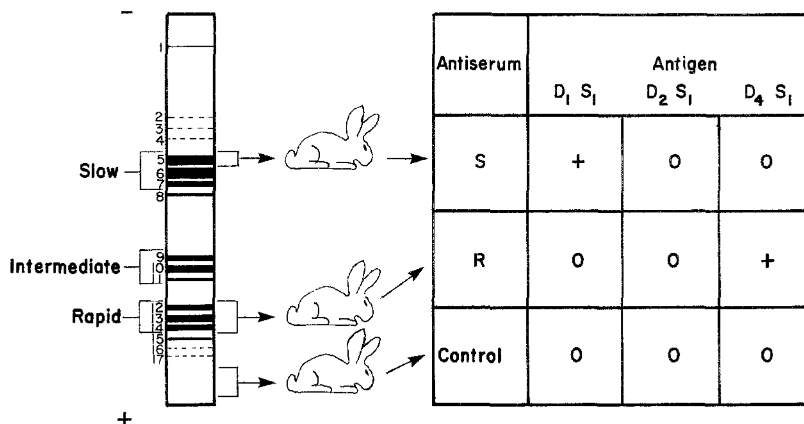
When the SP-Sephadex fractions were examined by urea gel electrophoresis at acid pH (system III) similar patterns were obtained.

In each of the three SP-Sephadex columns a second major peak was eluted with high concentration of salt. These peaks could not be resolved on urea gels, where most of the material remained at the origin, but gel electrophoresis in SDS showed several bands corresponding to polypeptides of molecular weights 15,000 to 45,000 in addition to a moderate amount of protein that did not enter the gel.

The polypeptides in fraction D_5 were chromatographed on the cation exchange column CM-Sephadex. Three peaks were obtained. The polypeptides in all three peaks remained at the origin in acid and alkaline urea gel electropherograms. SDS gels showed each of these peaks to contain a large amount of material that migrated with the "major" band. Some polypeptides failed to enter the gel and minor bands of more rapid mobility were present in all fractions.

Immunoprecipitation Studies

Further evidence that the partially purified fractions were biochemically distinct was obtained from immunoprecipitation studies (Fig. 6). Antiserum directed against the top band of the slowly migrating group reacted only with the purified fraction containing this band (D_1S_1). In a similar manner,



Antiserum	Antigen		
	D_1S_1	D_2S_1	D_4S_1
S	+	O	O
R	O	O	+
Control	O	O	O

Fig. 6. Precipitin tests were performed as described in *Methods*. Antiserum S-Fraction D_1S_1 (Fig. 3) was electrophoresed according to *Method II* and the band corresponding to band 5 (above) was used as immunogen as described in *Methods*. Antiserum R-Fraction D_4S_1 (Fig. 3) was electrophoresed (*Method II*) and the region containing bands 12–14 (above) was used as immunogen. Control antiserum was obtained by immunizing with an area of the gel that contained no protein. The gel pattern shown above was obtained with the total urea-soluble fraction to indicate the relative positions of the immunizing fractions. +, positive reaction; ○, negative reaction

antiserum directed against the rapidly migrating fraction reacted solely with its corresponding column fraction (D_4S_1). Neither serum reacted with fraction D_2S_1, containing the bands of intermediate migration. No cross-reactions were noted.

Attempts to produce precipitating antibody by immunizing rabbits against the intermediate group (Fig. 6) were unsuccessful.

Inner and Outer Membrane Proteins

The cell envelope fraction of *E. coli* contains two membrane structures, the inner and outer membranes, separated by the cell wall region (Osborn, 1969). The inner and outer membranes were isolated and solubilized individually with guanidinium thiocyanate and then dialyzed against urea in the usual manner. The two urea-soluble fractions then were compared by urea and SDS gel electrophoresis (Fig. 7). The SDS gel patterns confirm that the "major" band is located predominantly in the outer membrane. The rapidly migrating and intermediate polypeptides were mainly localized in the outer membrane while the slowly migrating polypeptides were present in both membrane fractions.

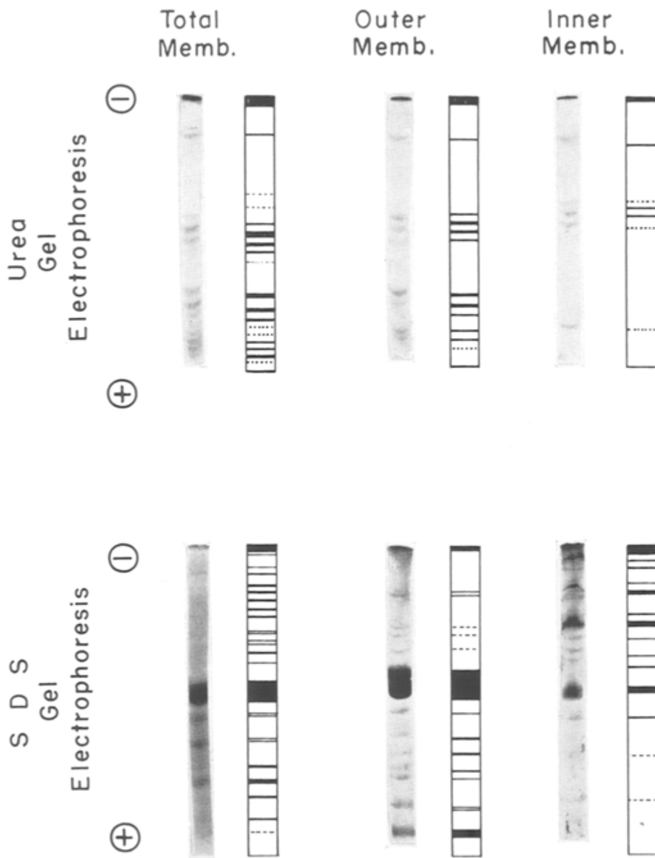


Fig. 7. Comparison of inner and outer membranes. Electrophoresis was performed by *Methods I* and *II*. The urea gels shown in this illustration were run for 8 hr. The combination of the patterns obtained with isolated inner and outer membranes was identical to that observed when the total cell envelope was examined in a similar fashion

Discussion

In the present study, effective solubilization of tightly bound membrane proteins was achieved by the use of the chaotropic agent, guanidinium thiocyanate. Further protein fractionation is difficult in the presence of the high ionic concentrations used. This problem was overcome by the finding that the polypeptides remained soluble when guanidinium thiocyanate was removed and replaced with urea thereby making possible the use of fractionation techniques depending on charge differences of the different polypeptides, such as ion exchange chromatography and gel electrophoresis. The similarity of the gel patterns of the total membrane and the urea-soluble fractions suggests that few of the major membrane polypeptides were lost,

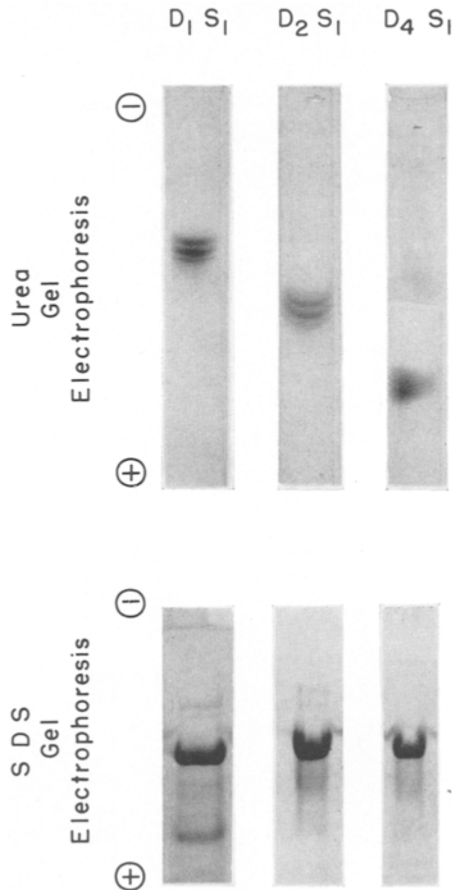


Fig. 8. Comparison of SP-Sephadex fractions. Electrophoresis was performed by *Methods I* and *II*

within the limitation of resolution of the acrylamide gel electrophoretic systems used.

The thiocyanate anion is known to react with free amino groups forming thiocarbamyl derivatives. This could possibly produce a heterogenous population of polypeptide molecules that differ only in their degree of *derivatization*, resulting in artificial fractionation. There are several indications that this did not occur. The most compelling evidence came from the studies with [^{14}C]thiocyanate, which showed that less than one in 5,000 amino acid residues in the purified fractions were thiocarbamylated. This small amount of protein-bound [14C]thiocyanate was equally distributed among the various chromatographic fractions as shown by their similar specific radioactivity. The original urea-soluble fraction was thiocarbamylated to a

greater extent, with loss of most of the bound radioactivity occurring during purification presumably due to the lability of the thiocarbamyl bond. Despite this, the electrophoretic mobilities of the polypeptides in the final fractions were identical to those of the corresponding bands in the initial urea-soluble fraction. Nevertheless, the occurrence of even minor degrees of thiocarbamylation is a disadvantage if renaturation of the proteins is to be attempted, and the replacement of thiocyanate with an equally effective but less reactive chaotropic anion would be desirable.

These and other studies have localized certain groups of polypeptides in the outer membrane of the *E. coli* cell envelope. These include the "major" band present in SDS gels of *E. coli* and *Salmonella* membranes (Schnaitman, 1970*a*; Osborn *et al.*, 1972). Each of the chromatographically isolated fractions contained significant amounts of polypeptides that migrated in the position of the "major" band (Fig. 8). However, despite the similarity of the SDS gels, the urea gel patterns and chromatographic behavior indicated that the polypeptides of these fractions differed in charge. It therefore seems unlikely that this band represents a single structural protein.

The finding that a single band in SDS gels may be resolved into several components suggests that analogous situations may occur when other complex mixtures of polypeptides of similar molecular weight are analyzed by this technique.

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