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Dissociation and Reassembly of *Escherichia coli* Outer Membrane and of Lipopolysaccharide, and Their Reassembly onto Flagellar Basal Bodies

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Purified lipopolysaccharide vesicles dissociate when treated with ethylenediaminetetraacetic acid (EDTA) and then reassemble when dialyzed against Mg^{2+}. Purified outer, lipopolysaccharide membrane (L membrane) is partially dissociated by treatment with EDTA and fully dissociated upon further treatment with Triton X-100. Both the partially and fully dissociated L membrane can be reassembled by dialysis against Mg^{2+}. Reassembly of lipopolysaccharide or L membrane in the presence of intact flagella results in specific attachment of flagellar basal bodies to vesicles via the L and sometimes the M ring. Lipopolysaccharide and L membrane appear to be composed of substructures bound together by both Mg^{2+} (divalent cation)-mediated and hydrophobic bonds.

DePamphilis and Adler previously reported the purification (2) of intact flagella from *Escherichia coli* and the structure of the flagellar basal body (3), which is composed of four rings mounted on a rod. Beginning with the ring closest to the hook, they are referred to as the L, P, S, and M rings. They also reported a procedure for purifying the outer membrane (4), a complex layer primarily composed of lipopolysaccharide (LPS) and protein with some lipid present. The outer membrane, referred to as L membrane, is specifically attached to the L ring of flagellar basal bodies (4), and the inner, cytoplasmic membrane is specifically attached to the M ring.

The chemical, physical, and serological properties of purified LPS were extensively studied (17). Such data are also available for special forms of L membrane such as the material excreted during lysine-limited growth of *E. coli* (10, 11, 24, 27), extracted from cells with ethylenediaminetetraacetic acid (EDTA) treatments (12) or prepared from spheroplasts extensively dialyzed against EDTA (15).

The purpose of this paper is to report what appears to be the first description of in vitro self-assembly of purified L membrane vesicles and of LPS vesicles. Purified L membrane is solubilized in Triton X-100 after treatment with EDTA. The solubilized material reassembles into vesicles upon removal of the detergent in the presence of Mg^{2+}. When intact flagella are present during reassembly, the resulting vesicles are attached to flagellar basal bodies via the L ring and sometimes the M ring. The evidence presented strongly indicates that L membrane is composed of substructures bound together by both Mg^{2+} (divalent cation)-mediated and hydrophobic bonds. Similar experiments done with purified LPS lead to similar results.

MATERIALS AND METHODS

Bacteria. AW330, a motile strain of *E. coli* K-12 (2), was grown on tryptone broth as previously described (2).

Purification of LPS. The preparation of LPS from *E. coli* was based on the method of Westphal and Jann (26) as modified by Osborn et al. (18). Frozen cells, harvested from 6 liters of culture grown to an optical density at 590 nm (OD_{590}) of 1.2, were suspended in 40 ml of 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer containing 0.3 M KCl (*p*H 7.6, 26 C) and put through a French pressure cell four times at 5 C. The lysate was diluted to 300 ml with this same buffer containing KCl, and the cell walls were purified by differential centrifugation (18). The final cell wall pellet was suspended in 50 ml of doubly distilled water and extracted twice with 90% phenol for 10 min at 68 C; the extracts were cooled to 5 C. The aqueous layers were washed with ether to remove residual phenol and then dialyzed for 16 hr against three 1-liter changes of 10 mM $MgCl_2$. The material was then concentrated to 5 ml by ultrafiltration (Diaflo; Amicon Corp.).

Purification of outer, LPS membrane. A procedure was previously described (4) for the purification of outer, LPS membrane (L membrane) from *E. coli* cell envelopes with flagella still attached. In this procedure, spheroplasts produced with lysozyme and EDTA were incubated in Mg^{2+} and then lysed with Triton X-100.

After incubation in deoxyribonuclease I, L membranes with attached flagella were then purified by $(NH_4)_2SO_4$ precipitation, differential centrifugation, and CsCl gradient centrifugation. The resulting vesicles are referred to as "native" L membrane.

In the present report, a modification of this procedure was also used in which spheroplasts were first lysed with Triton X-100 in the absence of Mg^{2+}. $MgCl_2$ (1 M) was then added to give a molar ratio of Mg^{2+}/EDTA = 6. Deoxyribonuclease I was added, and the lysate was incubated for 20 min at 30 C. L membrane is readily solubilized by Triton X-100 if Mg^{2+} has first been chelated by EDTA. The initially translucent lysate becomes turbid upon incubation because of the reassembly of solubilized L membrane in the presence of Mg^{2+}. The procedure for purification of this "reconstituted" L membrane was the same as for "native" L membrane except that the reconstituted material readily sedimented during the step requiring 5,000 × *g* for 10 min (4). This sediment yielded a single band in a CsCl gradient with a density of 1.40 g/ml and was identified by its chemistry and structure as a form of L membrane. Only trace amounts of the L membrane band were found when the supernatant from the above sedimentation was applied to a CsCl gradient. The supernatant from the 10,000-rev/min-for-1-hr step (4) yielded a band of flagella at 1.30 g/ml and a dispersed floculent band of material at a lighter density, but no L membrane band was found. Therefore, these two supernatant fractions were discarded.

Chemical assays. The purpose of the chemical assays was to identify the presence of LPS in L membrane preparations and estimate the amounts of LPS and protein. Therefore, LPS purified from the same strain of *E. coli* used in L membrane preparations served as a chemical standard for comparison.

Dry weights were determined on samples dried to a constant weight in a vacuum oven at 80 C. Heptose was determined by the cysteine-H_2SO_4 reaction (6) as modified by Osborn (16). Under these conditions, 1.0 μmole of glucoheptose (Pfanstiehl Labs) gave a value of $OD_{505} - OD_{545} = 1.6$. Osborn reported a value of 1.07 for 1 μmole of L-glycerol-D-mannoheptose, the isomer identified in LPS. After establishing a standard curve with glucoheptose, the heptose content in these preparations was adjusted to equivalents of the L-glycerol-D-mannoheptose isomer. Glucose was determined with the glucose oxidase assay (Worthington Glucostat reagents) after hydrolysis of the samples in 2 N H_2SO_4 for 1 hr at 100 C. The solution was then cooled and neutralized with $BaCO_3$ (16). Glucose standards were treated similarly to compensate for the destruction of glucose during the hydrolysis. The amount of 2-keto-3-deoxyoctonate (KDO) was determined by using a modification (16) of the method of Weissbach and Hurwitz (25) in which 1 μmole of KDO was reported to give an absorbance of 19.0 at 548 nm. The color faded on addition of 1 M NaOH as expected (25). Total inorganic phosphate was determined by the method of Chen et al. (1) on samples ashed with concentrated H_2SO_4 and 72% $HClO_4$. Protein was assayed with the method of Lowry et al. (13) by using bovine serum albumin as a standard.

Dialysis tubing. The dialysis tubing used in all experiments was boiled for 1 hr in doubly distilled water containing 0.01 M NaCl and 0.001 M EDTA before use.

Electron microscopy. Materials were negatively stained with phosphotungstate (*p*H 7.2) or uranyl acetate (*p*H 4.5) as previously described (3). Phosphotungstate was superior for revealing L membrane vesicles. All preparations were viewed in a Siemens Elmiskop I at 80 kv by using a liquid nitrogen decontamination device and a 50-μm thin-metal objective aperture.

RESULTS

This report deals with three types of preparations: (i) LPS purified from cell walls by extraction with 90% phenol, (ii) outer, LPS membrane ("native" L membrane) purified from spheroplasts incubated in Mg^{2+} and then lysed with Triton X-100, (iii) "reconstituted" L membrane purified from spheroplasts lysed with Triton X-100 in the absence of Mg^{2+} and then incubated in Mg^{2+}.

Properties of purified LPS and L membrane. Chemical analysis (Table 1) of purified LPS and L membrane, both native and reconstituted, demonstrated the presence of heptose, glucose, KDO, and phosphate, compounds characteristic of LPS (17). The molar ratios of these compounds in the LPS preparation, the absence of detectable protein, and the density are typical for this material (7, 10, 14, 19, 21, 27). Both L membrane preparations have higher ratios of glucose and phosphate than LPS. However, the most obvious difference is the presence of protein in L membrane, some of which is flagella and pili. Based on the heptose content relative to that found in purified LPS, native L membrane con-

TABLE 1. *Chemical analysis of lipopolysaccharide (LPS) and L membranes*

Sample	Heptose		Glucose		KDO[c]		Phosphate		Protein (%)	Density[d]
	Per cent[a]	Molar ratio[b]	Per cent	Molar ratio	Per cent	Molar ratio	Per cent	Molar ratio		
LPS	12.0	1.0	4.8	0.47	1.14	0.08	6.6	1.2	<0.1	1.48
Reconstituted L membrane	8.0	1.0	7.3	1.0	0.90	0.10	5.8	1.5	32.8	1.40
Native L membrane	6.6	1.0	7.6	1.35			6.2	2.1	43.8	1.34

[a] Per cent is based on dry weight of the sample.
[b] Molar ratios are relative to a heptose value of 1.0.
[c] 2-Keto-3-deoxyoctonate.
[d] Buoyant density in CsCl, 0.1 M Tris (*p*H 7.8, 26 C), 10 mM $MgCl_2$.

tains about 55% LPS and reconstituted L membrane contains about 67% LPS, the remaining material being essentially protein. Reconstituted L membrane has approximately 25% less protein than the native L membrane. The differences in relative composition of the three materials are also reflected in their buoyant densities in CsCl (Table 1). The higher molar ratios of phosphate and glucose found in L membrane compared to purified LPS indicate the presence of phospholipid and an additional carbohydrate. The buoyant density also indicates some lipid is present since the densities are slightly lighter than expected for the amounts of LPS and protein present. The presence of phospholipid is reported by Leive et al. (12) and Knox et al. (10) on a similar material from *E. coli*. The possibility also exists that Triton X-100 has altered the chemistry of L membrane either by extracting some lipid or protein, or by binding to the membrane. In the case of reconstituted L membrane, components from both the cytoplasmic and L membranes may conceivably be present.

Examination of LPS (Fig. 1), native L membrane (Fig. 1 of reference 4), and reconstituted L membrane (Fig. 2) with an electron microscope after negative staining showed that all of the three materials were composed of vesicles. All of the preparations of L membrane also contain flagella. L membrane prepared by other workers (11, 15) was also vesicular. Thin sections of LPS (5, 9) and of native (4) and reconstituted L membrane all show the trilaminar structure typical of membranes.

A distinguishing property of reconstituted L membrane preparations compared to native L membrane is that the material is readily sedimented at 5,000 $\times$ *g* for 10 min, or that it will settle out of suspension upon standing for several hours. Examination of this material in a phase-contrast microscope showed the presence of large aggregates approximately 20 to 40 μm wide. Aggregates, 5 to 10 μm wide, seen in an electron microscope were composed of densely packed vesicles similar in appearance to Fig. 2.

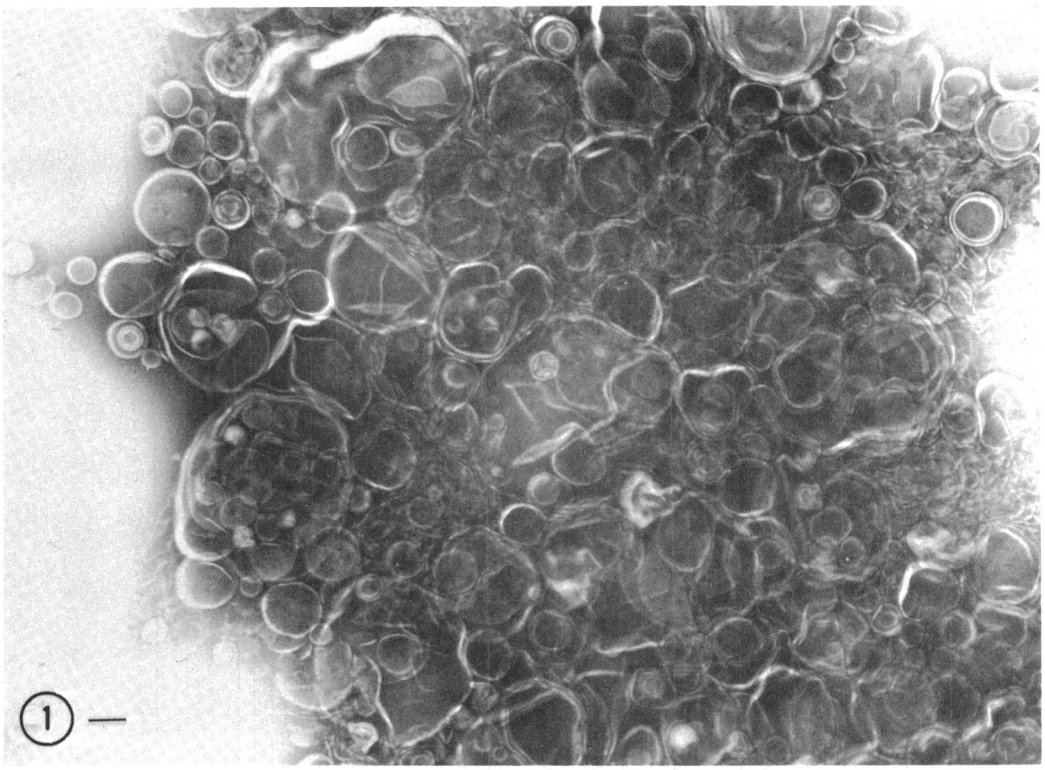
Dissociation and reassembly of purified LPS. LPS purified in the presence of Mg^{2+} had a vesicular structure (Fig. 1). Dialysis of 1 to 5 mg of these vesicles per ml against 500 volumes of Tris-EDTA buffer [0.1 M Tris, 5×10^{-3} M EDTA (*p*H 7.8, 26 C)] for 12 hr at either 5 or 28 C resulted in complete dissociation of the vesicles and the appearance of disc (14 to 70 nm diameter)- and rod (10 to 20 nm long, 7 nm wide)-shaped substructures (Fig. 3). Some of the rod-shaped structures may represent discs standing on their edge. Vesicular structures reformed (Fig. 4) when this material was dialyzed against 500 ml of Tris-Mg^{2+} buffer [0.1 μM Tris, 0.02 M $MgCl_2$ (*p*H 7.8, 26 C)] for 12 hr at either 5 or 28 C.

Dissociation and reassembly of native L membrane. When the above experiment was performed on native L membrane, both vesicles and substructures were found after dialysis against Tris-EDTA buffer (Fig. 5). In this EDTA-dissociated L membrane, the substructures (substructures A) appeared both as rods (14 to 28 nm long, 7.5 nm wide) and discs (14 to 70 nm wide) similar to those seen after LPS dissociation. Figure 6 is a guide to this and subsequent experiments. Neither dialyzing for an additional 12 hr nor increasing the EDTA concentration to 10^{-2} M resulted in further degradation of the vesicles. Besides the use of electron microscopy, the extent of vesicle degradation was estimated from the change in OD_{400}, where OD was a function of light scattering and the results were expressed as per cent of the initial OD of untreated material. By this criterion, native L membrane was 25% dissociated.

Reassembly of vesicles from EDTA-dissociated native L membrane was accomplished by dialysis against Tris-Mg^{2+} buffer for 12 hr at 5 or 28 C (Fig. 7). The OD_{400} returned to 100%. However, unlike the original vesicles, the reassembled material settled to the bottom of the dialysis bag. Electron microscopy of this material showed large aggregates of vesicles indistinguishable from Fig. 2. Vesicles also reformed when the Tris-Mg^{2+} buffer was made 2.0 M in KCl (Fig. 8); the OD_{400} returned to 60%. This suggests that L membrane substructures are not held together by ionic bonds. The function of Mg^{2+} is apparently to form coordinate bonds (chelates) between substructures.

Complete solubilization of EDTA-dissociated L membrane required the presence of a detergent, Triton X-100. Addition of Triton X-100 to a final concentration of 0.2% dropped the OD_{400} to 7.0%, and neither vesicles nor disc-shaped substructures were found. The only material detectable with the electron microscope was small rod-shaped particles 5 nm wide and 14 to 25 nm long (Fig. 9) which are referred to as substructures B. Possibly substructures A and B are the same but appear different after negative staining as a result of binding with Triton X-100. The detergent had no effect on the vesicular configuration of native L membrane in the presence of Mg^{2+}. This fact is the basis of the purification method for native L membrane (4).

Reassembly of vesicles from EDTA-Triton X-100-solubilized native L membrane occurred in two steps (Fig. 6). When $MgCl_2$ was added directly to give a Mg^{2+}/EDTA ratio of 6, the OD_{400} increased to 70% in 1.5 hr. However, examina-



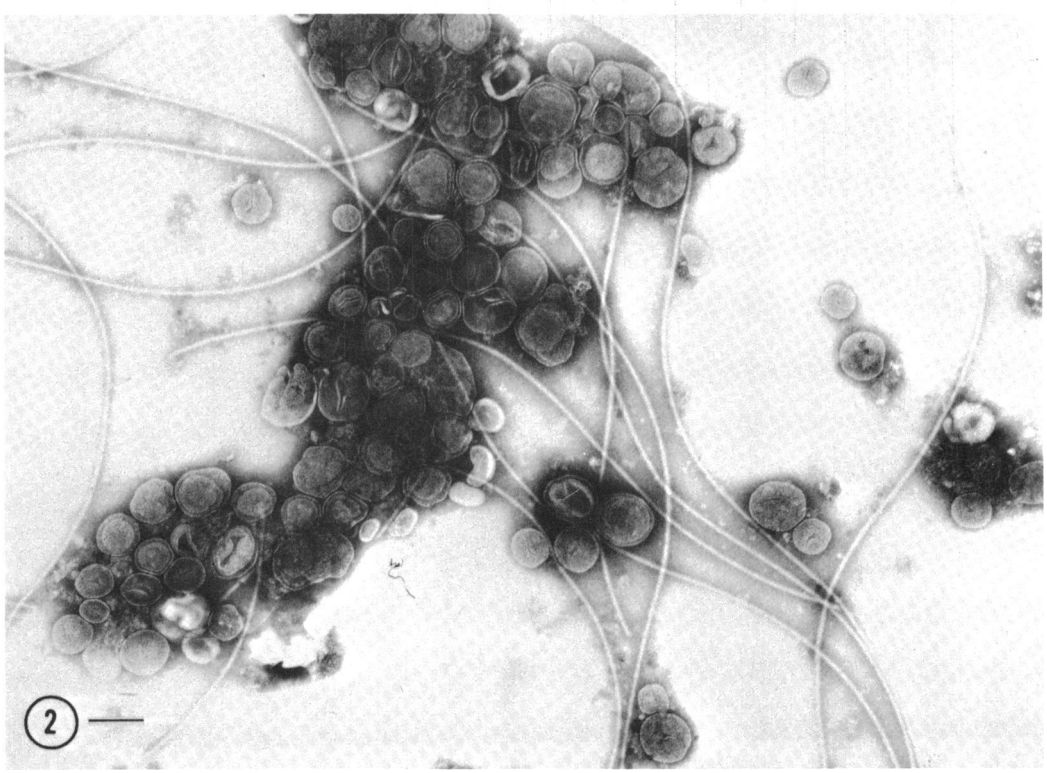
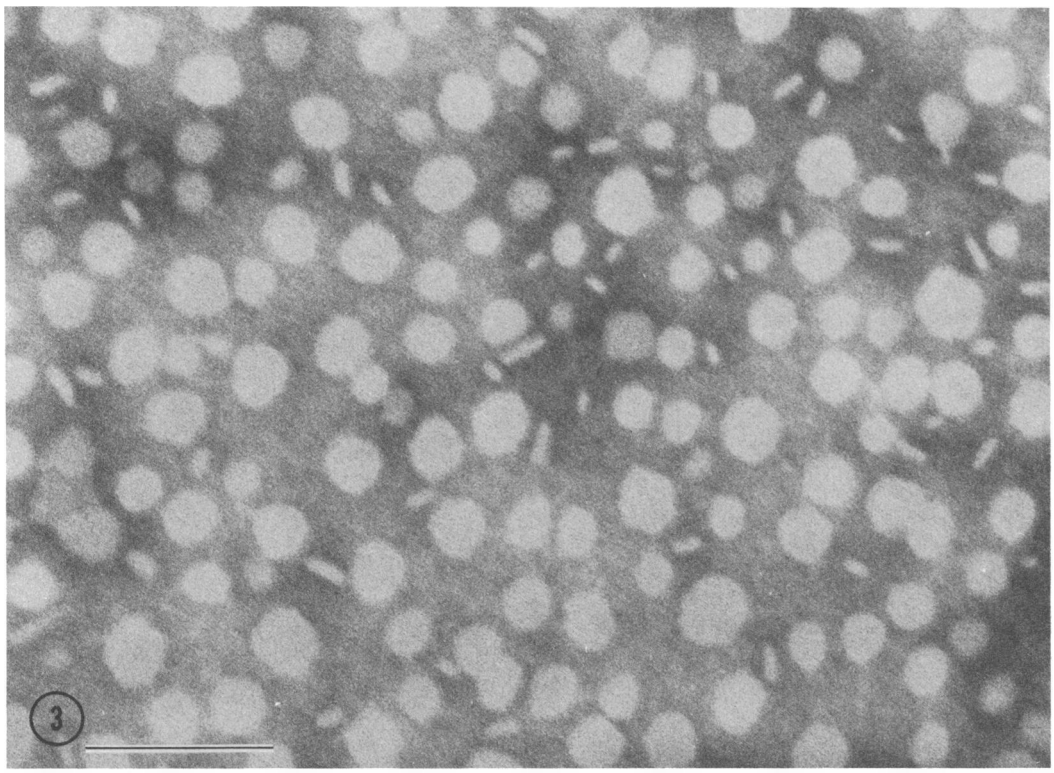


FIG. 1. *Purified lipopolysaccharide after dialysis against $MgCl_2$. Phosphotungstic acid. ×50,000. The bar in this and subsequent figures represents 100 nm.*

FIG. 2. *Preparation of reconstituted L membrane with flagella attached. Phosphotungstic acid. ×74,000.*



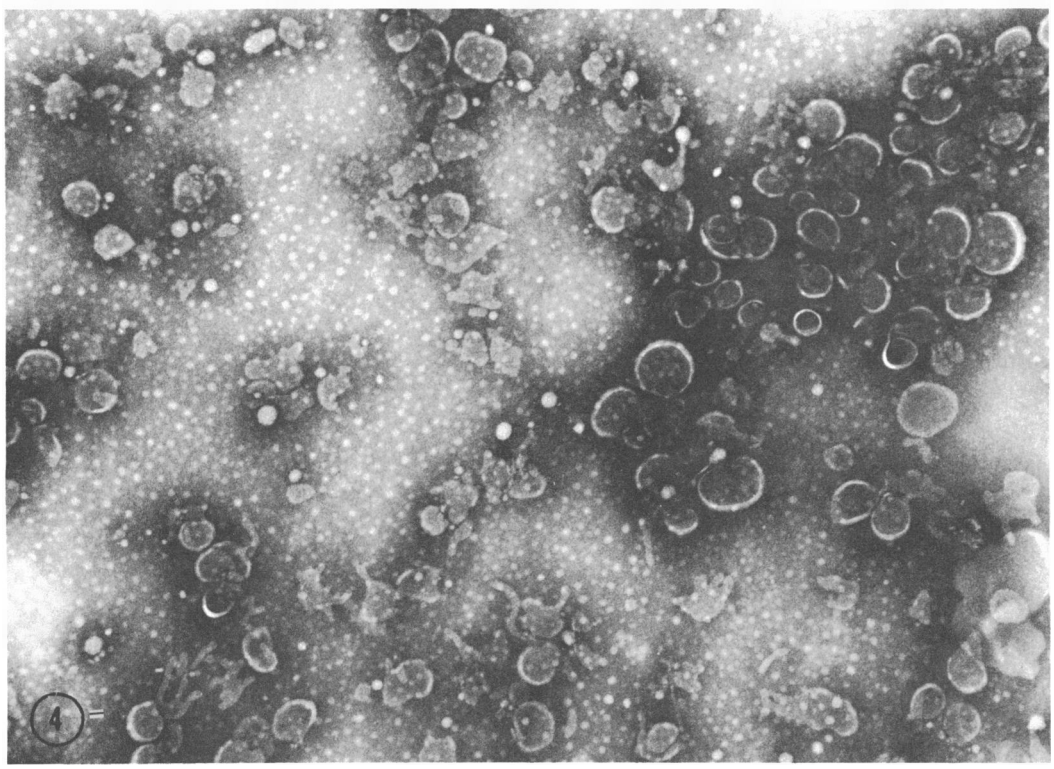


FIG. 3. *Purified lipopolysaccharide after dialysis against Tris-EDTA buffer. Both disc- and rod-shaped substructures are present. Phosphotungstic acid.* ×*250,000.*

FIG. 4. *EDTA-dissociated lipopolysaccharide reassembled by dialysis against Tris-*Mg^{2+} *buffer. Phosphotungstic acid.* ×*14,300.*

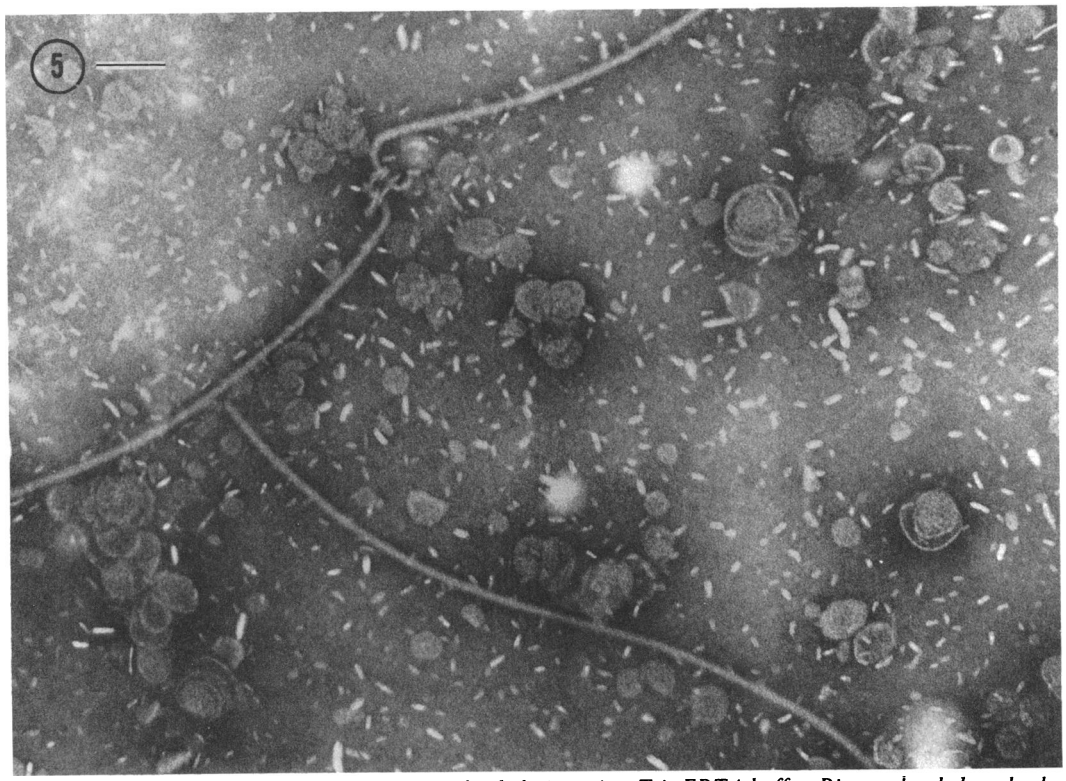


FIG. 5. *Native L membrane after dissociation by dialysis against Tris-EDTA buffer. Disc- and rod-shaped substructures (substructures A) are present. Phosphotungstic acid. ×89,000.*

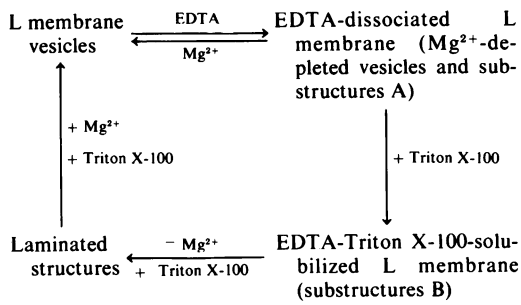
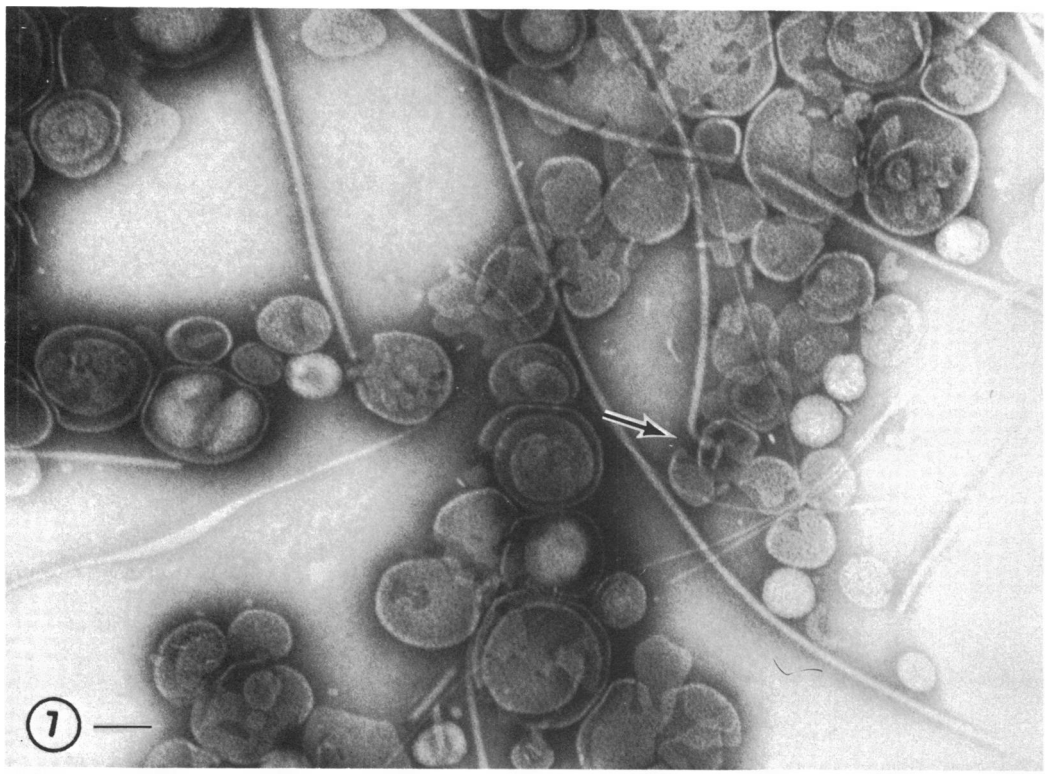


FIG. 6. *Dissociation and reassembly of purified L membrane.*

tion of the material after negative staining showed the presence of laminated structures (Fig. 10) rather than vesicles. In some cases a laminated structure was surrounded by a pool of substructures (Fig. 11) similar to substructures A (Fig. 5) and EDTA-dissociated LPS (Fig. 3). When a sample of EDTA-Triton X-100-solubilized L membrane was dialyzed against 500 volumes of Tris-Mg^{2+} buffer for 12 to 24 hr at 26 C, the sample became turbid within 3 hr and contained laminated structures like those seen in Fig. 10 and 11. However, extending the dialysis to 48 hr and then changing the dialysate and continuing for an additional 48 hr resulted in vesicles indistinguishable from those in Fig. 7. This reassembled material, like that reassembled from EDTA-dissociated L membrane, rapidly settled out of suspension.

The release of Triton X-100 from a dialysis bag was assayed by following the OD_{280} in the dialysate. In 48 hr of dialysis at 26 C, 60 to 70% of the detergent was removed. Therefore, Mg^{2+} evidently causes the rapid reaggregation of substructures B into laminated forms despite the presence of Triton X-100. Extended dialysis then removes sufficient Triton X-100 to allow a transformation of the laminated structures to vesicular forms.

The presence of 2.0 M KCl in the dialysate prevented the reassembly of vesicles although laminated structures still appeared. However, they were smaller, more uniform in appearance (Fig. 12) with some disc-shaped substructures visible. The material remained in a uniform suspension. Since removal of Triton X-100 occurred at half the rate observed in the absence of KCl, the presence of 2.0 M KCl may prevent the release of bound detergent and in this way block the formation of vesicles.



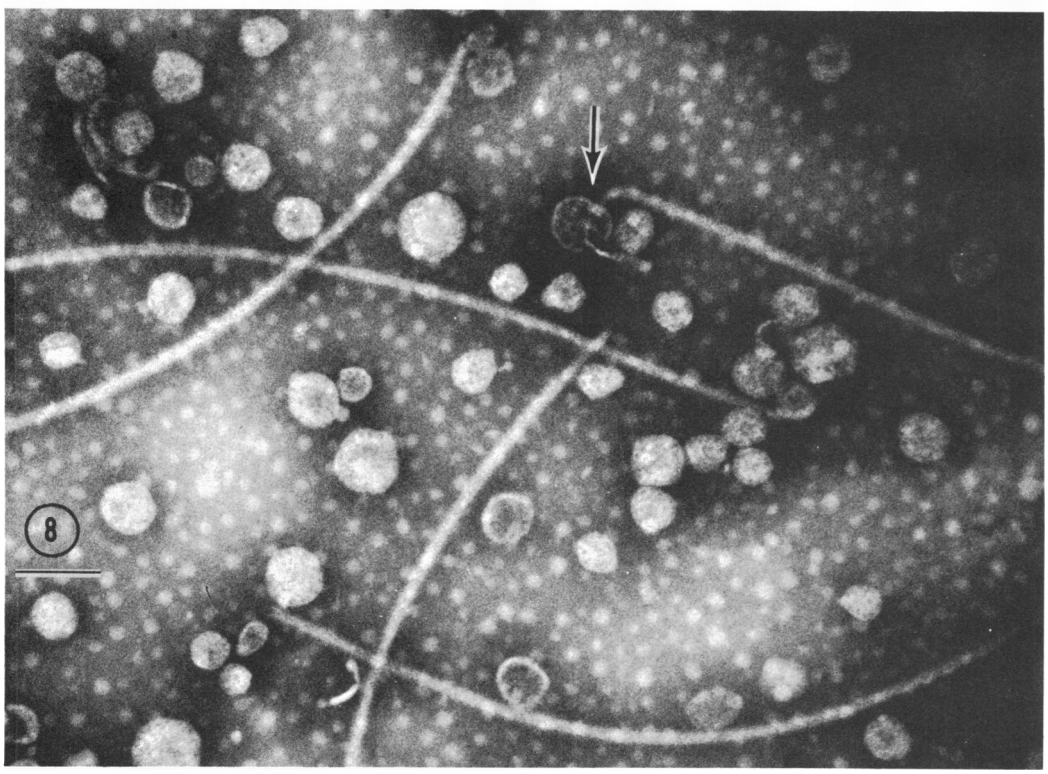


FIG. 7. *EDTA-dissociated native L membrane reassembled by dialysis against Tris-Mg^{2+} buffer. Arrow shows flagellar basal body. Phosphotungstic acid.* ×*74,200.*

FIG. 8. *Same as Figure 7 except that dialysis buffer also contained 2.0* M *KCl. Arrow shows flagellar basal body. Phosphotungstic acid.* ×*111,000.*

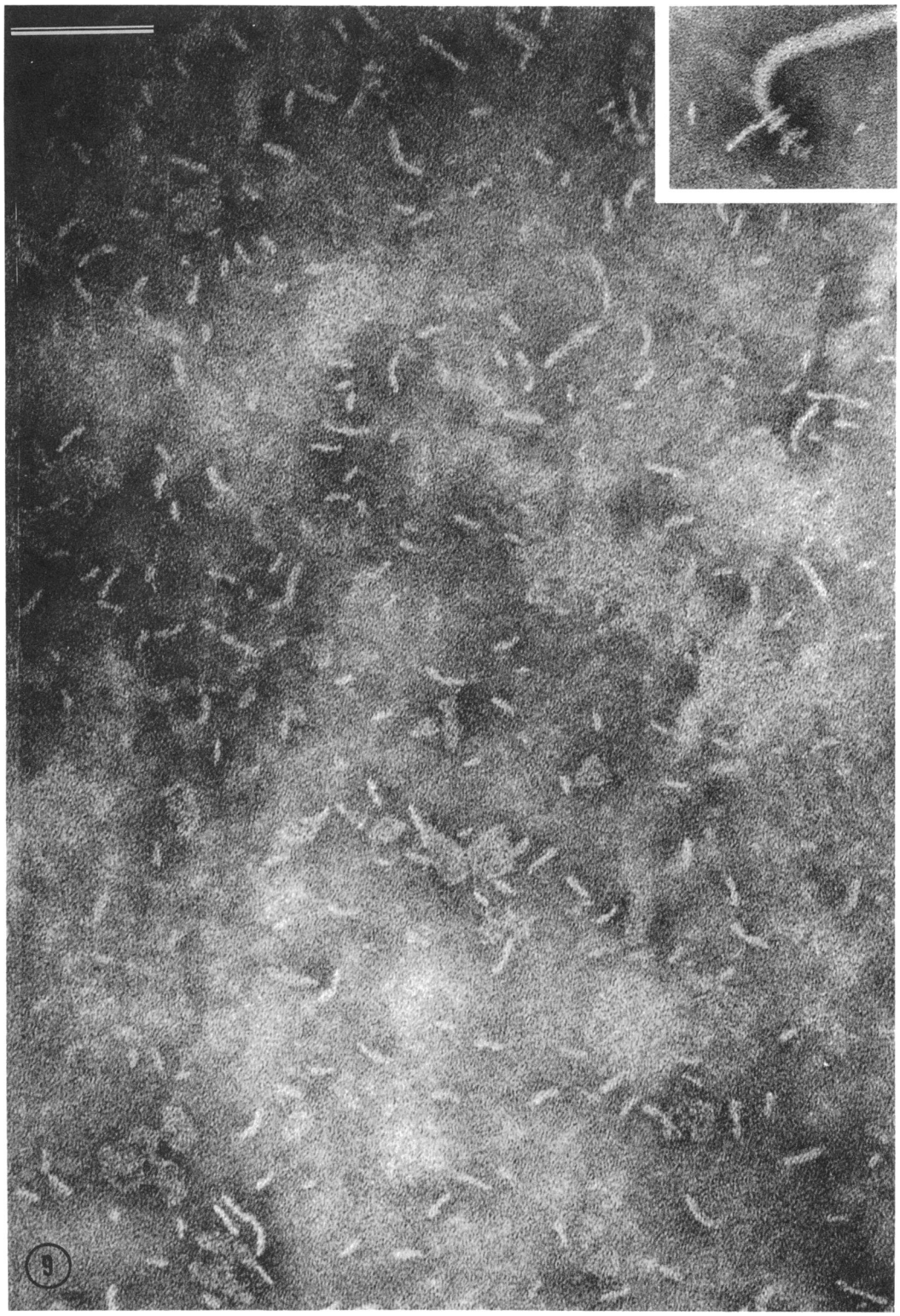
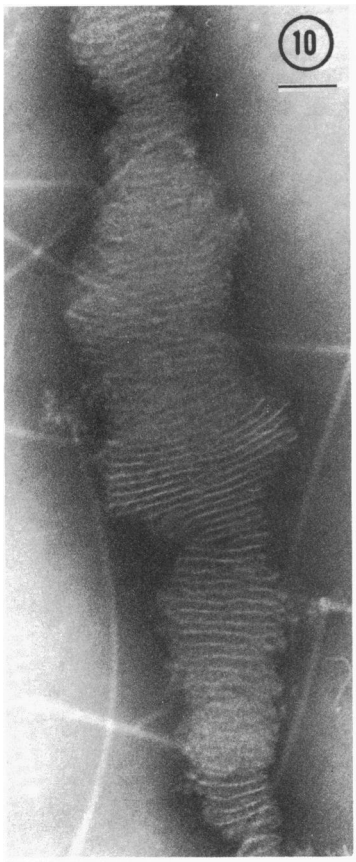
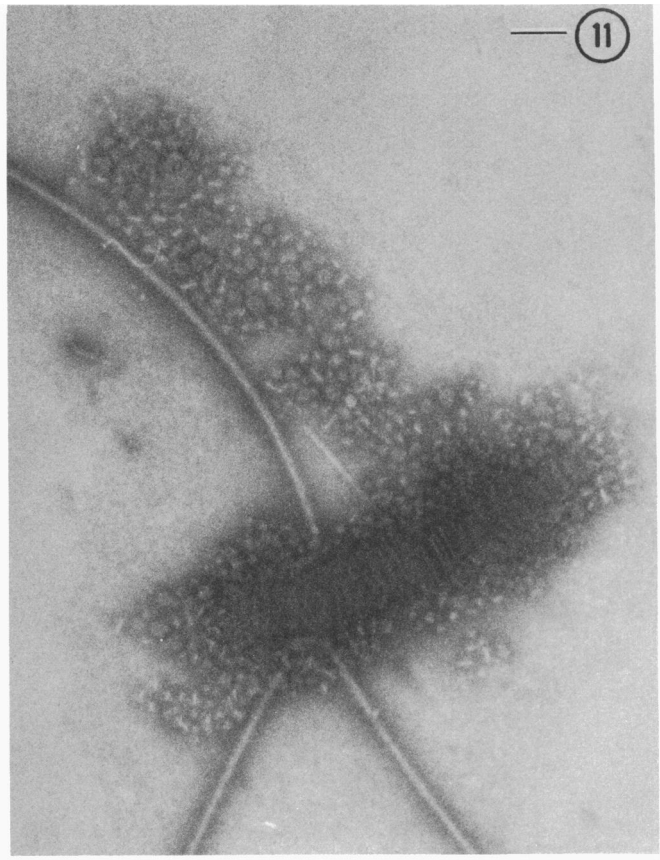


FIG. 9. *Native L membrane after solubilization in EDTA and Triton X-100. Only rod-shaped substructures (substructures B) are present. The insert shows some substructures have associated with the L ring of a flagellar basal body. Phosphotungstic acid.* ×222,000.





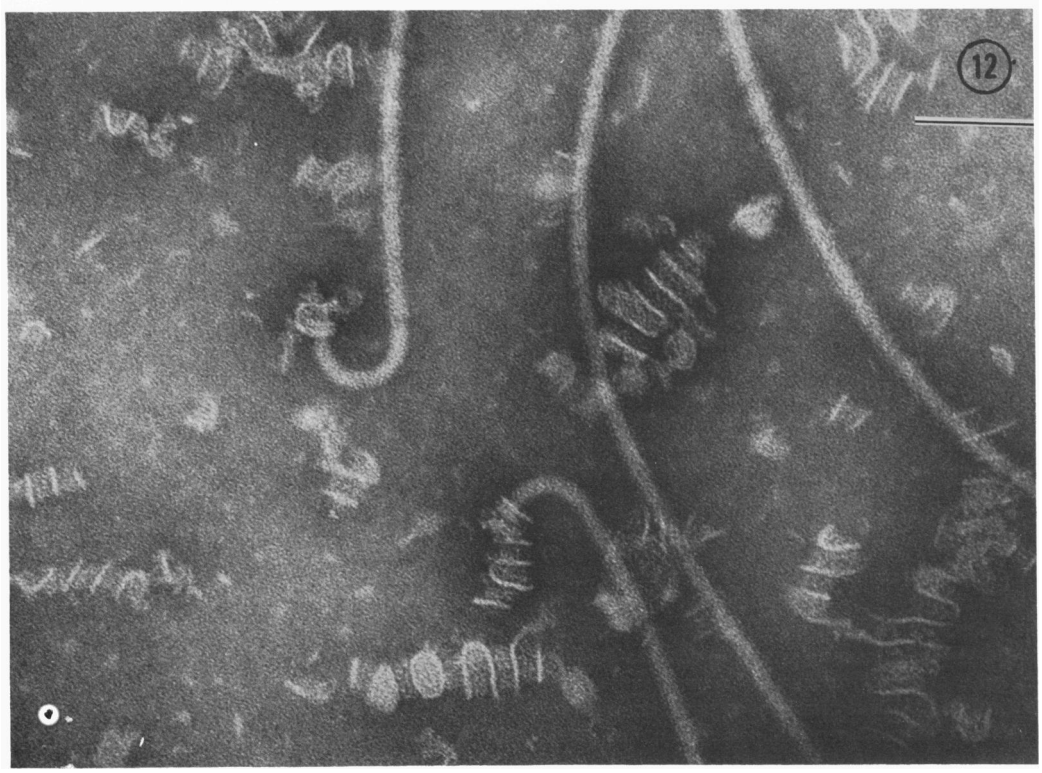


FIG. 10. *Laminated structure found after $MgCl_2$ was added to EDTA-Triton X-100-solubilized native L membrane. Phosphotungstic acid.* ×*74,200.*

FIG. 11. *Same as Fig. 10 except L membrane substructures appear associated with the laminated structure. Phosphotungstic acid.* ×*74,200.*

FIG. 12. *EDTA-Triton X-100-solubilized native L membrane after dialysis against Tris-Mg^{2+} buffer containing 2.0 M KCl. Phosphotungstic acid.* ×*163,000.*

$CaCl_2$ was only about 60% as effective as $MgCl_2$ in causing reassembly of L membrane under the conditions described above.

Dissociation and reassembly of reconstituted L membrane. When the above experiments were performed on purified reconstituted L membrane, the results were essentially the same. However, dialysis of the purified reconstituted L membrane against Tris-EDTA buffer degraded 60% of the reconstituted L membrane (Fig. 13), twice as much dissociation as occurred with native L membrane. A predominance of discs is visible, 14 to 60 nm in diameter. Therefore, based on electron microscopic observations and decreases in OD_{400}, reconstituted L membrane was more sensitive than native L membrane to the Tris-EDTA treatment, and, in this respect, is similar to purified LPS.

Preparation of reconstituted L membrane as previously described is an additional demonstration that L membrane solubilized in EDTA and Triton X-100 can reassemble. Before the addition of Mg^{2+}, no vesicles were found in the Triton X-100 lysate.

Demonstration of L membrane substructures in Triton X-100 lysates and the requirement of Mg^{2+} for reassembly. To demonstrate further the essential role of Mg^{2+} in the structure of L membrane and to show the presence of L membrane substructures in Triton X-100 spheroplast lysates containing no Mg^{2+}, samples of lysates with or without $MgCl_2$ were directly added to CsCl gradients as described next. *E. coli* spheroplasts were prepared as described previously (4) and divided into two 2-ml portions containing 3×10^9 cells/ml. One portion was lysed in the presence of 0.5% Triton X-100 without $MgCl_2$ and diluted to 5.0 ml with 0.1 M Tris at *p*H 7.8; 2.18 g of CsCl was rapidly dissolved in the solution. This is analogous to the preparation of reconstituted L membrane described above. The other portion was first incubated in 20 mM $MgCl_2$ for 10 min at 26 C and then lysed with Triton X-100. This is analogous to the preparation of native L membrane. After dilution into Tris-Mg^{2+} buffer, CsCl was rapidly dissolved in the solution. In both cases the concentration of CsCl was 2.4 M. The two portions were centrifuged in a Spinco SW-39 rotor for 50 hr at 30,000 rev/min. A third centrifuge tube, containing only 0.1 M Tris (*p*H 7.8), 0.1% Triton X-100, and CsCl, served to locate the position of the detergent at the end of centrifugation. The gradients were collected from the bottom in 10 fractions, dialyzed overnight against the buffer present, and examined in an electron microscope.

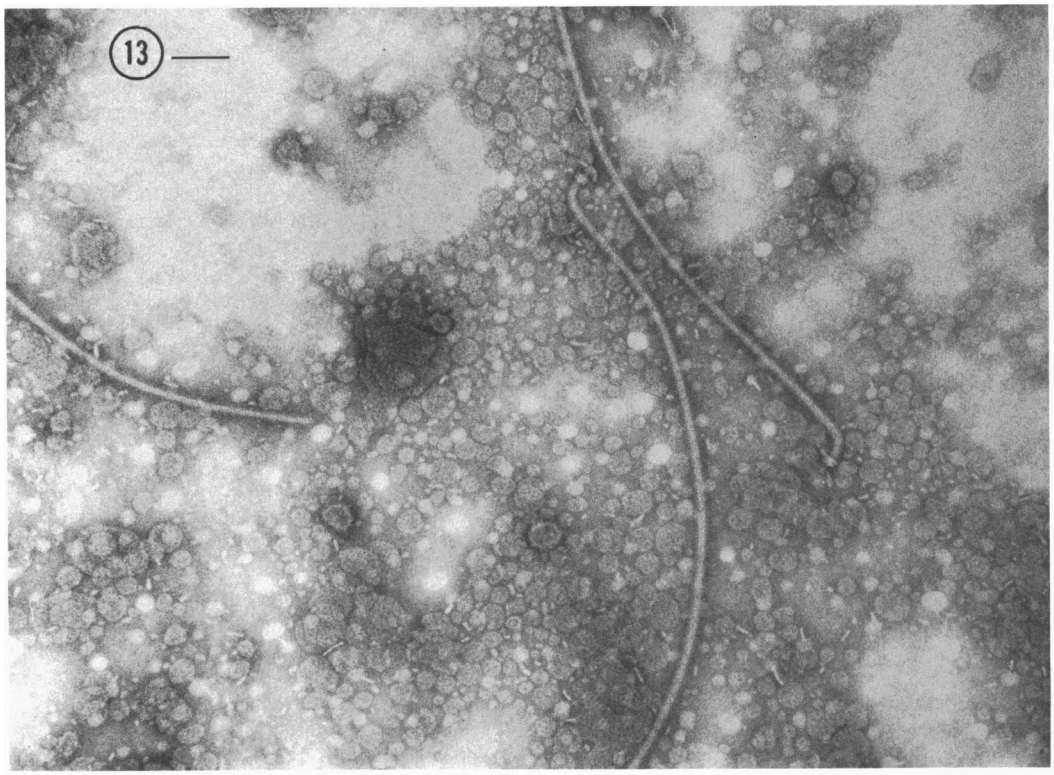
The results were that fractions 2 and 3 (density: 1.36 to 1.34 g/ml) from the Mg^{2+}-treated portion contained vesicles (Fig. 14), whereas the same fractions from the portion prepared without Mg^{2+} contained disc-shaped substructures 20 to 60 nm in diameter and stacks of rod-shaped substructures (or discs on edge) with a thickness of 5 to 7 nm and a length of 20 to 60 nm (Fig. 15). Flagella were in fraction 6. Triton X-100 was found in fractions 9 and 10 as assayed by the OD_{280}. The isolated substructures formed densely packed aggregates of vesicles within 1 hr after the solution was adjusted to 20 mM $MgCl_2$.

Reassembly of dissociated LPS and L membrane onto flagellar basal bodies. Purified intact flagella (2) were added to EDTA-dissociated LPS, and the material was then dialyzed against Tris-Mg^{2+} buffer for 12 hr at 25 C. The reassembled LPS vesicles were attached to flagellar basal bodies predominantly at the L ring with a small LPS fragment attached to the M ring (Fig. 16). Occasionally, vesicles were seen attached to the M ring (Fig. 17). Unlike native L membrane vesicle-basal body complexes (4), where the basal bodies were always found pointing to the inside of the vesicle, reassembled complexes showed both possible orientations (Fig. 16, 17, 18).

Reassembly of native or reconstituted L membrane vesicles after dissociation in EDTA (Fig. 7, 8, 19) or solubilization in EDTA and Triton X-100 (Fig. 20, 21) gave the same result. In these preparations intact flagella were already present. Again the predominant point of attachment was the L ring, but sometimes vesicles attached to the M ring. No attachment of L membrane or LPS to P or S rings of basal bodies was ever found.

Figure 9 shows that substructures B still occasionally associate with L rings despite the presence of EDTA and Triton X-100.

Initiation of L membrane assembly by flagellar basal bodies. A surprising discovery was the occurrence of L membrane vesicles attached to the L ring of basal bodies in two preparations (2) of "purified" intact flagella (Fig. 22) in which complete separation of solubilized L membrane substructures from flagella had evidently not occurred. These vesicles had reconstituted despite the presence of 5×10^{-4} M EDTA throughout the purification. The conditions responsible for vesicle formation were the concentration of flagella in the presence of L membrane substructures with the concomitant extraction of Triton X-100 as a result of the CsCl gradient (Triton concentrates at the meniscus). Vesicles were not present before the CsCl step, and such a large number (up to 16) of closely grouped basal bodies found in these vesicles was never seen in studies of cell envelopes (4) or native L membrane (4) from peritrichously flagellated *E. coli*. Furthermore, all of the vesicles had basal bodies attached. Dialysis against



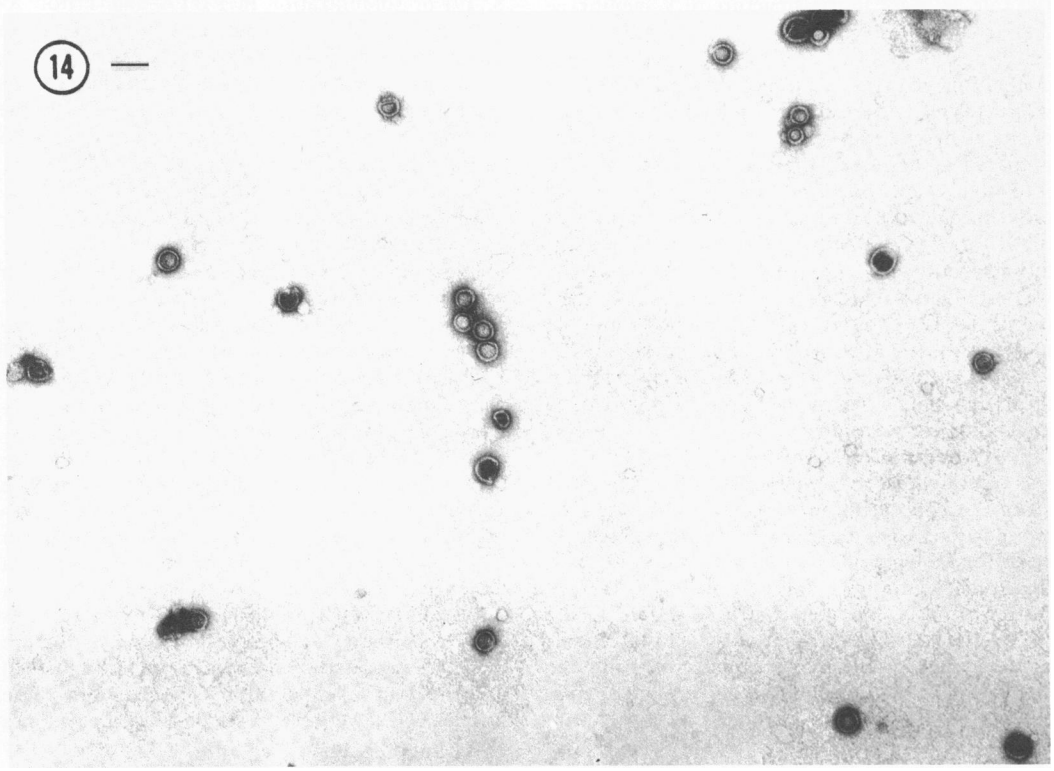


FIG. 13. *Reconstituted L membrane after dissociation in EDTA. Phosphotungstic acid.* ×*74,200.*

FIG. 14. *L membrane vesicles from the* Mg^{2+}*-treated lysate described under Results: Uranyl acetate.* ×*50,000.*

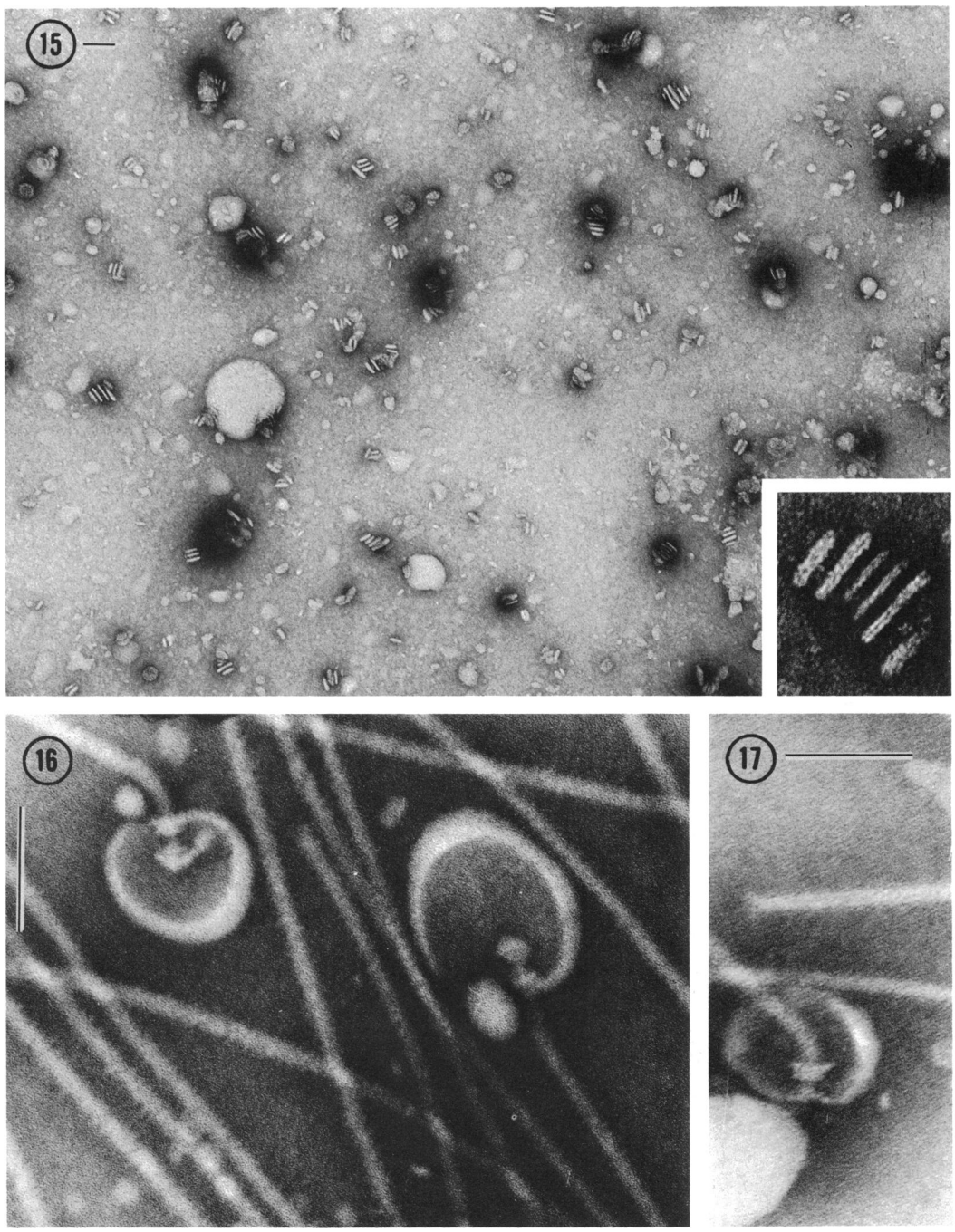
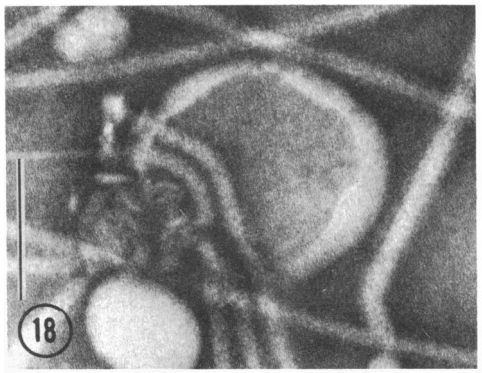
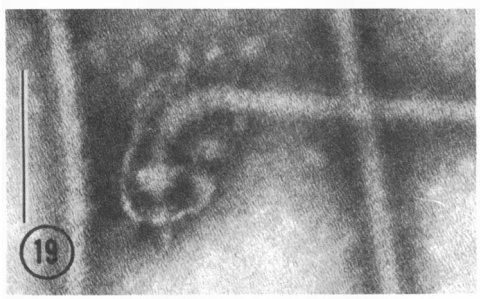


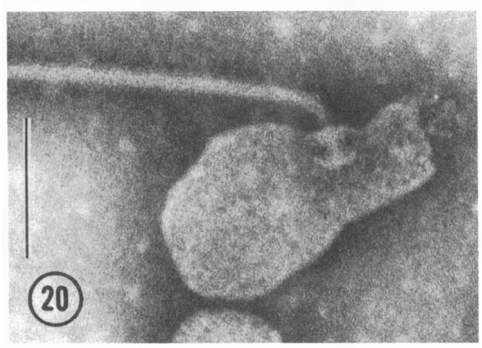
FIG. 15. *L membrane substructures from the lysate prepared without Mg^{2+}. Uranyl acetate. ×50,000. Insert, ×248,000. Note that the stacks of rods (or discs standing on their edge) have a trilaminar appearance.*

FIG. 16. *Flagellar basal bodies attached to lipopolysaccharide vesicles after reassembly of EDTA-dissociated lipopolysaccharide in the presence of intact flagella. Phosphotungstic acid. ×185,000.*

FIG. 17. *Same as Figure 16 except vesicle is attached to M ring of basal body. Phosphotungstic acid. ×185,000.*







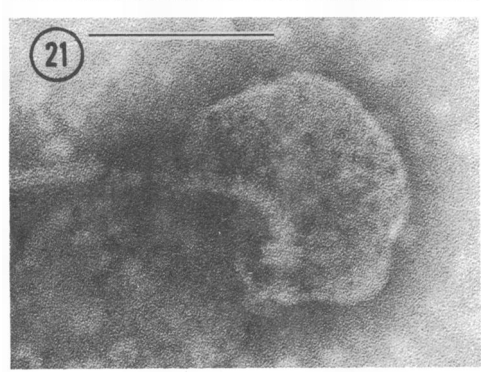


FIG. 18. *Same as Fig. 16 except that basal bodies are "outside" the vesicle. Phosphotungstic acid.* ×*185,000.*

FIG. 19. *L membrane attached to both L and M rings of a flagellar basal body after reassembly from EDTA-dissociated native L membrane. Phosphotungstic acid.* ×*200,000.*

0.01 M EDTA in 0.1 M Tris at *p*H 7.8 showed no effect on the structure of these complexes, but the vesicles were degraded when Triton X-100 was added to a final concentration of 0.2%. However, although the basal bodies were released, some vesicle fragments remained bound.

In the previous experiment (demonstration of L membrane substructures in Triton X-100 lysates) substructures did not vesiculate when Triton X-100 was removed by a CsCl gradient (Fig. 15) in the absence of Mg^{2+} and flagella.

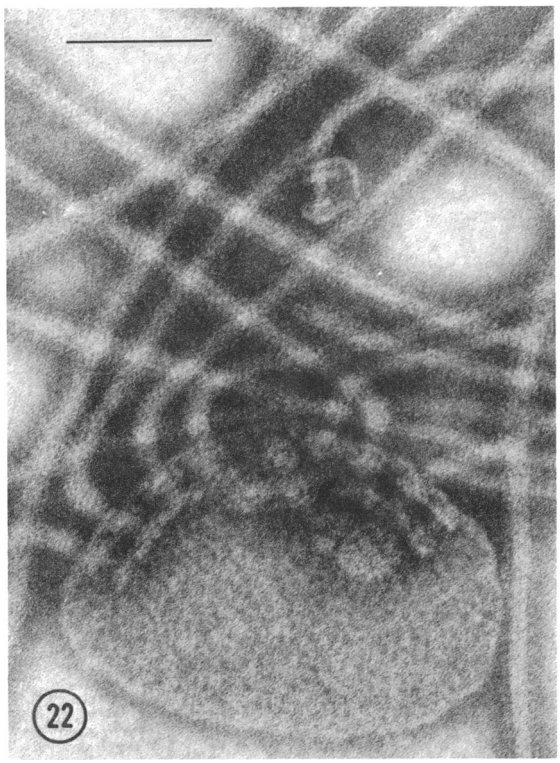
The initiation of L membrane assembly by flagellar basal bodies in the absence of Mg^{2+} is analogous to the previously reported phenomenon (2, 3) of spontaneous aggregation of basal bodies of purified intact flagella. This aggregation also occurs in EDTA and is prevented only by the presence of Triton X-100. Examination of such basal body aggregates showed that the binding was clearly between the rings (Fig. 23, 24; and Fig. 18, 19 in reference 3). Commonly, basal bodies lay in the same direction with all of their rings in register (Fig. 23), or they were rotated 180° so that only the L ring of each basal body was collinear with the M ring of the adjacent basal body (Fig. 23, 24). In the latter case, the P and S rings were then no longer in position to associate directly with the rings on an adjacent basal body. Aggregates of basal bodies were occasionally found where the association clearly involved only the L ring of one basal body and the M ring of another, or only the L rings. No examples were found which demonstrated interactions between only the following: P and P, S and S, or P and S. Hooks without basal bodies showed no tendency to aggregate.

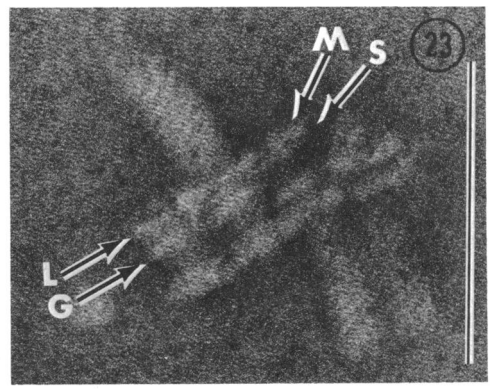
These data suggest that only the L and M rings are responsible for the aggregation of basal bodies. In studies on flagellar basal bodies from *Bacillus subtilis* (3), it was noted that basal bodies aggregated very little compared to *E. coli*. Since *B. subtilis* basal bodies do not have the top rings (L and P) found on *E. coli* basal bodies, it appears that the L ring has a stronger tendency to aggregate than the M ring. This is analogous to the results described above on reassembly of LPS or L membrane onto basal bodies where the L ring is the primary attachment site.

Thus, L rings spontaneously bind to one another unless Triton X-100 is present. This bonding does not require M^{2+}. If L membrane

FIG. 20. *L membrane vesicle attached to the L ring of a flagellar basal body after reassembly from EDTA-Triton X-100 solubilized native L membrane. Phosphotungstic acid.* ×*185,000.*

FIG. 21. *L membrane vesicle attached to the M ring of a flagellar basal body after reassembly from EDTA-Triton X-100 solubilized native L membrane. Phosphotungstic acid.* ×*245,000.*





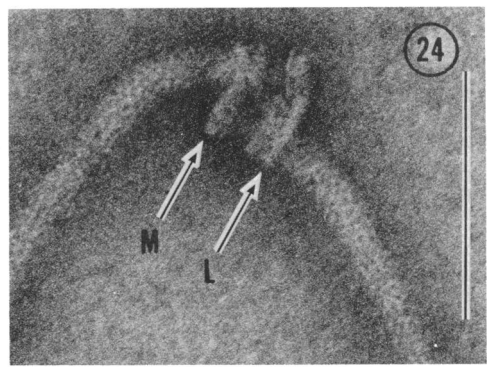


FIG. 22. *Reassembled L membrane vesicle specifically attached to the L ring of several flagellar basal bodies. Phosphotungstic acid.* ×*190,000.*

FIG. 23. *E. coli flagellar basal ends isolated from intact flagella by treatment with 4.5* M *urea (3). Note that the basal bodies are oriented so that their L and M rings are in register with either themselves or each other. Uranyl acetate.* ×*400,000.*

FIG. 24. *Intact flagella from E. coli (2, 3) with their basal bodies oriented so that their L and M rings are in register with each other. Uranyl acetate.* ×*330,000.*

substructures are also present, the L rings spontaneously bind to these substructures as well and in some way cause them to assemble into vesicles in the apparent absence of Mg^{2+}.

DISCUSSION

L membrane preparations contained primarily LPS and protein. Some phospholipid has also been reported (10, 12). Many of the structural properties described for L membrane are evidently the properties of LPS. LPS examined after positive staining (23) or thin sectioning (5, 9) shows a trilaminar structure typical of L membrane. Like L membrane, LPS purified from *E. coli* as described here is vesicular in the presence of Mg^{2+}. A similar result was reported by others for *E. coli* (10, 27) and for a galactose-deficient LPS from *Salmonella* (22) prepared by Mg^{2+} precipitation. However, other investigators generally reported the configuration of purified LPS as long slender ribbons (14, 21, 23) rather than vesicles. Since LPS in those reports was purified in the absence of Mg^{2+}, configurational differences are probably due to the amount of Mg^{2+} or other divalent cations bound to the LPS in various preparations. Data of Taylor et al. (24) show that LPS prepared by Mg^{2+} precipitation assumes a ribbon configuration during the process of dissolving in EDTA. As in the case of L membrane, removal of Mg^{2+} reversibly dissociates LPS vesicles into disc- and rod-shaped substructures (Fig. 3), and the substructures reassemble to give vesicles when Mg^{2+} is added back. When reassembly was done in the presence of purified intact flagella, the LPS vesicles were attached to flagellar basal bodies in the same manner that was found with reassembled L membrane. Therefore, LPS is evidently the primary determinant of L membrane structure.

The additional components of L membrane, besides LPS, apparently add stability to the structure. Removal of Mg^{2+} from native L membrane "unlocks" the substructures, but these vesicles, unlike purified LPS, do not completely dis-

sociate. Reconstituted L membrane, containing approximately 25% less protein than native L membrane, was dissociated twice as much as native L membrane by removal of Mg^{2+}. The possibility also exists that reconstituted L membrane derived from EDTA-Triton X-100 lysates of spheroplasts may contain components of both the cytoplasmic and L membrane.

L membrane vesicles are apparently assembled from substructures bound together by both Mg^{2+}-mediated and hydrophobic bonds. The evidence for L membrane substructures is that discs or rods, or both, were found under the following conditions: (i) L membrane was dissociated by EDTA treatment (Fig. 5, 13) or solubilized by further treatment with Triton X-100 (Fig. 9). EDTA-dissociated LPS also had the same appearance (Fig. 3). (ii) Laminated structures were formed (Fig. 11) on addition of Mg^{2+} to EDTA-Triton X-100-solubilized L membrane. (iii) Reassembly of L membrane vesicles from EDTA-Triton X-100-solubilized material was inhibited by 2 M KCl (Fig. 12). (iv) L membrane material was isolated in the absence of Mg^{2+} (Fig. 15).

Such structures were also seen by others after dissociation of purified LPS by detergents (14, 21) or ether (23).

Further studies are required to determine how many different substructures exist. The rod-shaped substructures found in EDTA-Triton X-100-solubilized L membrane (Fig. 9) may represent a basic subunit, from which larger rods and discs may be formed. However, it is likely that in some cases (Fig. 15, for example) the rods are discs standing on edge as suggested by Shands et al. (23) in his study of LPS.

The primary role of Mg^{2+} (and possibly other divalent cations) in maintaining L membrane structure is clearly evident from the following data.

(i) Purified L membrane is partially dissociated into substructures by dialysis against EDTA. Dialysis of the dissociated material against Mg^{2+} reassembles the vesicles. The same is true for purified LPS vesicles except that dissociation by EDTA is complete.

(ii) Purified L membrane is resistant to solubilization by the nonionic detergent Triton X-100 in the presence of Mg^{2+}, but is completely solubilized to substructures by Triton X-100 after Mg^{2+} has been chelated by EDTA.

(iii) Addition of Mg^{2+} to completely solubilized L membrane in the presence of Triton X-100 causes reaggregation of L membrane substructures into laminated forms. Transformation of the laminated forms into vesicles requires the removal of the detergent.

(iv) L membrane vesicles were isolated from lysozyme-EDTA spheroplasts by incubating them in Mg^{2+} before lysing with Triton X-100 and then directly applying the lysate to a Mg^{2+}-containing CsCl gradient which removed the detergent. The same experiment without Mg^{2+} yielded only substructures of the same buoyant density as L membrane vesicles.

(v) No vesicles were ordinarily found in preparations of intact flagella (2, 3) in which lysozyme-EDTA spheroplasts were lysed with Triton X-100 and the purification carried out in the presence of EDTA and Triton X-100. The same procedure, but with Mg^{2+} present after Triton X-100 lysis, resulted in purifying reconstituted L membrane. The same procedure, but incubating the spheroplasts in Mg^{2+} before Triton X-100 lysis and then keeping Mg^{2+} present, resulted in purifying native L membrane (4).

The presence of hydrophobic bonding in L membrane structure is suggested by the following data, but experiments to determine the effect of temperature and nonpolar solvents must be done to definitely demonstrate such bonding.

(i) Mg^{2+}-depleted purified L membrane vesicles are completely solubilized by Triton X-100.

(ii) Triton X-100 prevents reassembly of vesicles on addition of Mg^{2+} although aggregation of substructures into laminated forms does occur (Fig. 10). Vesicles reform only on removal of the detergent.

(iii) In the absence of Mg^{2+} and Triton X-100, L rings of flagellar basal bodies can initiate assembly of L membrane vesicles (Fig. 21). EDTA neither inhibits assembly nor dissociates the reconstituted vesicle-basal body complexes, but Triton X-100 does.

Experiments with purified LPS in a ribbon configuration showing that LPS can be reversibly solubilized in sodium dodecyl sulfate (14) or deoxycholate (21) or dispersed in ether (23) suggest that LPS is also made by substructures that can aggregate by using hydrophobic bonds.

The specificity of L membrane attachment to the flagellar basal body L ring was first shown by DePamphilis and Adler (4) in preparations of purified native L membrane and in preparations of osmotically lysed spheroplasts. The fact that dissociated purified native L membrane or LPS can reassemble onto the basal body L ring further demonstrates that one function of the L ring is to specifically and spontaneously attach to L membrane. The chemistry of the M ring, which in vivo is attached to the cytoplasmic membrane (4), is probably similar to that of the L ring since reassembled native L membrane or LPS was sometimes found attached to M rings.

The properties of L membrane and purified LPS described above suggest that in vivo syn-

thesis of L membrane could occur via synthesis of substructures that spontaneously assemble to form the outer membrane including correct attachment to flagellar basal bodies.

L membrane dissociation and reassembly is similar to that of cytoplasmic membranes from *Mycoplasma* (8, 20) which can be dissociated in sodium dodecyl sulfate but require removal of detergent in the presence of Mg^{2+} for reassembly of lipid and protein aggregates into membrane vesicles. Therefore, the study of L membrane structure may provide insight to the general structure of membranes.

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