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Solubilization of the Cytoplasmic Membrane of *Escherichia coli* by Triton X-100

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Treatment of a partially purified preparation of cell walls of *Escherichia coli* with Triton X-100 at 23 C resulted in a solubilization of 15 to 25% of the protein. Examination of the Triton-insoluble material by electron microscopy indicated that the characteristic morphology of the cell wall was not affected by the Triton extraction. Contaminating fragments of the cytoplasmic membrane were removed by Triton X-100, including the fragments of the cytoplasmic membrane which were normally observed attached to the cell wall. Treatment of a partially purified cytoplasmic membrane fraction with Triton X-100 resulted in the solubilization of 60 to 80% of the protein of this fraction. Comparison of the Triton-soluble and Triton-insoluble proteins from the cell wall and cytoplasmic membrane fractions by polyacrylamide gel electrophoresis after removal of the Triton by gel filtration in acidified dimethyl formamide indicated that the detergent specifically solubilized proteins of the cytoplasmic membrane. The proteins solubilized from the cell wall fraction were qualitatively identical to those solubilized from the cytoplasmic membrane fraction, but were present in different proportions, suggesting that the fragments of cytoplasmic membrane which are attached to the cell wall are different in composition from the remainder of the cytoplasmic membrane of the cell. Treatment of unfractionated envelope preparations with Triton X-100 resulted in the solubilization of 40% of the protein, and only proteins of the cytoplasmic membrane were solubilized. Extraction with Triton thus provides a rapid and specific means of separating the proteins of the cell wall and cytoplasmic membrane of *E. coli*.

Several studies have indicated that the nonionic detergent Triton X-100 has a specific solubilizing effect on the cytoplasmic membrane of *Escherichia coli*. Fox and Kennedy (6) solubilized the M protein involved in β-galactoside transport from envelope fragments derived from *E. coli* with Triton X-100, and Birdsell and Cota-Robles (3) observed that this detergent lysed spheroplasts of *E. coli* which contained exposed regions of cytoplasmic membrane. Recently, DePamphilis and Adler (5) utilized extraction with Triton X-100 in the presence of Mg^{2+} to isolate the outer, or L membrane from *E. coli* spheroplasts.

Recent studies in this laboratory (8) have indicated that the cell wall of *E. coli* contains approximately 60% of the protein of the cell envelope, including one major structural protein with a molecular weight of about 44,000 daltons. Cell wall-enriched and cytoplasmic membrane-enriched fractions obtained from *E. coli* envelope preparations have been compared by polyacrylamide gel electrophoresis, and this has permitted the localization of many of the proteins of the envelope in either the cytoplasmic membrane or the cell wall (8). This report is concerned with the distribution of these proteins in Triton-soluble and Triton-insoluble fractions derived from the *E. coli* envelope.

MATERIALS AND METHODS

Cultures and growth conditions. *E. coli* J-5, a uridine diphosphate-galactose-4-epimerase-negative mutant derived from *E. coli* $O111_{B4}$, was used in this study. Late log-phase cultures were grown in a minimal salts medium containing 0.5% glucose, as previously described (7). Cultures were labeled with ^{3}H-leucine and ^{3}H-tyrosine or ^{14}C-leucine and ^{14}C-tyrosine as previously described (7), and radioactive protein was determined as previously described (7).

Reagents and chemicals. Triton X-100 was obtained from Calbiochem, Los Angeles, Calif. All other reagents were obtained from the sources described previously (7).

Electron microscopy. All samples were fixed with glutaraldehyde and OsO_4 in Beckman microfuge tubes and processed as previously described (8).

Preparation of envelope fractions and extraction with Triton X-100. Cells were broken by passage through a French pressure cell and the envelope fraction was isolated as previously described (7). When envelope fractions were used without further fractionation, they were washed by suspension in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2'-ethane sulfonic acid (HEPES) buffer (*p*H 7.4) and centrifugation in a Spinco 50Ti rotor for 45 min at 50,000 rev/min. Separation of the envelope into cell wall-enriched and cytoplasmic membrane-enriched fractions was accomplished by centrifugation in discontinuous sucrose gradients as previously described (8).

Suspensions of the unfractionated envelope or the various envelope fractions were incubated at a protein concentration of 1 to 10 mg/ml in 2% Triton X-100 in 10 mM HEPES buffer (*p*H 7.4) for 10 to 20 min at 23 C. At the end of the incubation period, the suspensions were chilled and then centrifuged in a Spinco 50Ti rotor for 1 hr at 50,000 rev/min to sediment the Triton-insoluble material. Because the removal of divalent cations by ethylenediaminetetraacetic acid has a significant effect on the solubility of the cell wall proteins (5), it is important to note that the envelope fractions used in this study were previously exposed to Mg^{2+}-containing buffers, either after breakage with the French pressure cell (7) or after removal of the fractions from sucrose gradients (8). Addition of Mg^{2+} to the incubation mixture at a final concentration of 10 mM had no effect on the solubility of the cytoplasmic membrane proteins in Triton X-100.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis of lipid-free membrane proteins was carried out as previously described in sodium dodecyl sulfate (SDS)-containing gels (7), with the minor modification that the protein samples were concentrated by pressure ultrafiltration through a Diaflow PM-10 membrane (Amicon Corp., Lexington, Mass.) before electrophoresis. Split gels were prepared by overlayering the running gels with a stacking gel containing 2.5% acrylamide, 0.62% bisacrylamide, 5 mg of riboflavine/ml, 0.5 M urea, 10% sucrose, and 0.1% SDS in the same buffer as in the running gels. A lucite divider was inserted into the stacking gel to separate the upper portion of the gel tube into two compartments, and the gel was photopolymerized. Identical amounts of samples to be compared were placed on either side of the divider. Resolution of low-molecular-weight proteins was improved by using a two-layered running gel consisting of a 9-cm lower layer containing 12% acrylamide and 0.32% bisacrylamide and a 2-cm upper layer containing 6% acrylamide and 0.16% bisacrylamide. The lower layer was polymerized before the upper layer was added. These gels were run for 8 hr at 5 ma per gel. The discontinuity of the two layers resulted in restacking of the protein at the interface and increased resolution of some of the rapidly migrating components.

Details of the removal of Triton X-100 from samples prior to gel electrophoresis are given below. The same procedures were used for both Triton-soluble and Triton-insoluble fractions.

RESULTS

Triton X-100 extraction of the cell wall-enriched fraction. The cell wall of *E. coli* has a much greater density on sucrose gradients than does the cytoplasmic membrane (8). It is therefore interesting that the cell wall-enriched fraction obtained by centrifugation on sucrose gradients contains a substantial amount of cytoplasmic membrane contamination (8). Preliminary experiments indicated that this contamination cannot be removed by repeated gradient centrifugation. Coupled with the observation that the cell wall fragments are primarily "open" fragments (8), this indicates that the contamination is not due to cytoplasmic membrane fragments which are merely trapped or swept along with the cell wall fragments.

Figure 1 illustrates the nature of the cytoplasmic membrane contamination observed in the cell wall-enriched fraction. Careful examination of the cell wall-enriched fraction indicates that virtually all of the fragments of cytoplasmic membrane visible in this preparation are attached to fragments of cell wall. This attachment appears specific, in that the membrane is always attached to the inner (peptidoglycan) surface of the cell wall, and in most cases a region of attachment is observed in which a constant gap is maintained between the cell wall and the cytoplasmic membrane (Fig. 1C and 1D). Evidence for the attachment of the cell wall and cytoplasmic membrane was reported first by Cota-Robles (4) and later by Bayer (1) in studies of plasmolyzed *E. coli* cells, although the size of the attached regions in plasmolyzed cells appears much smaller than that shown in Fig. 1. The "fused" regions shown in Fig. 1 are similar to the "islands" of cell wall attached to the cytoplasmic membrane observed with the freeze-etching technique by Bayer and Remsen (2).

Figure 2 shows a cell wall-enriched preparation after extraction with Triton X-100. The five-layered appearance of the cell wall is still evident, but all material identifiable morphologically as cytoplasmic membrane has been removed. The amount of protein which is solubilized from the cell wall-enriched fraction is variable, ranging from 15 to 25% of the total protein of the fraction. In general, the amount of protein solubilized from the cell wall-enriched fraction obtained from logarithmic-phase cultures is greater than the amount solubilized from the same fraction isolated from stationary-phase cultures, indicating that there may be more of the cytoplasmic membrane attached to the cell wall sites in rapidly growing cells. The amount of protein solubilized from the cell wall-enriched fraction is independent of the Triton concentration over a wide range (Fig. 3). The solubilization is much more effective at 23 C than at 0 C. Other temperatures have not been tested.

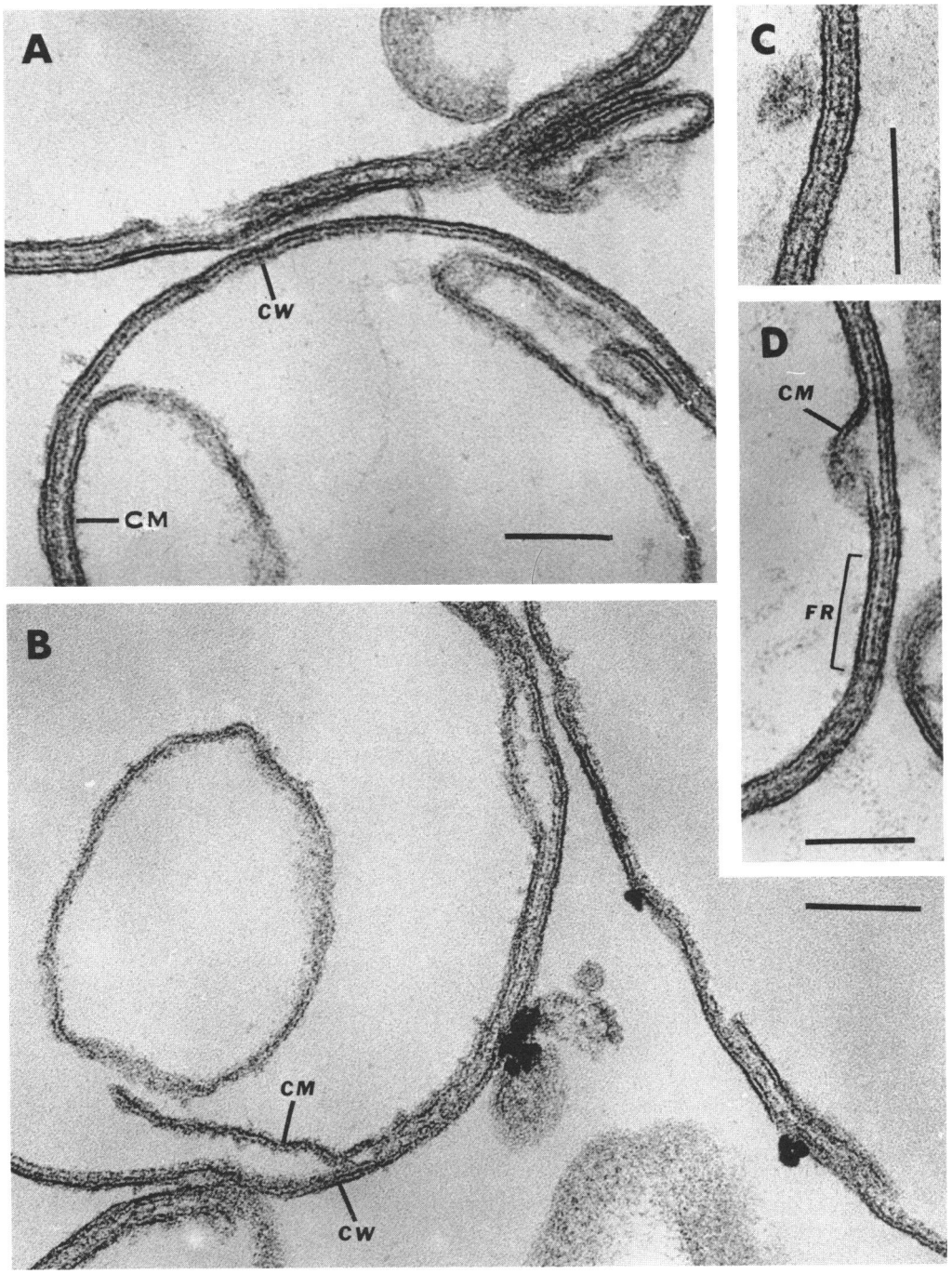

FIG. 1. *Micrographs illustrating the appearance of cytoplasmic membrane contamination observed in the cell wall-enriched fraction obtained from an Escherichia coli envelope preparation by sucrose gradient centrifugation (8). A, Appearance of large cell wall (CW) fragments containing attached cytoplasmic membrane fragments (CM). The cell wall is readily identified by the presence of the peptidoglycan layer on the inner surface. B, Similar field showing large fragments of cell wall with cytoplasmic membrane contamination. C, Fragment in which the cell wall and cytoplasmic membrane are fused. It is not possible to distinguish the outer membrane of the cell wall from the cytoplasmic membrane. D, Fragment showing a similar fused region (FR) in which the cytoplasmic membrane can be identified. Bars indicate 0.1 μm.*

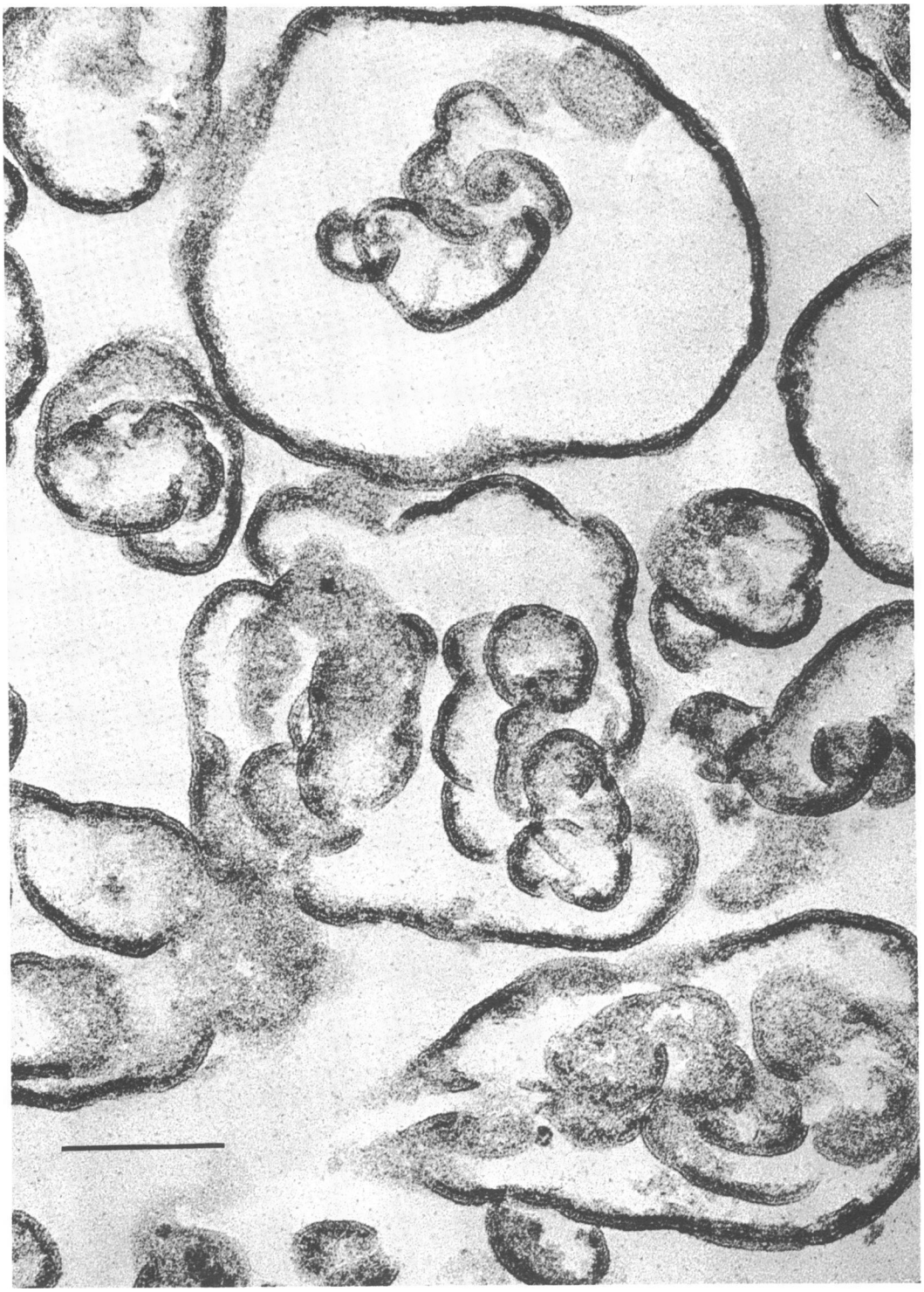

FIG. 2. *Appearance of the cell wall-enriched fraction after extraction with Triton X-100. This preparation consists of relatively intact fragments of cell wall. No fragments of cytoplasmic membrane are visible after Triton treatment. Bar indicates 0.2 μm.*

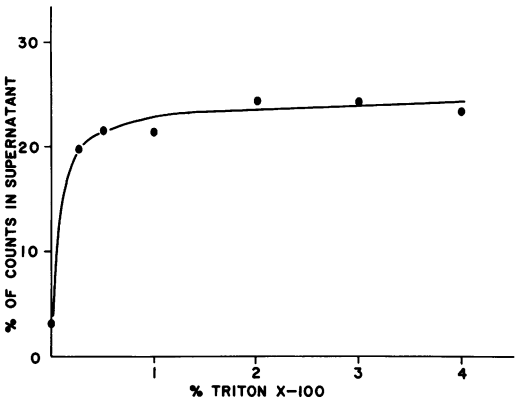

FIG. 3. *Effect of Triton X-100 concentration on the solubilization of protein from the cell wall-enriched fraction of the Escherichia coli envelope. The cell wall-enriched fraction of the envelope was isolated as previously described (8) from a late log-phase culture grown on medium containing ^{3}H-leucine and ^{3}H-tyrosine. The cell wall-enriched fraction was suspended in 10 mM HEPES buffer and incubated in the presence of various amounts of Triton X-100 for 10 min at 23 C. The protein concentration in the incubated suspension was 2 mg/ml. At the end of the incubation period, the samples were chilled and centrifuged at 50,000 rev/min in a Spinco 50Ti rotor for 1 hr. Portions were removed for scintillation counting before and after centrifugation to calculate the percentage of total radioactivity remaining in the supernatant fluid.*

Examination of Triton-soluble and Triton-insoluble envelope proteins by polyacrylamide gel electrophoresis. As the experiments described above with the cell wall-enriched fraction indicated that the cytoplasmic membrane was solubilized by Triton X-100, the solubility of the cytoplasmic membrane-enriched fraction of the *E. coli* envelope (8) in Triton X-100 was examined. In contrast to the wall-enriched fraction, the solubilization of protein from the cytoplasmic membrane-enriched fraction was much greater. From 60 to 80% of the protein of the cytoplasmic membrane-enriched fraction could not be sedimented after treatment with Triton X-100 for 10 min at 23 C. The solubilization of protein from the cytoplasmic membrane-enriched fraction showed the same independence of Triton concentration as did the wall fraction (Fig. 3), provided that at least 2 mg of Triton X-100 was present per mg of protein.

Previous studies of *E. coli* envelope proteins by polyacrylamide gel electrophoresis in this laboratory have been carried out with lipid-free protein preparations obtained by solubilization of the protein in acidified dimethyl formamide followed by gel filtration on Sephadex LH-20 to remove lipid (7). This procedure is also effective in removing Triton X-100 from Triton-treated samples. Elution of Triton X-100 and Triton-solubilized protein from an LH-20 column are compared in Fig. 4. The total amount of ultraviolet-absorbing material which eluted at the void volume coincident with the radioactivity peak was identical to the ultraviolet absorbance of the protein applied to the column. All of the radioactivity applied to the column was recovered in this peak, indicating that little if any Triton X-100 remained associated with the protein. This is fortunate, as it permits direct comparison of Triton-soluble and Triton-insoluble proteins by polyacrylamide gel electrophoresis without complications introduced by Triton or lipid. The Triton X-100 treatment does not appear to introduce any artifacts in the gel electrophoresis patterns, because a pattern identical to that of the starting material was obtained when Triton-soluble and Triton-insoluble fractions were mixed in the right proportions after the removal of lipid and Triton by gel filtration in acidified dimethyl formamide.

Figure 5 illustrates split-gel comparisons of the Triton-soluble and Triton-insoluble fractions obtained from the cell wall-enriched and cytoplasmic membrane-enriched fractions. The relative location of the bands is based on split gels, with 7.5% running acrylamide gels and 6 to 12% double-layered gels used as described in Mate-

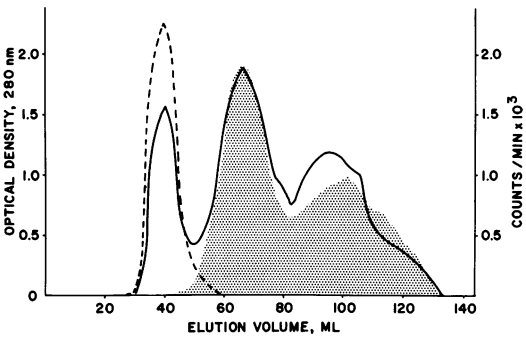

FIG. 4. *Elution of protein and Triton X-100 from a Sephadex LH-20 column equilibrated with acidified dimethyl formamide. A preparation of unfractionated envelope from a culture grown in medium containing ^{3}H-leucine and ^{3}H-tyrosine was extracted with 2% Triton X-100. A 1-ml amount of the Triton-soluble protein containing approximately 10 mg of protein was mixed with 7 ml of a solution containing 19 parts of N,N'-dimethyl formamide and 1 part of 0.24 N HCl. This was applied to a column (2.5 by 40 cm) of Sephadex LH-20 equilibrated with a solvent containing nine parts N,N'-dimethyl formamide and one part 0.12 N HCl and eluted with the same solvent. The dashed line indicates ^{3}H-labeled protein and the solid line indicates optical density at 280 nm. The stippled area indicates the optical density at 280 nm when 1 ml of 2% Triton X-100 was mixed with solvent and chromatographed under the same conditions.*

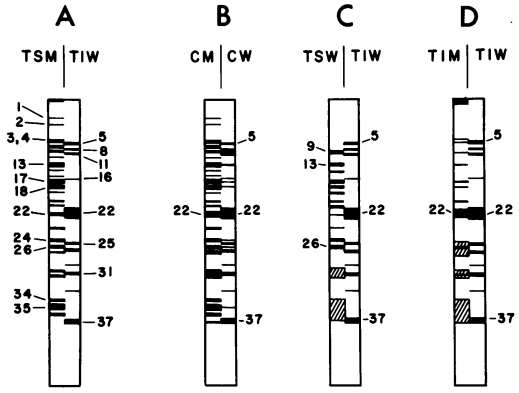

FIG. 5. *Split-gel comparison of various envelope fractions. A, Comparison of Triton-soluble protein from the cytoplasmic membrane-enriched fraction (TSM) and Triton-insoluble protein from the cell wall-enriched fraction (TIW). B, Comparison of the cytoplasmic membrane-enriched fraction (CM) and the cell wall-enriched fraction (CW) without Triton treatment. C, Comparison of the Triton-soluble protein from the cell wall-enriched fraction (TSW) with the Triton-insoluble protein from the same fraction (TIW). D, Comparison of Triton-insoluble protein from the cytoplasmic membrane-enriched fraction (TIM) with Triton-insoluble wall (TIW). The width of the lines is proportional to the relative intensity of the bands; hatching indicates diffuse bands. The bands are shown as they would appear on 7.5% gels, although the relative order of the faster-moving components was obtained from comparison on 6 to 12% double-layered gels.*

rials and Methods. The split-gel pattern obtained with Triton-soluble protein from the membrane-enriched fraction and Triton-insoluble protein from the wall-enriched fraction (Fig. 5A) is essentially identical to the pattern obtained when the membrane-enriched and wall-enriched fractions are compared without Triton extraction (Fig. 5B), with the exception that cross-contamination between major cell wall and cytoplasmic membrane proteins is greatly reduced or eliminated. The Triton-soluble protein from the cell wall-enriched fraction (Fig. 5C) appears to consist entirely of cytoplasmic membrane proteins, with the exception of protein 9, which is found in only trace amounts in either the membrane-enriched fraction or the Triton-soluble protein from the membrane-enriched fraction. The Triton-insoluble protein from the cytoplasmic membrane-enriched fraction (Fig. 5D) has two major components: cell wall protein 22 and a considerable amount of protein which remains at the top of the gel and is presumably denatured protein. Examination of sliced gels containing radioactive protein indicated that about 50% of the protein present in the Triton-insoluble fraction obtained from the cytoplasmic membrane-enriched fraction was accounted for by protein band 22. Material remaining at the top of the gel accounts for an additional 15 to 20% of this fraction.

Figure 6 shows optical-density scans of stained gels of the fractions shown in Fig. 5. The Triton-insoluble protein from the cell wall-enriched fraction shows essentially the same profile as the untreated wall-enriched fraction, indicating that the Triton has not extracted any of the major cell wall proteins. The Triton-insoluble protein from the cytoplasmic membrane-enriched fraction is seen to consist primarily of protein 22, the major protein of the cell wall. It is possible that the Triton-insoluble material present in the cytoplasmic membrane-enriched fraction is due to tiny fragments of the outer membrane of the cell wall which were detached during the breakage of the cell and which have remained associated with cytoplasmic membrane fraction.

The most interesting feature of Fig. 6 is the comparison of the Triton-soluble protein obtained from the cytoplasmic membrane-enriched and cell wall-enriched fractions. As noted above, the Triton-soluble protein of the cell wall-enriched fraction is due to fragments of membrane which are attached to the cell wall. Protein 9 appears unique to this fraction, and this suggests that this protein is found only in those regions of the cytoplasmic membrane which are attached to the cell wall. In addition, the relative amounts of other cytoplasmic membrane proteins (for exam-

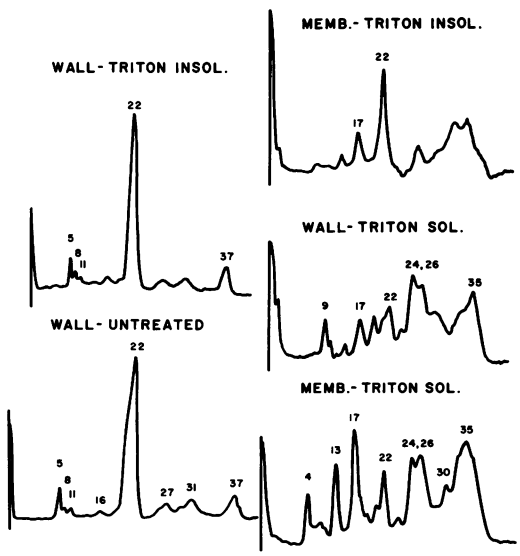

FIG. 6. *Optical density scans of 7.5% gels of various envelope fractions. Gels were fixed and stained with Coomassie blue and scanned as previously described. Numbering system as in Fig. 5.*

ple, proteins 13, 17, and 22) are quite different in the Trition-soluble protein from the two different sources. This is indicative that the cytoplasmic membrane of *E. coli* is not homogeneous but consists of a mosaic in which different proteins are enriched in different regions around the surface of the cell.

Extraction of the unfractionated E. coli envelope with Triton X-100. The results presented above indicate that Triton X-100 is quite specific in solubilizing only proteins of the cytoplasmic membrane and not of the cell wall, thus, providing a rapid and convenient method for separating the proteins of the cell wall and cytoplasmic membrane from unfractionated envelope preparations from *E. coli* (Fig. 7). Identical cultures were grown in the presence of ^{14}C- and ^{3}H-labeled amino acids, and the envelope was obtained from each culture under the same conditions. The envelope from the ^{14}C-labeled culture was extracted with Triton X-100, and the Triton-insoluble fraction was washed once and then mixed with the Triton-soluble fraction from the ^{3}H-labeled culture. The resulting radioactivity profile of the gel (Fig. 7) indicates a very clean separation of cytoplasmic membrane and cell wall proteins, with much less cross-contamination than was observed when a similar experiment was done by using crude wall-enriched and membrane-enriched fractions separated on sucrose gradients (8). It is interesting to note that the Triton-soluble fraction of the envelope contains a substantial amount of a protein which is coincident with protein 22, the major protein of the cell wall. This is always present, and it is possible that it represents a cytoplasmic membrane-bound precursor of the cell wall protein.

DISCUSSION

The results presented indicate that Triton X-100 is quite specific in its action on the *E. coli* cell envelope. When envelope preparations prepared in the presence of small amounts of Mg^{2+} are extracted with this detergent, proteins of the cytoplasmic membrane are selectively solubilized. The mechanism of this solubilization is not known. In this study, the term solubilization has been used to indicate only that certain particulate proteins can no longer be sedimented by preparative ultracentrifugation. Preliminary experiments have indicated that this "Triton-soluble" material is still in a high-molecular-weight form, as all of the "Triton-soluble" protein is excluded from Sephadex G-150. This may indicate that the solubilized material is still present in the form of small vesicles or aggregates or that it is in the form of mixed micelles with the detergent. Whatever the basis for the solubilization, it is operationally a very useful procedure, particularly in studies of the cytoplasmic membrane proteins in mutants which have altered membrane function.

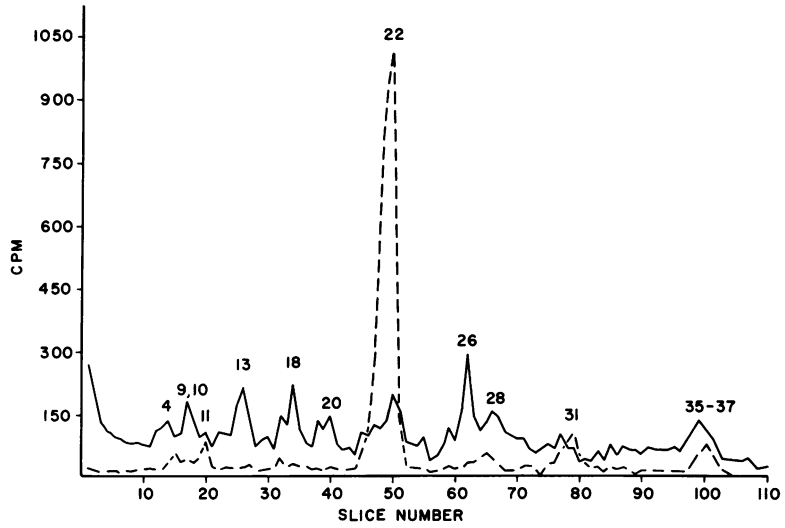

FIG. 7. *Protein profile of the Triton-soluble and Triton-insoluble fractions of the Escherichia coli envelope on 7.5% acrylamide gel. Unfractionated envelope preparations were obtained from late log-phase cultures grown on ^{3}H and ^{14}C amino acids (leucine and tyrosine) and extracted with 2% Triton X-100. The Triton-soluble protein from the ^{3}H-labeled culture and the Triton-insoluble protein from the ^{14}C-labeled culture were mixed after the removal of Triton and lipid on Sephadex LH-20 columns. Solid line indicates ^{3}H counts; broken line indicates ^{14}C counts. Numbering system as in Fig. 5.*

The resistance of the outer membrane of *E. coli* to solubilization by Triton X-100 is quite striking. In the accompanying report (9), results are presented which indicate that this resistance to solubilization is due at least in part to the action of divalent cations in stabilizing the structure of this cell wall.

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