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Effect of Ethylenediaminetetraacetic Acid, Triton X-100, and Lysozyme on the Morphology and Chemical Composition of Isolated Cell Walls of *Escherichia coli*

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Extraction of a partially purified preparation of cell walls from *Escherichia coli* with the nonionic detergent Triton X-100 removed all cytoplasmic membrane contamination but did not affect the normal morphology of the cell wall. This Triton-treated preparation, termed the "Triton-insoluble cell wall," contained all of the protein of the cell wall but only about half of the lipopolysaccharide and one-third of the phospholipid of the cell wall. This Triton-insoluble cell wall preparation was used as a starting material in an investigation of several further treatments. Reextraction of the Triton-insoluble cell wall with either Triton X-100 or ethylenediaminetetraacetic acid (EDTA) caused no further solubilization of protein. However, when the Triton-insoluble cell wall was extracted with a combination of Triton X-100 and EDTA, about half of the protein and all of the remaining lipopolysaccharide and phospholipid were solubilized. The material which remained insoluble after this combined Triton and EDTA extraction still retained some of the morphological features of the intact cell wall. Treatment of the Triton-insoluble cell wall with lysozyme resulted in a destruction of the peptidoglycan layer as seen in the electron microscope and in a release of diaminopimelic acid from the cell wall but did not solubilize any cell wall protein. Extraction of this lysozyme-treated preparation with a combination of Triton X-100 and EDTA again solubilized about half of the cell wall protein but resulted in a drastic change in the morphology of the Triton-EDTA-insoluble material. After this treatment, the insoluble material formed lamellar structures. These results are interpreted in terms of the types of noncovalent bonds involved in maintaining the organized structure of the cell wall and suggest that the main forces involved are hydrophobic protein-protein interactions between the cell wall proteins and to a lesser degree a stabilization of protein-protein and protein-lipopolysaccharide interactions by divalent cations. A model for the structure of the *E. coli* cell wall is presented.

Cell walls of *Escherichia coli* and other gram-negative bacteria are complex both in morphology and in chemical composition. When sectioned preparations of *E. coli* cells or cell walls are examined by electron microscopy, three distinct regions or structures can be identified. The outermost region, that of the lipopolysaccharide (LPS) carbohydrate chains, cannot be revealed by normal staining techniques. Staining with ferritin antibody has shown the LPS to be on the outer surface, and that O antigen-specific carbohydrate extends quite far out from the surface of the cell (19). Beneath this LPS coat is a membrane bilayer, identical in appearance to the cytoplasmic membrane (16). This structure is referred to as the outer membrane, or, because of its involvement with LPS, as the LPS membrane or L membrane (4). A granular layer identified as peptidoglycan (murein) is tightly attached to the inner surface of the outer membrane (16). The cell wall may be separated from the cytoplasmic membrane by centrifugation of broken envelope preparations in sucrose gradients, and the isolated cell wall has the same structure as observed in intact cells (16).

The peptidoglycan layer is the best understood of these three regions, largely because it can be readily isolated and studied. Weidel and Peltzer (20) isolated this structure by extracting cell walls with hot sodium dodecyl sulfate and

showed that it was a continuous "bag" studded with granules of protein and formed in the shape of the intact cell. The protein which is attached to the peptidoglycan has now been extensively studied by Braun and his associates (1, 2). This protein has a molecular weight of 7,000 daltons, has an unusual amino acid composition, contains covalently bound lipid, and is itself covalently linked to diaminopimelic acid residues on the peptidoglycan. Gross morphological changes are observed in intact envelopes when this protein is cleaved from the peptidoglycan by trypsin, indicating that this protein plays an important role in the maintenance of the organized structure of the cell wall.

The organization of the outer membrane and the attached LPS is less obvious. Recent studies on the chemical composition of the cell wall suggest that the outer membrane is a true membrane in that it contains phospholipid and protein in a ratio similar to that observed in other biological membranes (16). The protein composition is somewhat unique in that a protein with a molecular weight of about 44,000 daltons accounts for up to 70% of the total protein of the cell wall (16, 18). Both the cell wall from normal cells (18) and the outer membrane isolated from spheroplasts (4) are quite resistant to dissolution by the nonionic detergent Triton X-100. Part of this resistance to detergent lysis must be due to a stabilizing effect of divalent cations, since DePamphilis (3) has recently shown that treatment of the outer membrane isolated from spheroplasts with a combination of Triton X-100 and ethylenediaminetetraacetic acid (EDTA) leads to complete dissolution of this membrane, and that it can be reconstituted by dialysis against Mg^{2+}-containing buffer. EDTA has previously been shown to have a number of effects on *E. coli* cell walls. Treatment of intact cells with EDTA allows lysozyme to attack the peptidoglycan layer (11), results in a partial release of LPS from the cell (9), and promotes the release of periplasmic proteins by osmotic shock (7). All of these findings suggest a role for divalent cations in maintaining the integrity of the outer membrane and in the attachment of LPS to the outer membrane.

In this study, the effects of several mild treatments on isolated cell walls are examined. This study was undertaken for three reasons: first, to attempt to provide more information about the localization and organization of proteins in the cell wall; second, to obtain some information about the types of noncovalent bonds involved in maintaining the organized structure of the cell wall; and third, to provide some means for fractionating cell wall proteins.

MATERIALS AND METHODS

Bacteria and culture conditions. *E. coli* J-5 was used for all experiments except as noted. This organism was grown on minimal medium with glucose as a carbon source as previously described (15). The *E. coli* K-12 derivative AT1047 was obtained from A. L. Taylor, The University of Colorado Medical Center, Denver. This strain requires proline, thiamine, lysine, and diaminopimelic acid, and was grown on minimal medium as above, with 50 μg of proline, 20 μg of lysine, 50 μg of DL-diaminopimelic acid, and 1 μg of thiamine per ml of medium. When labeling of the diaminopimelic acid was required, 0.1 μCi of ^{3}H-labeled l-diaminopimelic acid was added per ml of medium. Late log-phase cultures were used in all experiments.

Preparation and extraction of envelope. Envelope was obtained by breakage with a French pressure cell, and the cell wall-enriched fraction was obtained by centrifugation on discontinuous sucrose gradients (16). The cell wall-enriched fraction from the gradient was extracted with 2% Triton X-100 for 10 min at 23 C followed by ultracentrifugation (18). The pellet after this extraction is referred to as "Triton-insoluble wall." This was further extracted for 10 min at 23 C with 2% Triton X-100 in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2'-ethane sulfonic acid (HEPES) buffer, *p*H 7.4, containing 5 mM EDTA adjusted to *p*H 7.4 with NaOH. After this extraction, the suspension was chilled and centrifuged for 1 hr in a Spinco 50Ti rotor at 50,000 rev/min. The pellet from this suspension is designated as the "Triton-EDTA insoluble wall" and the supernatant fraction is designated as the "Triton-EDTA supernatant."

Other methods. Electron microscopy was carried out as previously described on samples fixed in microcentrifuge tubes first with glutaraldehyde and then with OsO_4 (16). Preparations were block-stained after the osmium fixation with uranyl acetate in Veronal acetate buffer as described by Palade and Bruns (10). Sections were then post-stained with uranyl acetate and lead citrate (16).

Gel electrophoresis was carried out as previously described (14, 15) after removal of lipid and Triton X-100 by gel filtration on Sephadex LH-20 in acidified dimethyl formamide (18).

Reagents. Lysozyme and Triton X-100 were obtained from Calbiochem, Los Angeles, Calif. Uniformly ^{3}H-labeled l-diaminopimelic acid (specific activity 0.2 Ci/mmole) was obtained from Amersham-Searle, Inc., Des Plains, Ill.

RESULTS

Effect of lysozyme on the Triton-insoluble wall. It is likely that the protein associated with the cell wall is primarily localized in the outer membrane rather than in association with the peptidoglycan layer (16). This would be true even for the peptidoglycan-bound protein described by Braun et al. (1, 2) if this protein is in fact a "cement" holding the peptidoglycan to the outer membrane. To test this, the Triton-insoluble wall fraction was treated with lysozyme to see

whether protein was released when the peptidoglycan layer was broken down. Table 1 shows the effect of lysozyme on Triton-insoluble wall isolated from strain J-5. Neither lysozyme nor EDTA, nor a combination of these, resulted in any substantial release of protein. To demonstrate that lysozyme was active under these conditions, the experiment was repeated with strain AT1047 in which the peptidoglycan was labeled with ^{3}H-diaminopimelic acid. There was little or no release of the ^{3}H label in either the first Triton extraction or in the incubated control (Table 2), showing that autolysis of the peptidoglycan during the incubation periods at 23 C was minimal. When the Triton-insoluble wall was incubated with lysozyme, more than 60% of the ^{3}H label was released. Figure 1 shows the effect of lysozyme and EDTA treatment on the morphology of the Triton-insoluble wall. Lysozyme treatment resulted in the disappearance of the peptidoglycan as a discrete layer, and the inner surface of the outer membrane took on a ragged or "sawtooth" appearance (arrow, Fig. 1D). The most likely interpretation is that the "teeth" on the inner surface of the outer membrane represent fragments of peptidoglycan which are still attached to the inner membrane by the peptidoglycan-bound protein. This appearance is probably accentuated by the presence of lysozyme bound to these fragments, as examination of these preparations by gel electrophoresis has indicated that they contain a substantial amount of tightly bound lysozyme. EDTA had little effect on the morphology, other than to increase the tendency of fragments of outer membrane to roll up to form multilayered structures as seen in Fig. 1D.

In a previous study (15) it was noted that 10 to 15% of the protein of the envelope failed to dissolve in acidified dimethyl formamide. Because high-molecular-weight carbohydrates such as the peptidoglycan are insoluble in this organic solvent, it is probable that some of this solvent-insoluble protein represented the lipoprotein which is covalently bound to the peptidoglycan. Braun et al. (1, 2) have indicated that this protein accounts for about 10% of the total envelope protein. Breakdown of the peptidoglycan by lysozyme should permit this protein to dissolve in the solvent. To test this, the solubility of the Triton-insoluble cell wall protein in acidified dimethyl formamide was examined before and after lysozyme treatment. Before lysozyme treatment, about 15% of the cell wall protein was insoluble in acidified dimethyl formamide (Table 3), but this solvent-insoluble protein was reduced almost to zero by lysozyme treatment.

The size of the peptidoglycan-bound protein

TABLE 1. *Release of ^{3}H-labeled protein from Triton-insoluble wall of strain J-5*[a]

Treatment	Total protein[b]	Supernatant fluid[b]	Released (%)
Incubated control	6,840	92	1.3
Plus 5 mM EDTA	7,420	24	0.3
Plus lysozyme (0.2 mg/ml)	7,184	64	0.9
Lysozyme plus EDTA	7,212	48	0.7

[a] Strain J-5 was grown on glucose minimal medium containing ^{3}H-leucine and ^{3}H-tyrosine as previously described (15), and the Triton-insoluble cell wall was obtained by extraction with 2% Triton X-100 for 10 min at 23 C (18). The Triton-insoluble wall was incubated at a protein concentration of approximately 2 mg/ml for 20 min at 23 C in 10 mM HEPES buffer (*p*H 7.4) containing lysozyme and EDTA as indicated. The suspension was then chilled and centrifuged at 144,000 × *g* for 1 hr. Portions were removed for counting before centrifugation and from the supernatant after centrifugation to calculate the percentage of label released.

[b] Expressed as ^{3}H counts per minute per milliliter.

TABLE 2. *Release of ^{14}C-labeled protein and ^{3}H-labeled DAP from the wall-enriched fraction and Triton-insoluble wall of strain AT1047*[a]

Treatment	Protein (^{14}C counts per min per ml)			DAP (^{3}H counts per min per ml)		
	Total	Supernatant fluid	Released (%)	Total	Supernatant fluid	Released (%)
Triton extraction of wall-enriched fraction	13,660	2,194	16.1	14,892	764	5.1
Triton-insoluble wall						
Incubated control	5,120	94	1.8	6,254	152	2.5
Plus lysozyme (0.2 mg/ml)	5,034	40	0.8	6,996	4,312	61.9

[a] Strain AT1047 was grown on glucose minimal medium containing ^{3}H-labeled diaminopimelic acid (DAP) and ^{14}C-tyrosine. Triton extraction of the wall-enriched fraction was carried out as previously described (18). The Triton-insoluble wall was incubated, centrifuged, and sampled as above.

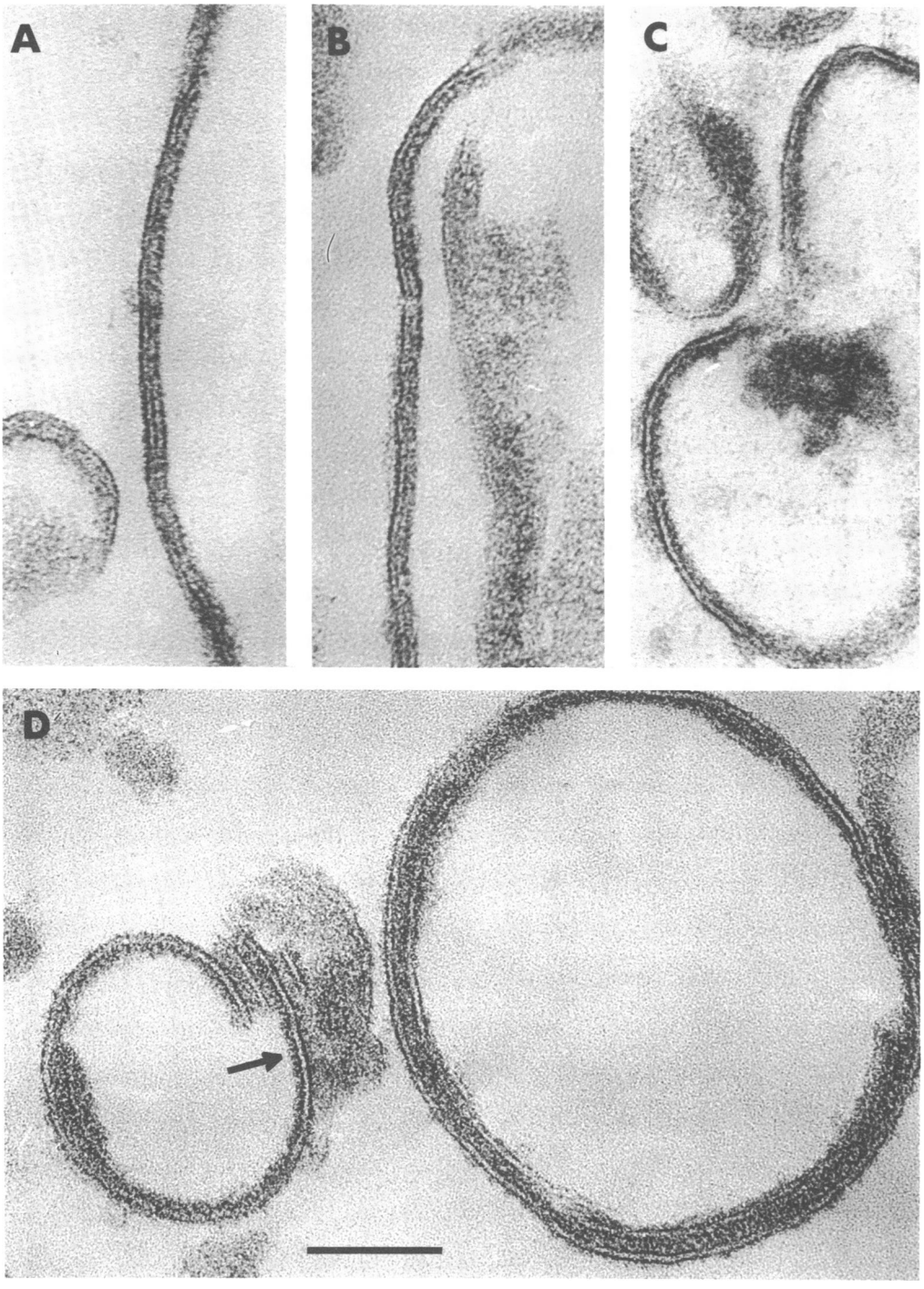

FIG. 1. *Effect of lysozyme and EDTA on Triton-insoluble cell wall. Samples of Triton-insoluble wall were treated as in Tables 1 and 2. A, Incubated control. B, Treated with EDTA only. Note that morphology is essentially identical to the incubated control. C, Treated with lysozyme only. D, Treated with lysozyme plus EDTA. Arrow indicates the characteristic "sawtooth" appearance of peptidoglycan layer after lysozyme treatment. Bar indicates 0.1 μm.*

TABLE 3. *Solubility of cell wall protein in DMF plus HCl after lysozyme treatment*[a]

Treatment	Total protein[b]	DMF-HCl insoluble[b]	Insoluble (%)
Incubated control	35.3	5.6	15.9
Plus 5 mM EDTA	30.8	5.7	18.5
Plus lysozyme (0.2 mg/ml)	35.5	0.3	0.8

[a] Triton-insoluble wall was isolated from a culture grown on ^{3}H-leucine and ^{3}H-tyrosine, and identical portions were incubated with buffer alone, lysozyme, and EDTA as described in Table 1. After incubation, the suspensions were centrifuged at 144,000 × *g* for 1 hr, and the pellets were dissolved in a solvent containing nine parts of dimethyl formamide (DMF) and one part of 0.12 N HCl. The final protein content of the solvent was approximately 2 mg/ml. This solution was allowed to stand for 30 min at 0 C and was then centrifuged at 20,000 × *g* for 15 min to sediment any insoluble protein. The insoluble pellet was suspended in water and counted.

[b] Expressed as counts per minute × 10^3.

after lysozyme treatment was examined by comparing the protein profiles of Triton-insoluble cell wall from control and lysozyme-treated samples on gel electrophoresis (Fig. 2). This experiment was done by preparing two identical preparations of Triton-insoluble wall, one labeled with ^{14}C-amino acids and the other with ^{3}H-amino acids. The ^{3}H-labeled cell wall was incubated with lysozyme, and the ^{14}C-labeled cell wall was incubated without lysozyme. The two preparations were dissolved in acidified dimethyl formamide, mixed, and prepared for gel electrophoresis. Any peaks labeled with ^{3}H but not with ^{14}C should represent peptidoglycan-bound protein which is now soluble in the solvent as a consequence of lysozyme treatment. A number of new small peaks appeared (Fig. 2), indicating that lysozyme had produced irregular breaks in the peptidoglycan resulting in a mixture of various sized fragments to which one or more of the 7,000-molecular-weight polypeptides was attached. This is in accord with the results of Braun and Sieglin (2) who found with isolated peptidoglycan preparations that lysozyme cleavage was not complete. The fastest moving peak seen in Fig. 2 probably represents the monomeric polypeptide attached to a minimum-sized peptidoglycan fragment. Peaks with this high a mobility are not normally observed in preparations of cell wall which have not been treated with lysozyme, indicating that little of this protein is free in the cell wall.

Reextraction of the cell wall with Triton X-100 plus EDTA. The results shown in Table 1 indicated that EDTA by itself did not release any protein from the cell wall. Reextraction with Triton X-100 under the conditions used to solubilize the cytoplasmic membrane resulted in no further release of protein. However, reextraction with Triton X-100 in the presence of EDTA resulted in a substantial solubilization of cell wall protein (Table 4). This is similar to the observation of DePamphilis (3), who observed that the outer membrane of spheroplasts was disaggregated by a combination of Triton X-100 plus EDTA. The amount of protein solubilized was somewhat variable, ranging from 35 to 50% of the total cell wall protein. The solubilization is much less effective at lower temperatures. Repeating the extraction with Triton X-100 or with EDTA, or with a combination of these, resulted in no further solubilization of protein.

Triton extraction of cell wall-enriched fractions in the absence of EDTA was previously shown to solubilize only proteins of the cytoplasmic membrane (18). The electropherograms shown in Fig. 3 show that reextraction with Triton plus EDTA solubilized proteins which are characteristic of the cell wall. The major structural protein of the cell wall (peak 22) appears to be present in both the Triton-EDTA-soluble and the Triton-EDTA-insoluble fractions. However, there is some specificity in solubilization of other proteins. Proteins 11 and 16 are enriched in the Triton-EDTA-soluble fraction, whereas

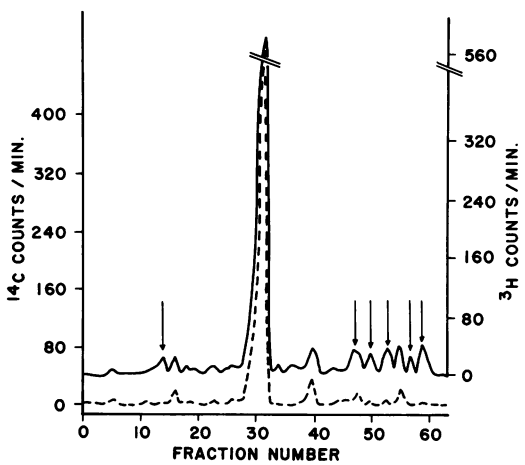

FIG. 2. *Effect of lysozyme on the electrophoretic profile of Triton-insoluble cell wall protein. Solid line indicates the ^{3}H-protein profile of a sample of Triton-insoluble wall treated with lysozyme as described in Table 3. Dashed line is the ^{14}C-labeled protein profile of an identical preparation incubated without lysozyme. The arrows indicate protein peaks observed only after lysozyme treatment. The total amount of radioactive protein in the areas under the peaks represented about 12% of the total ^{3}H-labeled protein. The samples were mixed after dissolving in acidified dimethyl formamide.*

TABLE 4. *Solubilization of protein and lipopolysaccharide (LPS) by Triton and Triton plus EDTA*[a]

Extraction	Protein (^{3}H-amino acids)			LPS (^{14}C-galactose)		
	Total	Supernatant fluid	Solubilized (%)	Total	Supernatant fluid	Solubilized (%)
First Triton extraction	1.36	0.29	21.3	6.02	2.51	41.7
Buffer wash	0.780	0.017	2.2	1.95	0.041	0.7
Second extraction						
Incubated control	0.947	0.015	1.6	2.28	0.009	0.4
EDTA only	0.829	0.016	1.9	1.97	0.018	0.9
Triton only	0.892	0.044	4.9	2.11	0.281	18.1
Triton + EDTA	1.014	0.325	32.1	2.37	2.05	86.5

[a] Results are expressed as counts per min per ml × 10^3. Strain J-5 was grown on succinate-minimal medium containing ^{14}C-galactose and ^{3}H-tyrosine (16). The inoculum was induced for galactose incorporation by growth overnight on minimal medium containing 2 mM L-fucose. The wall-enriched fraction (16) was incubated at a protein concentration of approximately 2 mg/ml for 10 min at 23 C in a solution containing 2% Triton X-100 and 10 mM HEPES buffer (*p*H 7.4) (18). The Triton-insoluble wall was then washed once with the same buffer. The solubilization of protein in these steps is given in the upper portion of the table. The Triton-insoluble wall was suspended at a concentration of 2 mg of protein/ml and extracted again for 10 min at 23 C in the same buffer containing 5 mM EDTA and 2% Triton X-100 as indicated. Centrifugation and sampling are the same as in Tables 1 and 2.

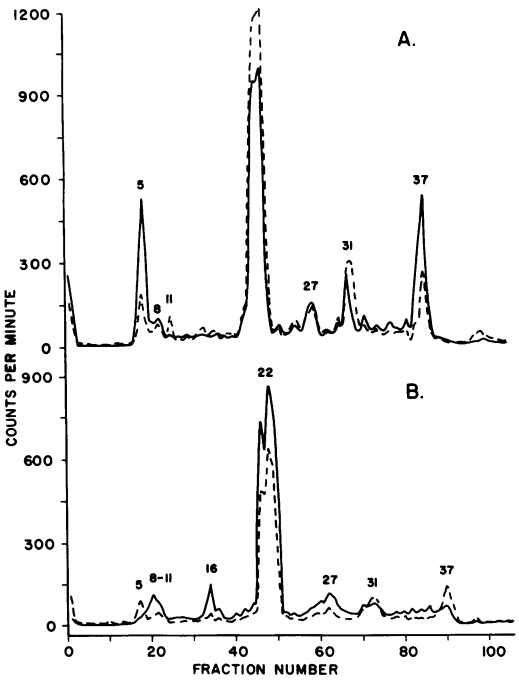

FIG. 3. *Comparison of the Triton-EDTA supernatant fluid and Triton-EDTA-insoluble fractions obtained from the Triton-insoluble cell wall. Fractionation was carried out as indicated in Table 4. Dashed line indicates the ^{14}C-labeled protein profile of unfractionated Triton-insoluble wall. Solid line in A indicates the ^{3}H-protein profile of the Triton-EDTA-insoluble fraction; solid line in B indicates the ^{3}H-protein profile of the Triton-EDTA supernatant. The ^{3}H- and ^{14}C-labeled samples were mixed and then dissolved in acidified dimethyl formamide. The gels are slightly overloaded to improve the resolution of minor peaks, resulting in a broadening and splitting of peak 22 (15). The protein peaks are numbered as previously described (18).*

proteins 5 and 37 are found almost entirely in the Triton-EDTA-insoluble fraction. Further evidence of the specificity of Triton-EDTA extraction is indicated by the recent observation that the receptors for colicins E3 and K are found primarily in the Triton-EDTA-soluble fraction obtained from *E. coli* C-600.

The morphology of the Triton-EDTA-insoluble fraction is shown in Fig. 4. This fraction still retains some of the morphological characters of the cell wall. Some of the outer membrane is still visible, although there are gaps appearing in the outer membrane as if blocks of material were removed from it (arrows, Fig. 4B and 4C). In other cases, the fragments have a "twisted ribbon" morphology, as seen between the arrows in Fig. 4A. The intact peptidoglycan layer appears to be holding the fragments together. When the Triton-insoluble wall was incubated with lysozyme and then extracted with Triton plus EDTA, a totally different morphology was observed (Fig. 5). Instead of long strips, the material is present in lamellar stacks, suggesting that some form of disruption and reaggregation has occurred. The amount of protein which was insoluble in Triton-EDTA was roughly the same whether lysozyme pretreatment was included or not. With the preparation shown in Fig. 5, 50% of the cell wall protein was found in the Triton-

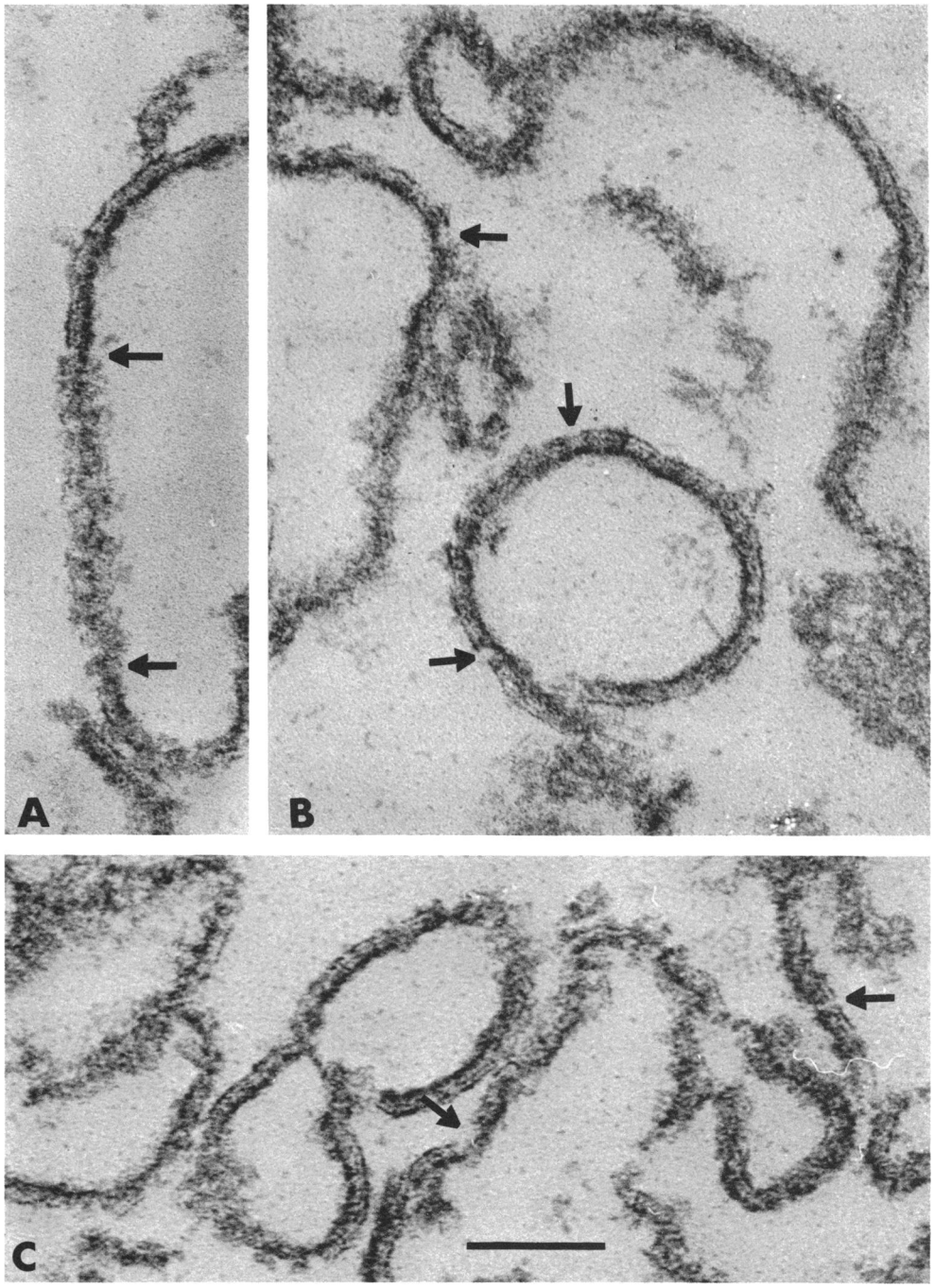

FIG. 4. *Triton-EDTA-insoluble cell wall fraction. This preparation was isolated as described in Materials and Methods and Table 4. The regions between the arrows in B and C show apparent gaps in the outer membrane. The bar indicates 0.1 μm.*

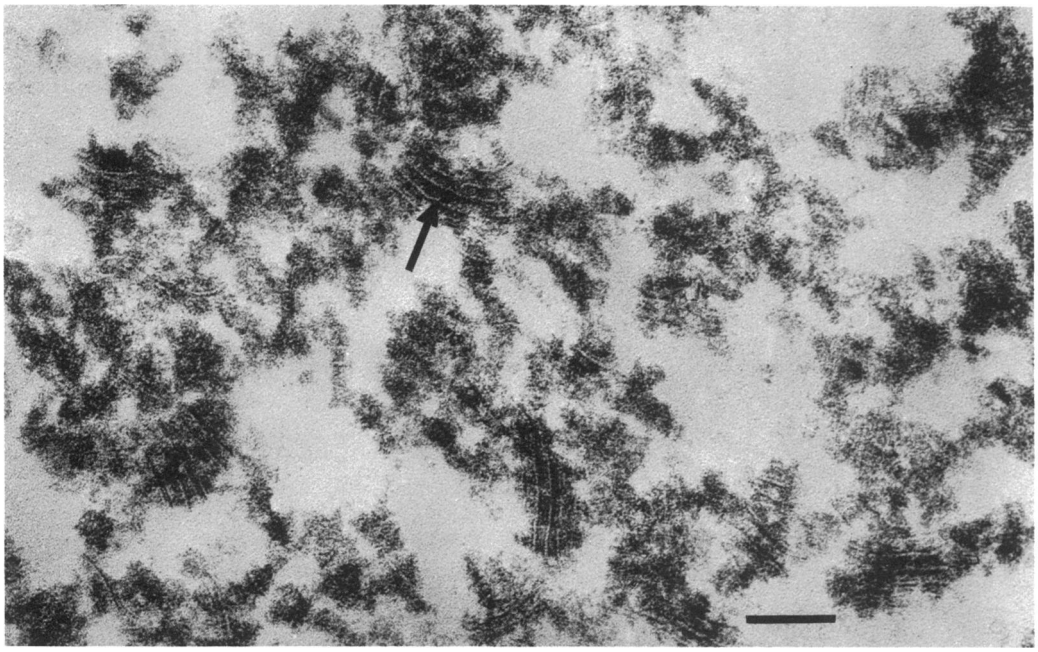

FIG. 5. *Triton-EDTA insoluble fraction prepared from a sample of Triton-insoluble wall which was treated with lysozyme as described in Tables 1 and 2 prior to Triton-EDTA extraction. The arrow indicates one of the lamellar regions. Bar indicates 0.1 μm.*

EDTA-insoluble pellet after lysozyme treatment. This indicated that the insolubility of cell wall protein in Triton plus EDTA was not due primarily to the presence of an intact peptidoglycan layer per se but to some form of interaction between the proteins themselves.

Extraction of lipid and LPS by Triton and Triton-EDTA. The cell wall contains lipid both in the form of phospholipid (16) and the lipid A component of LPS. Both of these components are extracted by Triton X-100 and Triton plus EDTA. Table 5 shows the phospholipid content of the cell wall-enriched fraction of the envelope and the Triton-insoluble and Triton-EDTA-insoluble preparations obtained from this fraction. About 80% of the lipid was removed by the first Triton extraction. Some of this lipid is accounted for by cytoplasmic membrane contamination of the cell wall-enriched fraction. In the experiment shown in Table 5, this contamination was unusually high, as indicated by the amount of protein removed by the Triton extraction. Nevertheless, the amount of phospholipid present in this cytoplasmic membrane contamination can be estimated based on the phospholipid content of the membrane-enriched fraction reported previously (16), and this would account for less than half of the phospholipid removed by Triton extraction of the cell wall fraction. Thus, about two-thirds of the phospholipid of the cell wall was also removed by Triton extraction. This had little effect on the morphology of the outer membrane, which is identical in the intact cell wall and in the Triton-insoluble wall (Fig. 1). This is analogous to the observations of Fleischer et al. on the inner mitochondrial membrane (5), where the morphology of the membrane was preserved after removal of much of the lipid.

The remaining lipid present in the Triton-insoluble wall was removed by Triton-EDTA extraction. Hence the remnants of outer membrane observed in the Triton-EDTA-insoluble fraction (Fig. 4) and the lamellar appearance shown in Fig. 5 cannot be due to the presence of phospholipid-protein bilayers. It is possible, however, that Triton has replaced the phospholipid in these structures.

A similar situation is seen with LPS. As indicated in Table 4, about 50% of the LPS is removed by the first Triton extraction with no alteration in morphology. This is similar to the observations of Levy and Leive (9), who reported that removal of up to half of the LPS from intact cells with EDTA had no effect on the morphology of the cell wall. The remainder of the LPS is also removed by the Triton-EDTA extraction. Thus, LPS is not replacing phospholipid in the bilayer or lameller structures seen after Triton-EDTA extraction. It is tempting to speculate that the gaps visible in the outer mem-

TABLE 5. *Removal of phospholipids by Triton*[a]

Sample	Amt of protein		Amt of phospholipid		Phospholipid/protein (μmoles/mg)
	mg	%	μmoles	%	
Crude wall	11.5	100	5.52	100	0.48
Triton-insoluble wall	7.35	63.9	1.15	20.8	0.156
Triton-EDTA-insoluble wall	3.72	32.3	0.134	2.4	0.036

[a] Samples were prepared as described in Materials and Methods and Table 4. Protein and lipid phosphate were determined as previously described (16).

brane as seen in Fig. 4B and 4C are due to removal of LPS-lipid-protein complexes from the outer membrane. Soluble LPS-lipid-protein complexes are released from intact cells of *E. coli* and *Salmonella* under some conditions of inhibited growth (8, 12).

DISCUSSION

A combination of the results described above with the recent results of DePamphilis (3, 4) and Braun et al. (1, 2) allows some general conclusions to be drawn about the organization of the *E. coli* cell wall and the types of interactions which serve to maintain the integrity of this structure. These are outlined below.

Role of the peptidoglycan in cell wall organization. The outer membrane appears to be attached quite firmly to the peptidoglycan layer. In no case was any separation of the peptidoglycan from the outer membrane (or the remains of the outer membrane after Triton-EDTA extraction) observed in this study. Digestion of the backbone of the peptidoglycan with lysozyme does not result in the release of any protein from the remainder of the cell wall. Complete separation of noncovalently linked cell wall protein from the peptidoglycan layer is accomplished only by rather severe treatment, for example the extraction with hot sodium dodecyl sulfate solution employed by Braun et al. (1) or the solubilization in acidified dimethyl formamide which we have employed in preparing cell wall proteins for gel electrophoresis. At the same time, cleavage of the covalently bound lipoprotein from the peptidoglycan with trypsin resulted in gross alteration in the properties of the cell envelope (1). This indicates very strongly that the lipoprotein described by Braun et al. (1, 2) is responsible for the attachment of the peptidoglycan to the outer membrane, presumably by very strong hydrophobic protein-protein interactions. Thus the peptidoglycan layer appears to be the skeleton on which the remainder of the cell wall is constructed.

Cell wall phospholipid. DePamphilis and Adler reported that the outer membrane isolated from spheroplasts by Triton extraction contained protein and LPS but little phospholipid (4). DePamphilis suggested that the Triton extraction may have removed phospholipid (3). The data in Table 5 indicate that this is probably correct. Recent studies in this laboratory on cell wall preparations purified without detergents indicate that the cell wall is the site of about half of the phospholipid of the cell. The limited effect of the removal of phospholipid on the morphology and protein composition indicates that the phospholipid plays only a minor role in the structural stability of the cell wall. However, this lipid may have an important role in determining the permeability of the cell wall, or it may provide a necessary lipid phase for cell wall biosynthetic reactions of the type described by Rothfield and Romeo (13). It may also provide the anchoring point for lipid A.

Attachment of LPS and the role of divalent cations. The requirement for EDTA in the formation of spheroplasts by lysozyme (11) and in the release of periplasmic proteins by osmotic shock (7) has indicated that divalent cations play a role in maintaining the impermeability of the cell wall to large molecules. The partial release of LPS from intact cells by treatment with EDTA (9) points to an involvement of divalent cations in stabilizing the attachment of LPS to the cell wall. In conjunction with Triton, EDTA promoted the solubilization of a considerable amount of protein, as well as the LPS and phospholipid of the Triton-insoluble wall fraction. Using somewhat different conditions and a different starting material, DePamphilis (3) was able to achieve complete dissolution of the outer membrane isolated from spheroplasts. This could be reversed by dialysis against Mg^{2+}-containing buffer. All of these phenomena indicate that divalent cations stabilize the cell wall, perhaps by forming ionic bridges between the phosphate groups in the LPS core and charged groups on adjacent proteins or phospholipids. However, EDTA does not cause any release of protein from the cell wall even after disruption of the peptidoglycan layer (Table 1), nor does it release LPS from the Triton-insoluble wall (Table 5). It is best to consider the effect of EDTA as a weak-

ening of the cell wall to a point where it is susceptible to mechanical damage or disaggregation of the LPS and protein components by detergents.

Hydrophobic protein-protein interaction. Several lines of evidence suggest that the major force holding the cell wall together is a hydrophobic interaction between the various protein components. This type of interaction is best illustrated by the appearance of the Triton-EDTA-insoluble protein shown in Fig. 5. Even in the absence of native lipid and LPS and in the presence of Triton X-100, the cell wall protein shows a tendency to aggregate into regular lamellar structures. Protein isolated from *E. coli* cell walls shows a very strong tendency towards aggregation. Dissolution of the cell wall in an organic solvent followed by removal of lipid and dialysis into sodium dodecyl sulfate-urea solutions has been the only method which in our hands has provided satisfactory resolution on gel electrophoresis. High concentrations of urea or guanidine hydrochloride or extremes of *p*H were not effective in preventing aggregation of either native or lipid-free preparations of cell wall proteins. The failure of these techniques attests to the strongly hydrophobic nature of the protein interactions.

Overall organization of the E. coli envelope. The model shown in Fig. 6 is an attempt to sum up the present knowledge concerning the cell wall of *E. coli*. The lipoprotein cytoplasmic membrane contains about half of the phospholipid of the envelope and is readily dissociable by Triton X-100 (18). It has a complex protein composition typical of other biological membranes (14). This is separated from the innermost layer of the cell wall by an electron-transparent region with a mininum dimension of about 10 nm, referred to in Fig. 6 as the periplasmic gap. The innermost layer of the cell wall is the peptidoglycan layer, which is visible by electron microscopy as a finely granular layer and which is presumably a continuous mesh (20), one molecule thick (6). This is shown attached to the outer membrane by the covalently bound lipoprotein described by Braun et al. (1, 2). The bond between the subterminal arginine and the terminal lysine residue which is linked to diaminopimelic acid residues on the peptidoglycan is very sensitive to trypsin (2). This lipoprotein can be visualized as extending into the phospholipid layer of the outer membrane. The outer membrane has a much simpler protein composition and is characterized by the presence of a single major structural protein with a molecular weight of about 44,000 daltons (16, 18). Similar major protein species are observed in envelopes of other

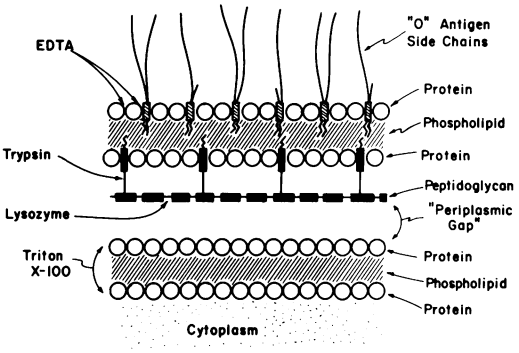

FIG. 6. *Simplified model of the cell envelope of E. coli. Various components of the envelope are listed on the right; probable sites of attack of various disruptive agents are indicated on the left.*

gram-negative species (17). The LPS is indicated to be on the outer surface of the outer membrane with the lipid A component inserted into the phospholipid layer and the O-specific side chains extending out into the external medium. This is consistent with the observation that LPS is capable of penetrating synthetic phospholipid monolayers (13). The primary site of action of EDTA (hence the site of divalent cation stabilization) is envisioned to be at the site of insertion of the LPS into the outer membrane. In this simplified model, flagella and pili have been omitted. DePamphilis and Adler (4) presented evidence that the "L" ring of the flagellar basal body is inserted into the outer membrane of the cell wall. In this model, both the cytoplasmic membrane and the outer membrane are illustrated as simple lipoprotein bilayers, without any attempt to show penetration of the various proteins into the phospholipid layers. Such penetration of protein into the lipid bilayer may well occur, but the actual organization of lipid and protein in these membranes is one of many problems which remain to be solved.

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