JOURNAL OF BACTERIOLOGY, Oct. 1972, p. 585-592
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Vol. 112, No. 1
Printed in U.S.A.

Specific Removal of Proteins from the Envelope of *Escherichia coli* by Protease Treatments

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Received for publication 1 May 1972

When the envelope fraction of *Escherichia coli* was treated by trypsin, about 40% of total envelope proteins were removed from the fraction without changing its phospholipid content. Analysis of envelope proteins by acrylamide gel electrophoresis in 0.5% sodium dodecyl sulfate revealed that trypsin treatment was very specific; one of the major proteins (molecular weight, 38,000) and all proteins of molecular weight greater than 70,000 were completely removed by the treatment. On the other hand, three other major proteins were found to be resistant to the treatment, including protein Y, which was previously shown to be related to deoxyribonucleic acid replication. The trypsin treatment of the envelope fractions composed of a five electron-dense layered structure formed vesicles with a triple-layered membrane (two electron-dense layers). Pronase treatment of the envelope fraction removed about 60% of the envelope proteins without changing its phospholipid content. A major protein of molecular weight of 58,000 was found to be the only protein resistant to the Pronase treatment. Application of these treatments is useful for purification and structural studies of envelope proteins.

The envelope of *Escherichia coli* appears in the electron microscope to be composed of a triple-layered outer membrane, an intermediate layer, and a triple-layered cytoplasmic membrane (6, 15). Recently, a freeze-etching technique has also revealed a multilayered structure for the envelope (1, 19). Separation and some characterization of the outer and inner (cytoplasmic) membranes of gram-negative bacteria have been attempted (5, 7, 8, 13, 14, 16-18). However, little is known about structural and functional properties of envelope proteins which are components of those membranes, except for a lipoprotein (molecular weight, about 7,000) which has been found covalently linked to peptidoglycan (2-4), and an envelope protein (molecular weight, 44,000) which has been shown to be related to deoxyribonucleic acid (DNA) replication (11, 12).

De Petris has shown that proteolytic enzymes detach the outer membrane from the intermediate layer and the inner cytoplasmic membrane (6). This result suggests that the enzymes digest proteins that exist between the outer membrane and central layer and link them together. Braun and his co-workers have found that trypsin specifically cleaves the lipoprotein from the peptidoglycan; as a result, the outer membrane is detached from the other parts of the envelope, although the protein still remains insoluble (2-4).

In the present paper, we have reexamined the effect of proteolytic enzymes on the envelope proteins of *E. coli*. We found that some envelope proteins show strong resistance to proteolytic digestion and cannot be removed from the envelope fraction, whereas the other envelope proteins are easily removed by the treatment. The different sensitivities of various envelope proteins to proteolytic enzymes suggest different structural and functional roles for the proteins in the envelope.

MATERIALS AND METHODS

Strains. *E. coli* MX74T2, MX74T2RO, and MX74T2ts27 (9) were used.

Media and growth condition. M9 medium supplemented with glucose (4 mg/ml), thiamine (2 μg/ml), and thymidine (4 μg/ml) was used in all experiments. For single or double label experiments, 20 μg of L-arginine per ml and 7.5 μg of L-leucine, L-histidine, L-tyrosine, or L-tryptophan per ml was also added.

Label experiments. Cells were labeled with various materials as follows: ^{32}P, ^{3}H-L-arginine (New England Nuclear Corp.), ^{14}C-L-arginine, ^{3}H-L-leu-

cine, ^{3}H-L-histidine, ^{3}H-L-tyrosine, ^{3}H-L-tryptophan, or ^{3}H-*N*-acetylglucosamine (Schwarz-Mann). The envelope fraction was prepared by differential centrifugation, and gel electrophoresis of the envelope proteins was carried out as described previously (11, 12).

Electron microscopy. The envelope fraction or the trypsinized envelope fraction was treated with 2% glutaraldehyde in 0.1 M phosphate buffer (*p*H 7.4) for 30 min at 4 C, washed five times for 5 min with the same buffer, postfixed in 1% OsO_4 in the same buffer overnight at 4C, washed three times for 5 min in Veronal acetate buffer, *p*H 7.4 (4.86 g of Na $C_2H_3O \cdot 3H_2O$, and 7.36 g of sodium Veronal/250 ml of water), and treated with 0.5% uranyl acetate in Veronal acetate buffer for 45 min at 4 C. Specimens were then dehydrated in a graded series of alcohols through propylene oxide and embedded in Epon 812. Sections were cut and double-stained with uranyl acetate and lead citrate.

Other materials used. Trypsin (type III), Pronase (type VI; fungal protease), and soybean trypsin inhibitor (type 1-S) were obtained from Sigma Chemical Co.

RESULTS

Trypsin treatment of E. coli envelope. A 20-ml culture of *E. coli* MX74T2 was labeled with various isotopes, and the envelope fraction was prepared by differential centrifugation as described previously (11, 12). The envelope fraction was resuspended in 2 ml of 0.01 M sodium phosphate buffer, *p*H 7.1, containing various amounts of trypsin. The mixture was incubated at 41 or 37 C for different lengths of time, as indicated in Table 1. Then the mixture was chilled and centrifuged at $10^5 \times g$ for 30 min. Radioactivity recovered in the pellet was measured and expressed as percent of total radioactivity before centrifugation. In experiment I in Table 1, trypsin inhibitor was added after the incubation to prevent from further digestion by trypsin. As shown in Table 1, treatment with 25 μg of trypsin per ml at 41 C for 5 min was enough to remove all trypsin-sensitive proteins from the envelope fraction, since further incubation (50 μg/ml, 41 C, 15 min) did not cause any further significant change of the radioactivity in the insoluble fraction (experiment I). The fact that about 60% of the total envelope proteins were resistant to trypsin treatment is also observed in experiment II in Table 1; a second trypsin treatment did not cause further digestion of the fraction. Experiment III in Table 1 shows that the same phenomena are observed with the envelope fractions labeled with leucine, histidine, tyrosine, or tryptophan as well as arginine. Radioactivity released by the first incubation without trypsin was usually about 5% of the total radioactivity (see experiment IV in Table 1). We also noticed that addition of trypsin inhibitor after the digestion was not necessary to prevent from further digestion. These results indicate that trypsin digestion is very specific; some envelope proteins are entirely resistant to digestion, while other proteins are easily digested by trypsin.

TABLE 1. *Recoveries of protein and phospholipid contents in the envelope fraction after the protease treatments*

Expt no.	Treatment[a]	Radioisotope[b]	Recovery in insoluble fraction[c] (%)
I	Trypsin, 25 μg/ml, 41 C, 5 min	^{3}H-arginine	60
	Trypsin, 50 μg/ml, 41 C, 15 min	^{3}H-arginine	57
II	Trypsin, 100 μg/ml, 37 C, 30 min	^{3}H-arginine	63
	Same as above, washed with buffer[d]		61
	Same as above, 2nd trypsin treatment as above		57
III	Trypsin, 100 μg/ml, 37 C, 30 min	^{14}C-arginine	49
		^{3}H-leucine	60
		^{3}H-histidine	55
		^{3}H-tyrosine	66
		^{3}H-tryptophan	69
IV	Trypsin, 100 μg/ml, 37 C, 30 min	^{3}H-arginine	58
	Pronase, 100 μg/ml, 37 C, 30 min	^{3}H-arginine	43
	None, 37 C, 30 min	^{3}H-arginine	95
V	Trypsin, 250 μg/ml, 37 C, 30 min	^{32}P	88
	Pronase, 250 μg/ml, 37 C, 30 min	^{32}P	86
	None, 37 C, 30 min	^{32}P	88

[a] All treatments were carried out in 0.01 M sodium phosphate buffer, *p*H 7.1.
[b] Radioisotopes used for labeling the envelope fraction.
[c] Recoveries in the pellets after centrifugation at $10^5 \times g$ for 30 min.
[d] Sodium phosphate buffer (0.01 M, *p*H 7.1).

When the envelope fraction was labeled with ^{32}P, there was no significant decrease of radioactivity in the insoluble fraction after the trypsin treatment, although about 12% of nonspecific solubilization of ^{32}P radioactivity was observed in experiment V (Table 1). In both control and trypsin-treated envelope fractions, about 85% of the total radioactivity was extractable with a mixture of ethanol and ether (3:1), indicating that a majority of the ^{32}P in

the fractions existed in phospholipids, which were not solubilized by trypsin treatment.

When the envelope fraction was labeled with ^{3}H-*N*-acetylglucosamine, no significant release of radioactivity by trypsin treatment over the control was observed (250 µg/ml, 37 C, 15 min). At least about 40% of the radioactivity was incorporated into peptidoglycan, judging from lysozyme sensitivity. The rest of the radioactivity may have been incorporated into glycoproteins or lipopolysaccharide, or both. In any case, the present result indicates that all fractions which incorporated *N*-acetylglucosamine were not solubilized by the trypsin treatment.

Acrylamide gel electrophoresis of trypsinized envelope. To examine whether trypsin treatment was specific or not, the envelope proteins after the treatment were analyzed by acrylamide gel electrophoresis in 0.5% sodium dodecyl sulfate (SDS). The envelope fraction was prepared from a mixture of two cultures, one from wild type (*E. coli* MX74T2RO) labeled with ^{3}H-arginine, and one from the temperature-sensitive *dnaB*$^{-}$ mutant (*E. coli* MX74T2ts27) (9) labeled with ^{14}C-arginine for 1 hr at 41 C, as described previously (11, 12). Analysis of half of this double-labeled envelope fraction by acrylamide gel electrophoresis showed a large difference at protein Y (peak 6) (Fig. 1A), a change previously reported to be related to DNA synthesis. Another half of the envelope fraction was treated with 50 µg of trypsin per ml at 40 C for 15 min, followed by centrifugation at $10^{5} \times g$ for 30 min. It was found that 43% of total radioactivity was solubilized into the supernatant fluid for both ^{3}H and ^{14}C. The pellet was solubilized with SDS and applied to a 7.5% acrylamide gel, and electrophoresis was carried out in 0.5% SDS as described previously (11, 12). As can be seen in Fig. 1B, the trypsin treatment was found to be very specific as follows: (i) proteins of molecular weight greater than 70,000 disappeared (larger than peak 3); (ii) peak 7 almost completely disappeared; (iii) peaks 4, 6, and 11 were clearly unchanged; and (iv) a new peak appeared at a molecular weight of 25,000 as indicated by an arrow in Fig. 1B. Since peak 6 in Fig. 1B still retained a large difference between ^{3}H and ^{14}C radioactivities after the treatment, peak 6 in Fig. 1B is protein Y and not a byproduct of the trypsin treatment. Recoveries of peaks 4, 6, and 11 were 92, 78, and 101%, respectively, for ^{3}H.

When the envelope fraction labeled with ^{14}C-arginine was treated with 200 µg of trypsin per ml at 37 C for 30 min, results similar to those mentioned above were obtained. It should be noticed that peak 4 was still completely resistant to the treatment (recovery, 100%), whereas peak 6 (protein Y) appeared to be partially digested by trypsin (recovery, 67%). The recovery of peak 11 was 140% under the conditions used. This increase of peak 11

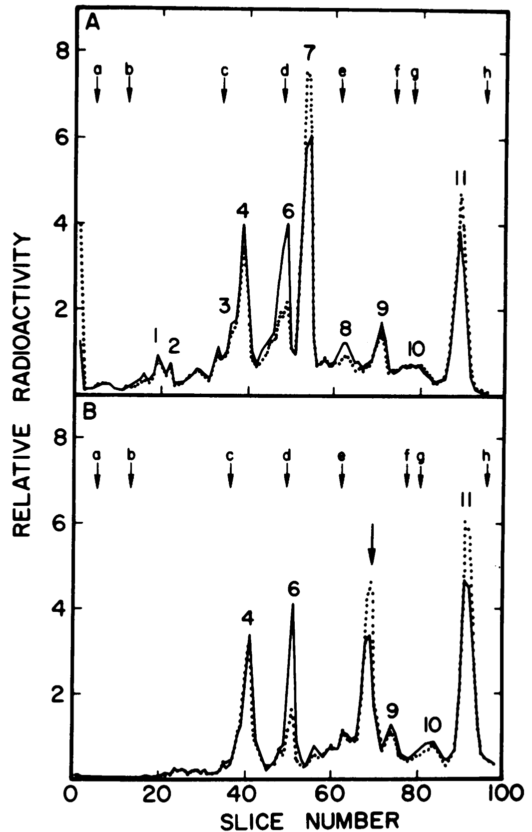


FIG. 1. *Gel electrophoresis of envelope fractions with or without trypsin treatment. The envelope fraction was prepared from a mixture of a culture of E. coli MX74T2RO labeled with ^{3}H-L-arginine and a culture of E. coli MX74T2ts27 labeled with ^{14}C-L-arginine for 1 hr at 41 C. Half of the fraction was treated with 50 µg of trypsin per ml at 41 C for 15 min in 0.01 M sodium phosphate buffer, pH 7.1. The insoluble fraction after the treatment was collected by centrifugation ($10^{5} \times g$ for 30 min). Both untreated (A) and treated (B) were analyzed by gel electrophoresis using 7.5% acrylamide gel in 0.5% SDS, as described previously (11). The solid lines represent ^{14}C-radioactivity and dashed lines represent ^{3}H-radioactivity. Small arrows with letters indicate positions of internal molecular weight standards (10): a, trimer; b, dimer; c, monomer of 1-dimethylamino-napthalene-5-sulfonyl (DANS)-bovine serum albumin; d, trimer; e, dimer; f, monomer of DANS-hen egg white lysozyme; g, cytochrome c; h, DANS-insulin. The number on each peak corresponds to those in the previous paper (10).*

was found to be due to the release of a specific protein which is covalently linked to peptidoglycan. We have recently found that this protein exists not only in a form bound to peptidoglycan as reported by Braum et al. (2-4), but also in a free form; that is, about two-thirds of this protein is not covalently linked to peptidoglycan but still exists in the envelope as an insoluble protein (Inouye, Shaw, and Shen, *manuscript in preparation*). After the bound fraction of the protein was specifically cleaved from peptidoglycan by trypsin (2-4), it converted to a free form, which could now be solubilized with SDS. As a result, recovery of peak 11 was greater than 100%. The fact that the recovery of peak 11 in Fig. 2B was 140% indicates that 68% (100/140) of the protein existed in the free form and 32% (40/140) in the bound form. It was shown (Inouye and Yee, *submitted for publication*) that peak 11 did not contain nonspecific byproducts of the trypsin treatment, since peak 11 did not change its extremely low content of histidine even after the trypsin treatment.

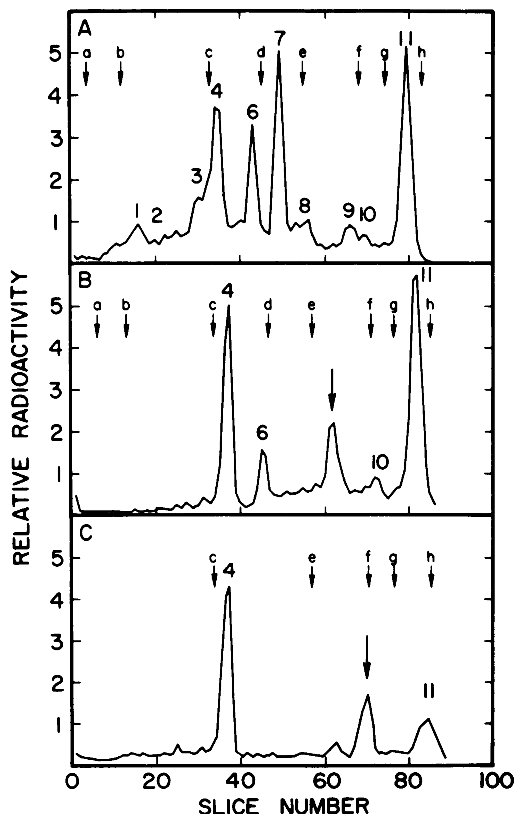


FIG. 2. *Gel electrophoresis of the envelope fractions treated with trypsin or Pronase. The envelope fraction labeled with ^{14}C-L-arginine was divided into three parts. One part was treated with 200 μg of trypsin per ml at 37 C for 30 min. Another third of the fraction was treated with 200 μg of Pronase per ml at 37 C for 30 min. After the treatment, insoluble fractions were collected by centrifugation ($10^5 \times g$, 30 min), solubilized with SDS, and applied on 7.5% acrylamide gel as in Fig. 1. A, Control without protease treatment; B, trypsin treatment; and C, Pronase treatment. Arrows and numbers on peaks are the same as in Fig. 1.*

Electron microscopy of trypsinized envelope. The envelope fraction before the trypsin treatment shows the typical structure with five electron-dense layers by electron microscopy (Fig. 3A). One can see fragments of the envelope but hardly any closed vesicles. However, after trypsin treatment, the five electron-dense layered structure almost completely disappeared, and vesicles of various sizes appeared (Fig. 3B). Higher magnification (Fig. 3C and D) shows that the membranes of these vesicles are composed of two electron-dense layers in contrast to the original envelope. The thickness of the membrane is between 6.9 and 11.4 nm. These vesicles were possibly a mixture of vesicles which were independently produced from the outer membrane and the inner membrane (cytoplasmic membrane) of *E. coli*. Figure 3E shows this possibility: two independent triple-layered membranes come from a still intact envelope structure. Occasionally one can observe a single electron-dense layer as shown by arrows in Fig. 3C and F. This may be a peptidoglycan layer, since De Petris has shown a similar layer of peptidoglycan by electron microscopy; it had been separated from both the outer and inner membranes by protease treatments (6).

Pronase treatment of the envelope fraction. Pronase digested the envelope fraction more extensively than trypsin. As shown in experiment IV in Table 1, about 60% of the total protein was solubilized by Pronase in contrast to 40% in the case of trypsin digestion under the same conditions. On the other hand, phospholipid content was not significantly decreased by Pronase treatment, just as was found for trypsin treatment (experiment V, Table 1). When the envelope fraction treated by Pronase (200 μg/ml, 37 C, 30 min) was analyzed by acrylamide gel electrophoresis, the pattern became much simpler than in the case of trypsin treatment (Fig. 2C).

Peak 4 was the only protein resistant to the Pronase treatment; its recovery was 95%. Peak 6, which was resistant to trypsin treatment, was completely sensitive to Pronase. Peak 11 also decreased strikingly; its recovery was found to be 41%. However, the actual recovery

of peak 11 appears to be smaller than 41%, since the peak was not as sharp as those in Fig. 2A and B, and furthermore, the peak in Fig. 2C was shifted toward smaller molecular weight, as judged by its relative position to those internal standards (g and h). Therefore the peak possibly contained not only the original protein but also degradative products from proteins of higher molecular weights.

As was seen in the case of the trypsin treatment, a new peak appeared as shown by an arrow in Fig. 2C. However, the molecular weight of the new peak was about 20,000, in contrast to 25,000 in the case of the trypsin treatment.

DISCUSSION

Trypsin and Pronase digested about 40% and 60% of total envelope proteins, respectively, without changing phospholipid content. This suggests that there are structurally two kinds of proteins in the envelope fraction; one resistant and the other sensitive to proteolytic digestion. Among the major envelope proteins, the one at 58,000 molecular weight (peak 4 in Fig. 1 and 2) was the only protein which was resistant to both enzymes. The reason for the resistance of this protein to proteolytic digestion may be that the protein is buried inside the membrane structure in such a way that it is protected from the enzymes, or that the protein itself is structurally resistant to the digestion. At any rate, this protein fraction accounts for 15 to 20% of total envelope proteins on the basis of arginine content and seems to play an important role in the structure of the *E. coli* envelope.

Peak 6 (protein Y) and peak 11 (Fig. 1 and 2) were resistant to trypsin but not to Pronase treatment. Peak 6 was less resistant to trypsin treatment than peak 4, so that longer incubation with more trypsin caused decrease of peak 6. With Pronase, the peak was completely removed. Recovery of peak 11 was found to be more than 100%, since a lipoprotein which was covalently linked to peptidoglycan was released by trypsin as discussed above. It should be noticed that the protein itself was insoluble even after the covalent linkage between the protein and peptidoglycan was cleaved by trypsin. Therefore the protein could be solubilized only in SDS.

Among the proteins which disappeared after digestion, the protein at 38,000 molecular weight (peak 7 in Fig. 1 and 2) was most striking. Although it was completely removed from its original position in gel electrophoresis, it is possible that a part of the protein removed by the enzymes resulted in a new peak at a position of lower molecular weight in gel electrophoresis. Actually new peaks appeared in both trypsin and Pronase digestions as shown by arrows in Fig. 2B and C, respectively. They may have been derived from peak 7. When the soluble fraction after the protease digestions (supernatant fluid at $10^5 \times g$ for 30 min) was analyzed by gel electrophoresis, no distinct peaks corresponding to those which disappeared after the treatments were observed, indicating that those removed from the envelope fraction by enzymes were degraded into smaller-molecular-weight materials.

Protease treatment has been shown to separate the outer membrane from the cytoplasmic membrane (2, 6). Figure 3 shows that the trypsin treatment of the isolated envelope induced formation of vesicles with triple-layered membranes. Judging from earlier results (2, 6), these vesicles probably consist of two different types; one produced from the outer membrane and the other from the cytoplasmic membrane. This is supported by Fig. 3F, in which two independent triple-layered membranes are being separated from the original envelope structure. It is also possible that vesicles produced from cytoplasmic membrane become gradually indistinct or disappear, because cytoplasmic membrane is more susceptible to trypsin digestion than the outer membrane (6). However, it is rather unlikely, because no gradual release of envelope protein(s) was observed by longer incubation of the envelope fraction with trypsin (Table 1).

E. coli cell wall fraction which consists of both the outer membrane and the peptidoglycan layer does not usually form closed vesicles (5, 17). However, after the trypsin treatment all membranes produced closed vesicles (Fig. 3B). This is probably because peptidoglycan which had prevented the vesicle formation was detached from the outer membrane by trypsin.

The present investigation has shown that the detachment of the outer membrane peptidoglycan and cytoplasmic membrane by proteolytic enzymes is accompanied by very specific changes in envelope proteins. Those proteins resistant to the treatment may be structural components for individual membranes, and those removed by the treatment may be more exposed, probably because they are required for the interaction with the outer membranes. Thus, the present method provides an excellent technique not only for structural study of the *E. coli* envelope but also for purification of certain envelope proteins.

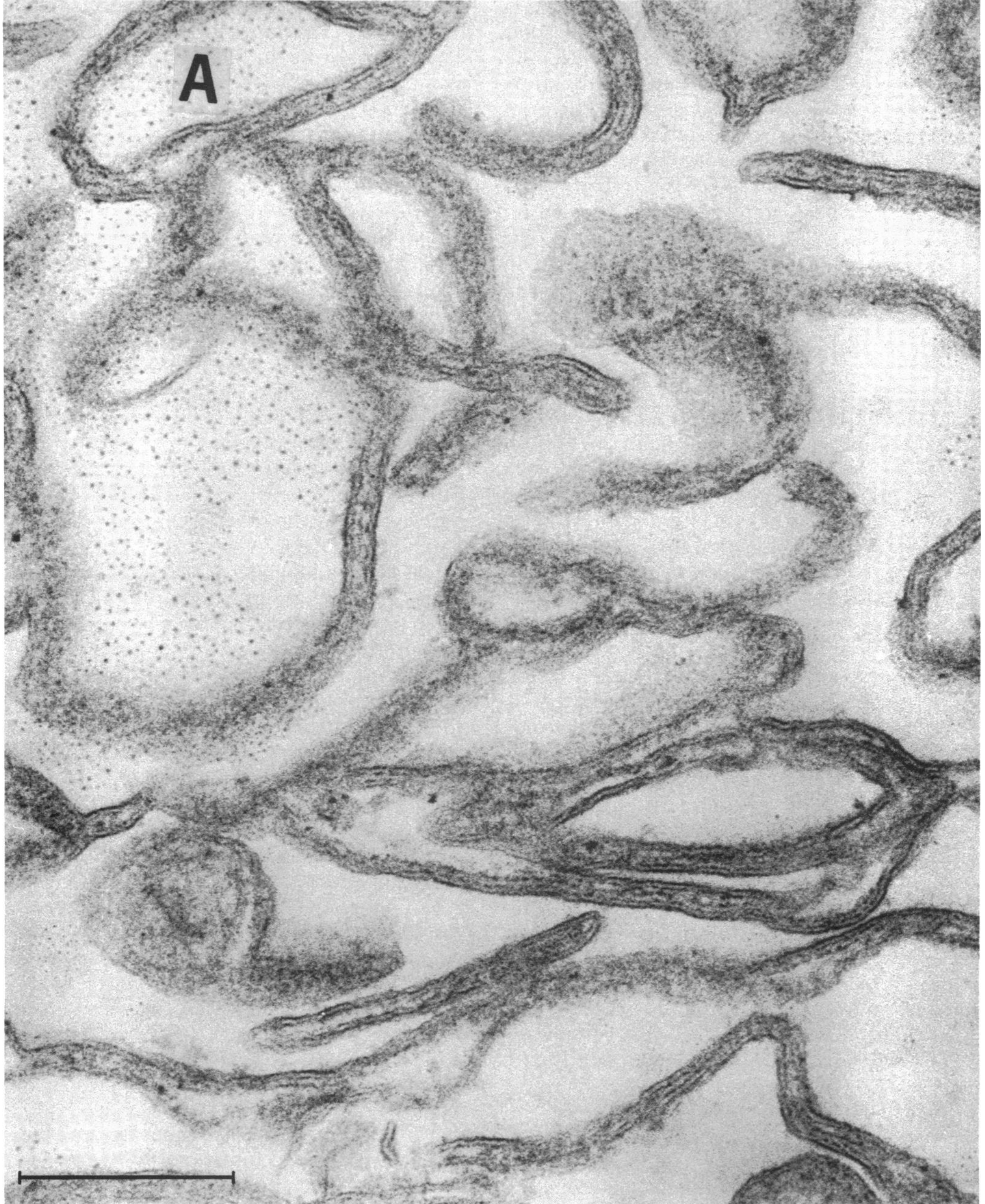
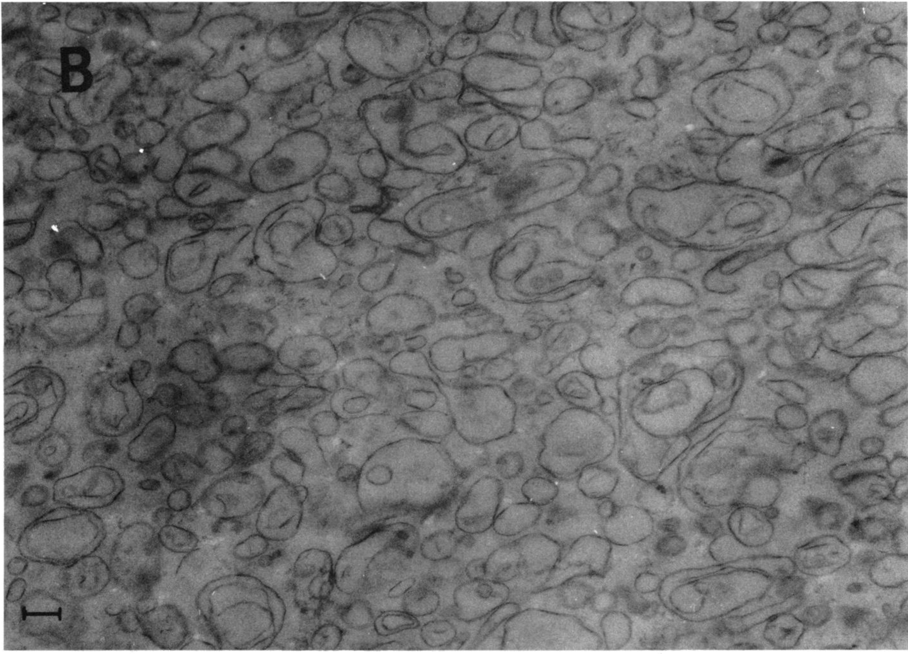
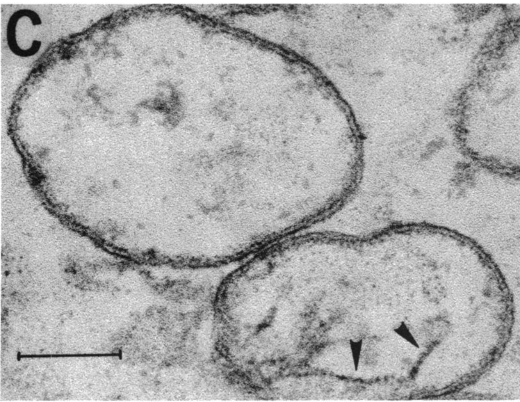
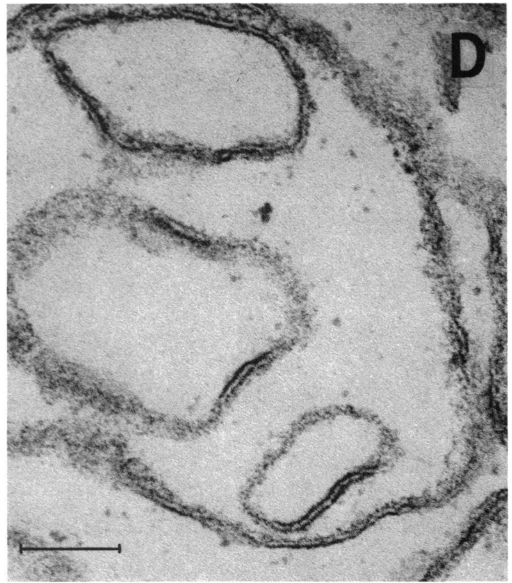
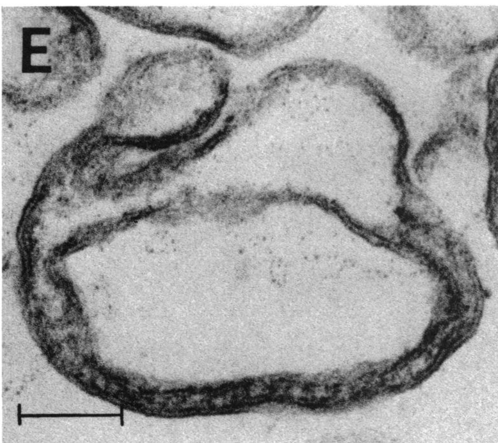


FIG. 3. *Electron microscopy of E. coli envelope fraction with or without trypsin treatment. A, Envelope fraction without trypsin treatment. B, Envelope fraction with trypsin treatment (200 μg/ml, 37 C, 30 min). C and D, Higher magnification of trypsinized envelope fractions. Arrows show a single electron-dense layer. F, Intermediate in vesicle formation. G, A single electron-dense layer attaches to two triple-layered membranes. Bars in A and B, 0.2 nm; in C, D, E, and F, 0.1 nm.*









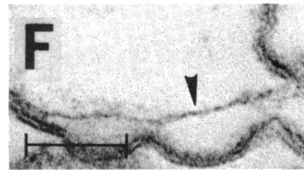


FIG. 3*B–F*

ACKNOWLEDGMENTS

We thank Jane L. Musser and Juli Haga of Princeton University for performing the electron microscopy. We also thank Rolf Sternglanz and Samuel Silberstein for reading the manuscript.

This investigation was supported by Public Health Service grant no. GM19043-01 from the National Institute of General Medical Sciences, by American Cancer Society grant no. BC-67, and by the Research Foundation of State University of New York.

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