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Reassociation of Purified Lipopolysaccharide and Phospholipid of the Bacterial Cell Envelope: Electron Microscopic and Monolayer Studies

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Phosphatidyl ethanolamine and lipopolysaccharide were extracted and purified from the cell envelope fractions of *Escherichia coli* and *Salmonella typhimurium*. The two components were studied separately and after recombination, by use of electron microscopy and monolayer techniques, and by measuring their ability to participate in the enzyme-catalyzed uridine diphosphate-galactose:lipopolysaccharide α,3 galactosyl transferase reaction, which requires a lipopolysaccharide-phospholipid complex as substrate. Electron microscopy of purified lipopolysaccharide showed a uniform population of hollow spheres, with each sphere bounded by a continuous leaflet. The diameter of the spheres was approximately 500 to 1,000 A, and the thickness of the enveloping leaflet was approximately 30 A. Phosphatidyl ethanolamine showed a regular lamellar structure. When lipopolysaccharide and phosphatidyl ethanolamine were mixed under conditions of heating and slow-cooling, the leaflet of the lipopolysaccharide spheroids appeared to extend directly into the phosphatidyl ethanolamine structure, with continuity between the two leaflets. Various stages of penetration were seen. At high concentrations of lipopolysaccharide, there were disruptive changes in phosphatidyl ethanolamine leaflets similar to those seen when saponin acts on cholesterol-lecithin leaflets. Monolayer experiments indicated that lipopolysaccharide penetrated a monomolecular film of phosphatidyl ethanolamine at an air-water interface, as revealed by an increase in surface pressure. The results indicate that a common leaflet structure containing lipopolysaccharide and phosphatidyl ethanolamine may be formed in vitro, and suggest that a similar leaflet may exist in the intact bacterial cell envelope.

The cell envelopes of mammalian and bacterial cells contain significant amounts of phospholipids and carbohydrate-containing lipids (glycolipids or lipopolysaccharides), but the relative locations of these lipids within the cell envelope and their possible structural or functional relationships are largely unknown. Gram-negative enteric bacteria contain unusually large amounts of phospholipid and lipopolysaccharide (19, 26, 30), together constituting 20 to 40% of the dry weight of the cell envelope fraction. Evidence for a close association of phospholipid and lipopolysaccharide in the cell envelope has recently arisen from studies of lipopolysaccharide biosynthesis in *Salmonella typhimurium*. There is a requirement for the phospholipid component of the cell envelope in at least two of the enzyme-catalyzed reactions leading to biosynthesis of the polysaccharide portion of the lipopolysaccharide (21):

glucose-deficient lipopolysaccharide

+ uridine diphosphate (UDP)-glucose

$$\xrightarrow[\text{phospholipid}]{\text{enzyme I,}} \text{glucosyl-lipopolysaccharide}$$

galactose-deficient lipopolysaccharide

+ UDP-galactose $\xrightarrow[\text{phospholipid}]{\text{enzyme II,}}$

galactosyl-lipopolysaccharide

When phospholipid is removed from the cell envelope fraction by extraction with lipid sol-

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vents, the lipopolysaccharide remains within the residual cell envelope but no longer acts as acceptor for sugar transfer, and acceptor activity can be stored by adding back the extracted phospholipid. The following sequence of reactions has previously been demonstrated by use of purified lipopolysaccharide and phospholipid (24):

(1) lipopolysaccharide + phospholipid → lipopolysaccharide·phospholipid
(2) lipopolysaccharide·phospholipid + soluble enzyme → enzyme·lipopolysaccharide·phospholipid
(3) enzyme·lipopolysaccharide·phospholipid + nucleotide sugar → glycosyl-lipopolysaccharide·phospholipid (+ enzyme).

The active lipopolysaccharide-phospholipid complex (step 1) can be formed in vitro by heating and slowly cooling a mixture of purified lipopolysaccharide and phospholipid. The existence of a similar lipopolysaccharide-phospholipid complex in the native cell envelope is suggested by the similar activity of the intact cell envelope and the complex formed in vitro as acceptors for sugar transfer in the enzymatic reactions. The reaction kinetics are almost identical whether intact cell envelope preparations or comparable concentrations of purified lipopolysaccharide and phospholipid are used, and the ratio of lipopolysaccharide to phospholipid in the intact cell envelope is similar to that required for enzyme activity when purified lipopolysaccharide and phospholipid are studied in the in vitro system (21).

Phosphatidyl ethanolamine was previously shown to be the active phospholipid of the cell envelope, and alterations of the fatty acid and polar portions of the phospholipid molecule were shown markedly to affect the activity of lipopolysaccharide-phospholipid mixtures in the transferase enzyme reactions (23).

The nature of the lipopolysaccharide-phospholipid interaction is unknown. The lipopolysaccharide is amphipathic, with a nonpolar lipid portion and a polar polysaccharide region, and should therefore form micellar or liquid crystalline structures in aqueous media (1, 7). The phospholipids of the cell envelope are also thought to exist in ordered liquid crystalline structures, predominantly as bimolecular leaflets. It is possible, therefore, that lipopolysaccharide and phospholipid molecules normally occur side-by-side in a common leaflet structure within the native cell envelope, and that the heating-cooling procedure promotes the formation of such a common leaflet or mixed micellar structure when the purified components are studied in vitro.

The studies of Fernandez-Moran (9), Bangham and Horne (2), and Lucy and Glauert (15) have shown that the leaflet structure of purified phospholipids can be observed by electron microscopy with the use of negative-staining techniques. We have employed similar techniques to study the interaction between purified lipopolysaccharide and phosphatidyl ethanolamine obtained from the bacterial cell envelope (25). The present studies were performed with the lipopolysaccharide-phospholipid complex which was active as an acceptor in the transferase enzyme system. The electron microscopic studies described below suggest that lipopolysaccharide is incorporated directly into the phospholipid leaflet structure. This conclusion is also supported by the results of parallel experiments in which monolayer techniques were used.

MATERIALS AND METHODS

Preparation of materials. Phosphatidyl ethanolamine was extracted from the cell envelope fraction of *Escherichia coli* strain AB 112 and was purified as previously described (23). Phosphatidyl cholines from beef brain and from egg were gifts from D. Papaphadjopolous and R. A. M. C. Dawson of the Agricultural Research Council Institute of Animal Physiology. All lipid preparations were dispersed in water by the method of Fleischer and Klouwen (11), and may therefore have contained trace amounts of sodium deoxycholate. Unless otherwise specified, lipopolysaccharide was derived from *S. typhimurium* G-30, a mutant lacking UDP-galactose-4-epimerase, and was purified from the cell envelope fraction by phenol extraction and Mg^{++} precipitation as previously described (18). This incomplete lipopolysaccharide acts as acceptor for galactose transfer in the UDP-galactose:lipopolysaccharide α,3 galactosyl transferase reaction (22). The lipopolysaccharides from *S. typhimurium* G-30 and *S. typhimurium* TV 119 (a rough B strain) were gifts from M. J. Osborn, and lipopolysaccharides from *S. minnesota* were gifts from Otto Lüderitz.

The purified lipopolysaccharide preparations were dialyzed against Tris-EDTA buffer [20 mM tris-(hydroxymethyl)aminomethane chloride buffer (*p*H 8.5), containing 1 mM ethylenediaminetetraacetate] for 10 days at 4 C prior to use. The lipid and polysaccharide components were prepared by heating lipopolysaccharide in 0.01 N HCl at 100 C for 45 min. The insoluble residue, "lipid A" (5), was collected by centrifugation and dialyzed for 10 days against Tris-EDTA buffer (see above). The supernatant fraction ("soluble polysaccharide") was taken to dryness several times from water and was then dissolved in Tris-EDTA buffer.

Experimental procedures. Assays of UDP-galactose:lipopolysaccharide α,3 galactosyl transferase activity were performed as previously described, in a final reaction volume of 0.25 ml (23). Lipopolysaccharide (0.4 mg, containing 0.18 μmole of heptose) and phosphatidyl ethanolamine (0.9 μmole) were

heated to 60 C for 30 min and slowly cooled to 25 C prior to addition of enzyme (0.2 to 0.4 mg of protein) and ^{14}C-labeled UDP-galactose (1.7×10^6 counts per min per μmole). The enzyme preparation was the 105,000 $\times$ *g* supernatant fraction prepared from sonic extracts of *S. typhimurium* EI-1 as previously described (22). Analytical ultracentrifugation was performed in a Spinco model E ultracentrifuge in Tris-EDTA buffer at 39,460 rev/min and 20 C. Sedimentation velocities were calculated (27) and were extrapolated to zero concentration. Other analytical procedures were performed as previously described (18, 23).

Thin-layer chromatography was performed on Silica Gel G (Brinkman Co., Westbury, N.Y.), with a solvent system of chloroform-methanol-water (65:25:4, v/v). The spots were visualized with iodine vapor.

In the monolayer experiments, phospholipids were dissolved in petroleum ether and were applied to the surface (20.5 by 4.5 cm) of a solution of 20 mM Tris buffer (*p*H 8.5)-0.5 mM EDTA (total volume, 70 ml) to reach the indicated initial surface pressures. Lipopolysaccharide from *S. typhimurium* G-30 (dissolved in the same Tris-EDTA buffer) was added to the bulk aqueous phase at zero time without change in total volume, the aqueous phase was stirred by a magnetic stirring device or by hand with a syringe, and the surface pressures were recorded continuously at constant area with a Wilhelmy dipping plate and force balance.

Electron microscopy. The materials to be examined were mixed in a total volume of 0.25 ml, containing 5 mM Tris acetate buffer (*p*H 8.5) and 0.5 mM EDTA. When mixtures of lipopolysaccharide and phospholipid were examined, the phospholipid concentration was 1.0 mg/ml, and the concentration of lipopolysaccharide varied from 6×10^{-4} to 3 mg/ml. Unless otherwise stated, mixtures containing lipopolysaccharide or lipid, or both, were heated to 60 C for 30 min and gradually cooled to 20 C over a 2-hr period prior to addition of negative stain.

The preparative methods for mounting specimens for electron microscopy were basically similar to those described by Bangham and Horne (2). Suspensions containing phospholipid, lipopolysaccharide, or mixtures of both were mixed with an equal volume of negative stain and deposited directly on either carbon-coated nitrocellulose or carbon films. Droplets from small Pasteur pipettes or sprayed material were placed on the prepared grids and allowed to dry at room temperature. Spraying methods were found to be satisfactory for depositing lipopolysaccharide when sprayed in the presence of negative stains.

For the reasons discussed by Horne (13), a variety of negative stains were used. It was found that 2% aqueous solutions of potassium phosphotungstate, ammonium molybdate, and lithium tungstate, when mixed in equal volumes with solutions of lipid or lipopolysaccharide, gave the best results in terms of contrast and preservation of structure (see Results).

Specimens were placed directly into electron microscope specimen holders and examined in Siemens Elmiskop or A.E.I. (E.M. 6B) electron microscopes. Micrographs were recorded at instrumental magnifications of either 40,000 or 80,000 times, previously calibrated from external reference standards in the form of gratings, platinum phthalocyanine, or catalase crystals (8).

Measurements were calculated from recordings obtained from a Joyce Loebl Mk IIIc microdensitometer.

RESULTS

Composition and properties of the lipopolysaccharide. The lipopolysaccharide preparation showed a single peak when examined by analytical ultracentrifugation (Fig. 1) with a corrected sedimentation constant of approximately 60*S* ($S_{20,w}$, extrapolated to zero concentration). Detailed studies of particle weight and sedimenta-

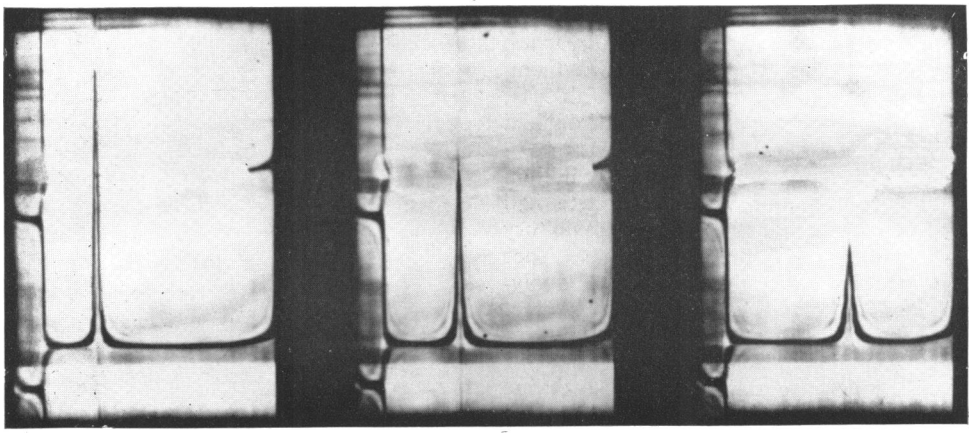


FIG. 1. *Ultracentrifugal pattern of lipopolysaccharide. Lipopolysaccharide of strain G-30 was examined as described in Experimental procedure. Photographs were taken at 8-min intervals. Centrifugation is from left to right.*

tion behavior in different solvents will be published elsewhere.

There was less than 0.7% contamination with nucleic acid, estimated by absorbance at 260 mμ. The molar ratios of heptose-glucose-ethanolamine-3-deoxyoctulosonate-phosphate in the soluble polysaccharide fraction after hydrolysis were 1.0:0.4:0.7:0.5:1.3; these values are generally similar to those previously reported (12, 17). The lipid portion of the molecule ("lipid A") comprised 45% of the total dry weight. Thin-layer chromatography revealed no additional lipid components. The lipopolysaccharide contained a small amount of material which reacted in the Lowry test for protein.

Effect of different negative stains. The following negative stains were tested: potassium phosphotungstate, lithium tungstate, and ammonium molybdate. There was a marked difference in the electron microscopic appearance of phosphatidyl ethanolamine when different stains were used. Both lithium tungstate and ammonium molybdate revealed the same organized structure of concentric lamellae previously seen in studies of phosphatidyl choline (2, 15). With potassium phosphotungstate, however, there was appreciable disruption of the basic lamellar structure; leaflets were irregularly spread apart, and there was loss of the usual fingerprint appearance of closely apposed lamellar structures. Lipopolysaccharide appeared identical when studied with all three stains.

Effect of negative stains on UDP-galactose: lipopolysaccharide α,3 galactosyl transferase reaction. Since the lipopolysaccharide-phospholipid interaction is required for activity in the galactosyl transferase reaction, interference with the normal interaction can be detected by a loss of enzyme activity. The three negative stains were therefore tested directly in the enzyme reaction mixture to determine whether the stains themselves interfere with the interaction (Table 1). Potassium phosphotungstate markedly inhibited the enzyme reaction at all concentrations tested. Moderate inhibition was seen with lithium tungstate, whereas ammonium molybdate had little effect on the reaction. These inhibitory effects cannot be ascribed solely to interference with phospholipid structure or with the lipopolysaccharide-phospholipid interaction, since direct effects on the enzyme are also likely. The enzyme studies also do not rule out the possibility of other effects of the negative stains during the drying procedure. However, because of the interference with the enzyme reaction and the abnormal appearance of phosphatidyl ethanolamine stained with potassium phosphotungstate, this stain was not used in further studies. All electron microscopic experiments were repeated with ammonium molybdate and lithium tungstate with identical results.

TABLE 1. *Effect of negative stains on UDP-galactose:lipopolysaccharide α,3 galactosyl transferase reaction*

Addition	Concn	Enzyme activity	
		A	B
	%		
Potassium phosphotungstate	0.1	1.69	1.2
	0.5	0.02	0.02
	1.0	0.01	0.01
Lithium tungstate	0.1	4.9	4.2
	0.5	1.0	2.56
	1.0	0.28	0.37
Ammonium molybdate	0.5	5.8	7.7
	1.0	6.7	7.0

[a] Enzyme assays were performed as described in *Experimental procedures*. Negative stains were added to the reaction mixture, to the indicated concentration. (A) Negative stain present during the heating and slow-cooling procedure; (B) negative stain added after the lipopolysaccharide-phospholipid mixture was heated and slow-cooled, but before addition of enzyme. Enzyme activity is expressed as millimicromoles of ^{14}C-galactose incorporated in 10 min per milligram of protein. With no addition, the enzyme activity was 9.4.

Appearance of phosphatidyl ethanolamine. Electron microscopy of negatively stained preparations of phosphatidyl ethanolamine (Fig. 2) showed large areas of closely packed leaflets arranged in a regular laminated pattern similar to the "onion-skin" or "finger-print" appearance of phosphatidyl choline. Each white leaflet represents a region not penetrated by negative stain, and is thought to represent hydrophobic regions of the organized lipid structure. The polar head groups are thought to be located largely in the dark bands between the white leaflets. The average thickness of the white leaflet was approximately 30 A. Measurements were made from microdensitometer recordings of the original negatives. Those areas free from overlapping leaflets were used for microdensitometry and showed uniform dimensions in all areas. Part of a typical microdensitometer recording is shown in Fig. 3. The average peak-to-peak distances between the leaflets was calculated to be 50 A, and those regions penetrated by the negative stain also showed a peak-to-peak distance of 50 A.

These values are similar to the dimensions of

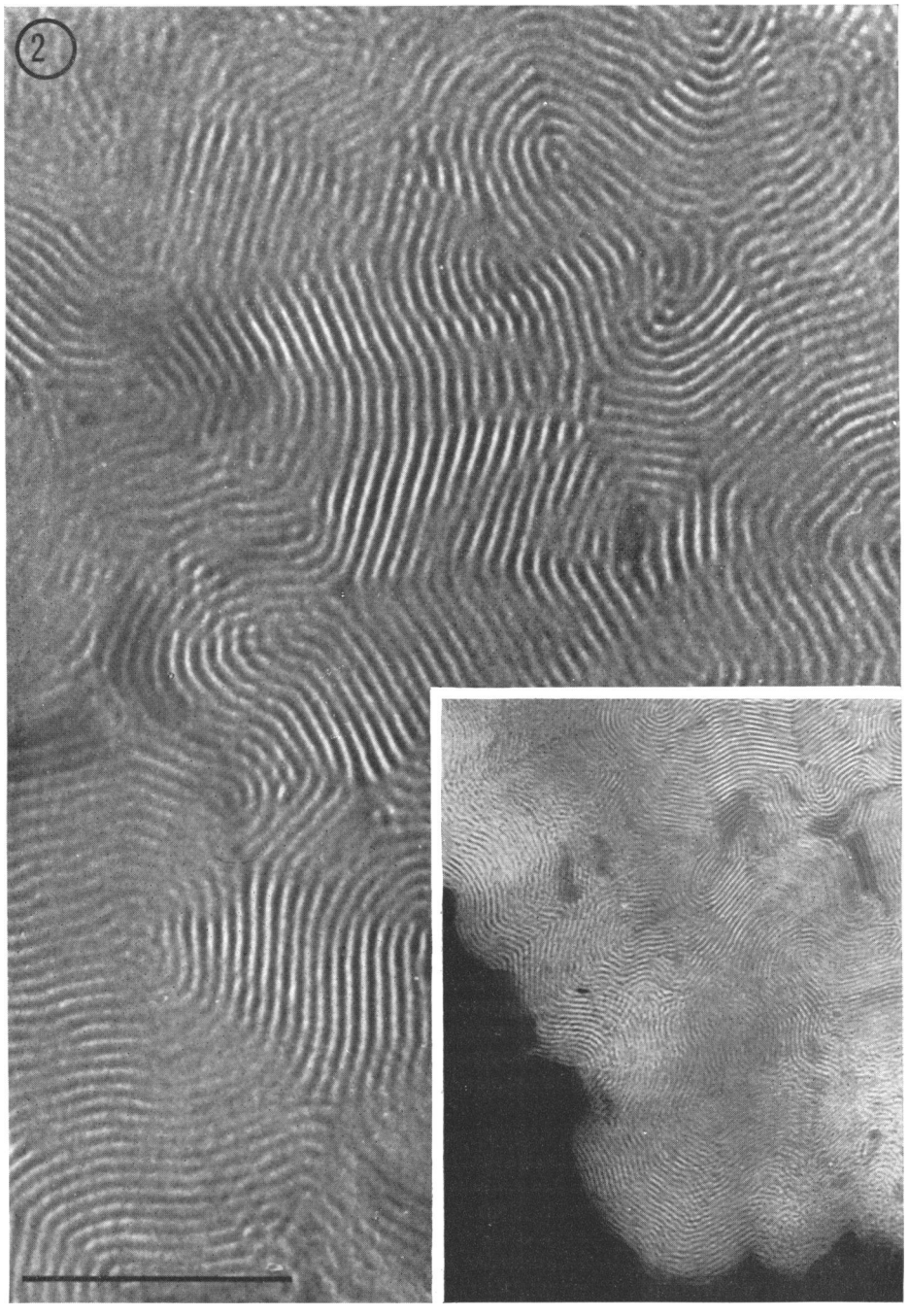


FIG. 2. *Electron micrograph of phosphatidyl ethanolamine negatively stained with lithium tungstate. The marker indicates 1,000 A.*

the organized structures of myelin, cephalins, and phosphatidyl choline previously revealed by X-ray diffraction and electron microscopy (2, 3, 9, 10, 15, 16, 28), assuming that the visible leaflets represent primarily the hydrocarbon portions of the lipid leaflets.

Appearance of lipopolysaccharide. Electron micrographs of lipopolysaccharide from *S.*

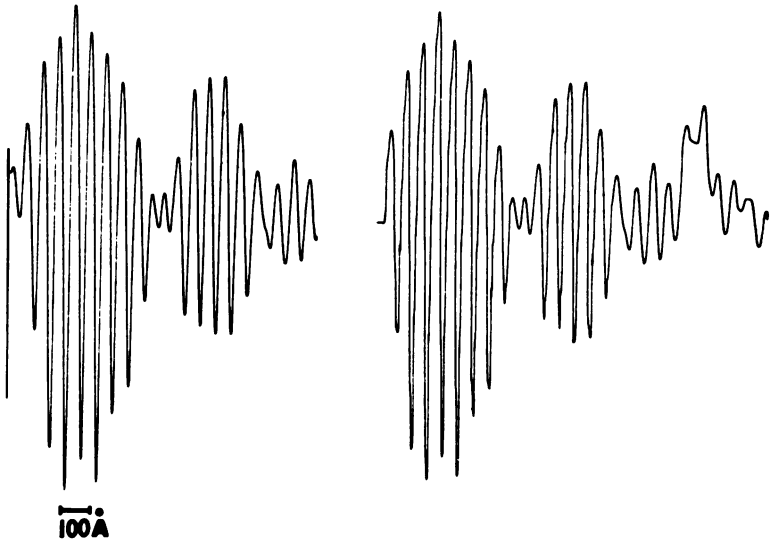


FIG. 3. *Microdensitometry of an electron micrograph of phosphatidyl ethanolamine negatively stained with lithium tungstate (See Fig. 2 and text).*

typhimurium G-30 showed a uniform population of oval bodies, each bounded by a continuous outer leaflet (Fig. 4a). The majority appeared spherical, but occasional spheroids were flattened or indented (Fig. 4b). The enveloping leaflet was almost always intact, and only a single broken leaflet (Fig. 4c) was seen of many thousands that were examined. In the ensuing discussion, the structures are assumed to be spherical, although the obvious plasticity of the envelope makes it clear that this is an oversimplification, and the actual shape of the particles on the grid may be closer to a flattened disc.

Measurements of the diameters of the spheroids were made directly from the electron micrograph negatives, and the size range and approximate distribution are shown in Fig. 5. Few particles were smaller than 250 A or larger than 2,500 A in diameter. The average thickness of the enveloping leaflet was approximately 30 A (200 measurements). (Measurements of thickness of the leaflets were always made at the thinnest portion of the visible leaflet, since oblique views of a spherical leaflet would give erroneously high values.) There were occasional spheroids containing an inner structure of similar shape, and the thickness of the leaflet of the inner spheroid was similar to that of the outer leaflet. Careful examination of electron micrographs showing inner and outer spheroids suggested that these structures were not a result of superimposition of two separate spheroids, since the inner structure was never seen to overlap or extend beyond the leaflet of the outer spheroid. No other organized structures were seen in the interior of the spheroids.

On one occasion (Fig. 4c) when the surrounding leaflet was broken, possibly during drying, dense negative stain appeared to penetrate into the interior, indicating the relatively hollow nature of the spheroids.

When negative stain was allowed to remain in contact with lipopolysaccharide in solution for increasing periods of time before preparation and drying of the films, there was an obvious increase in the degree of penetration into the interior of the spheroids. After 10 sec of contact, many spheroids had relatively dense, white centers, indicating little penetration by negative stain, but with increasing time of contact there was penetration of stain into essentially all visible spheroids, giving a uniform population of spherical bodies, each filled with negative stain. Maximal penetration was seen after approximately 1 min, and, when stain and lipopolysaccharide were mixed in solution for longer times, there was no further change in appearance.

Examination of lipopolysaccharide from *S. typhimurium* TV 119 revealed similar spheroid structures.

The lipid ("lipid A") and polysaccharide ("soluble polysaccharide") portions of the lipopolysaccharide of strain G-30 were obtained by mild acid hydrolysis of the lipopolysaccharide and were also examined by electron microscopy. The lipid portion differed markedly in appearance from intact lipopolysaccharide, pre-

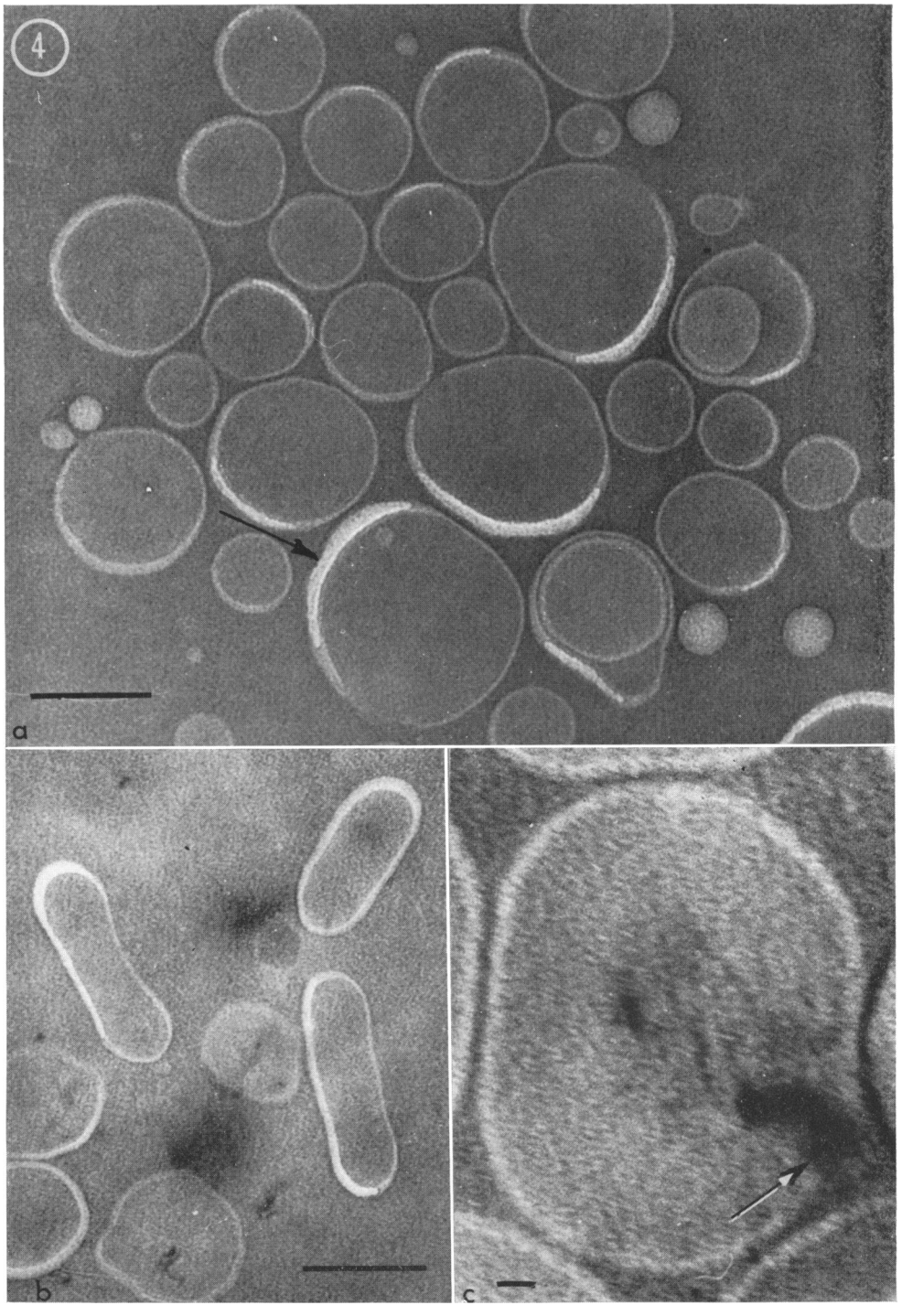


FIG. 4. *Electron micrographs of lipopolysaccharide of Salmonella typhimurium G-30, negatively stained with ammonium molybdate (25). (a) Typical lipopolysaccharide structures of generally spherical shape are visible. An inner structure of similar appearance can be seen within two spheroids. The opaque white areas seen along the rims of several spheroids (arrow) are believed to be artifacts of undetermined cause. The marker indicates 1,000 A. (b) Occasional spheroids are distorted or indented, indicating the relatively plastic nature of the structures. The marker indicates 1,000 A. (c) The surrounding leaflet is broken (arrow), and dense negative stain can be seen penetrating into the interior of the spheroid. The marker indicates 100 A.*

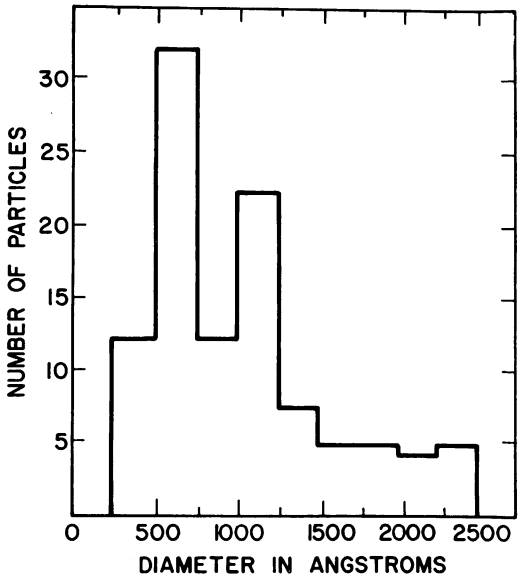


FIG. 5. *Histogram of external diameters of spheroids seen in electron micrograph of lipopolysaccharide from Salmonella typhimurium G-30, negatively stained with ammonium molybdate (see Fig. 4).*

senting a cobweb appearance made up of many individual leaflets (Fig. 6). The polysaccharide portion of the molecule showed poorly defined small aggregates of low molecular weight material without recognizable structure.

Interaction of lipopolysaccharide and phosphatidyl ethanolamine. Examination of the preparations containing the lipopolysaccharide-phospholipid complex provided direct evidence of the interaction of the two components (Fig. 7). In several fields, the leaflet of a lipopolysaccharide spheroid was seen to penetrate into the phosphatidyl ethanolamine leaflet structure with the normally continuous outer leaflet of the spheroid becoming discontinuous in one region and extending directly into the outermost leaflet of the adjoining phospholipid (Fig. 7a and b). In these regions, no clear demarcation between lipopolysaccharide and phospholipid was seen, and the continuous leaflet frequently extended a considerable distance from the original lipopolysaccharide spheroid. In many fields, the normally smooth outlines of the spheroids were markedly altered as the enveloping leaflet became disrupted in several regions (Fig. 7c).

In several regions, lipopolysaccharide spheroids

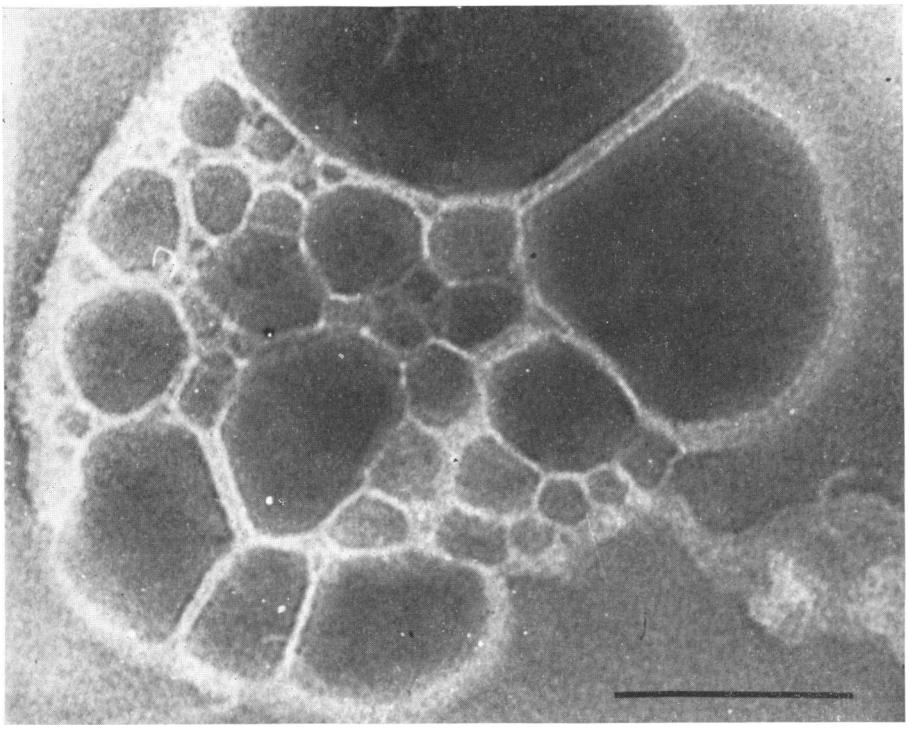


FIG. 6. *Electron micrograph of "lipid A" prepared from lipopolysaccharide of Salmonella typhimurium G-30, negatively stained with ammonium molybdate. The marker indicates 1,000 A.*

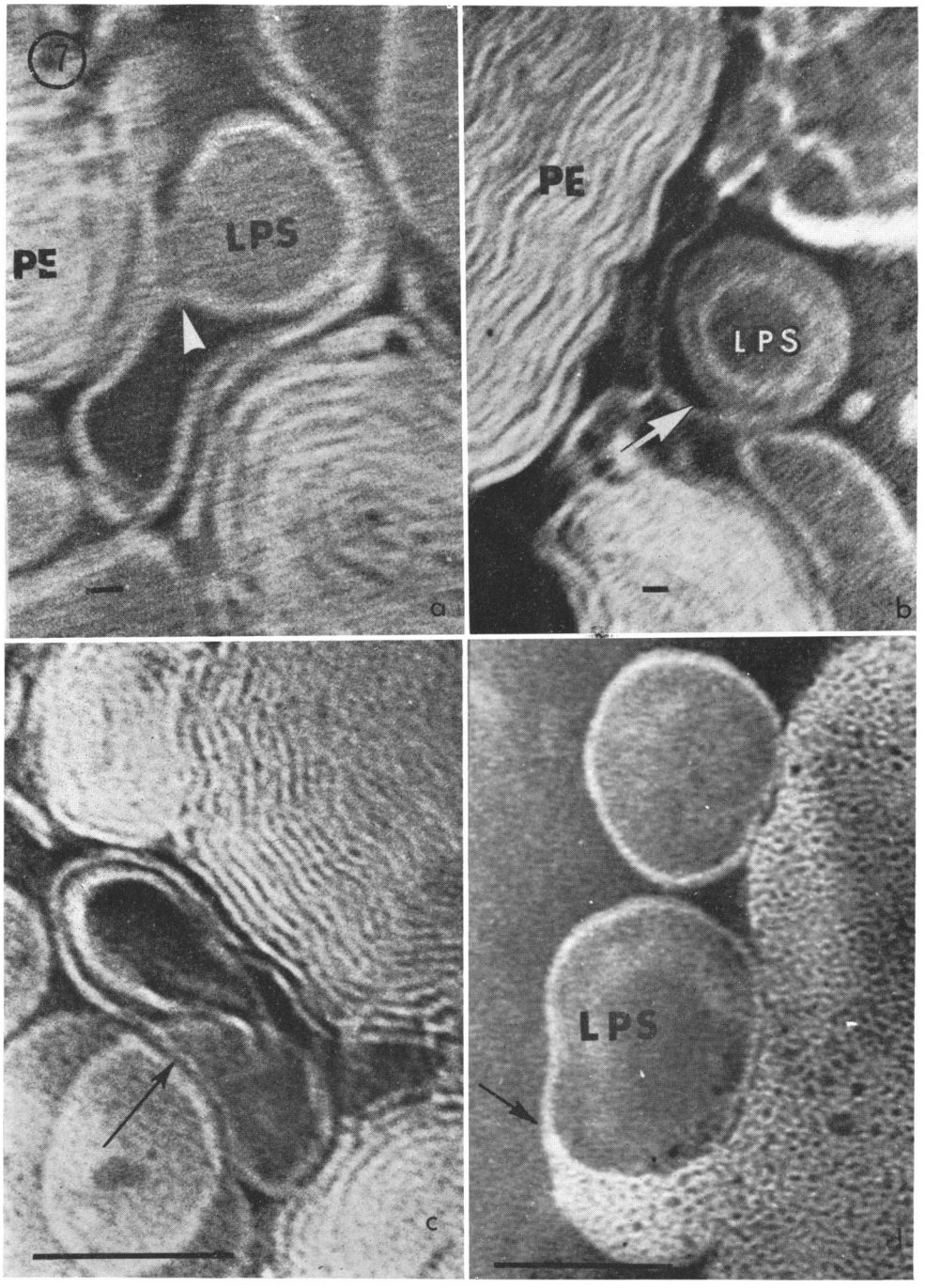


FIG. 7. *Electron micrographs of a mixture of lipopolysaccharide of Salmonella typhimurium G-30 and phosphatidyl ethanolamine, prepared as described in Experimental procedure, and negatively stained with lithium tungstate (25). The ratio of lipopolysaccharide to phosphatidyl ethanolamine was* 6×10^{-2}*:1 (w/w). The marker indicates 100 A in (a) and (b), and 1,000 A in (c) and (d). (a, b) The lipopolysaccharide (LPS) leaflet has opened (arrow) and appears to extend into a superficial leaflet of adjacent phosphatidyl ethanolamine (PE). (c) The arrow indicates what appears to be a lipopolysaccharide spheroid with disruption of the outer leaflet in several areas. (d) Lipopolysaccharide appears to penetrate directly (arrow) into phospholipid.*

were seen to merge directly with islands of phospholipid (Fig. 7d).

In other areas, no recognizable spheroids were seen, but there were characteristic alterations of the phospholipid leaflets, suggesting introduction of foreign molecules into the phosphatidyl ethanolamine leaflet structure. Surface leaflets peeled away from the underlying regular lamellar

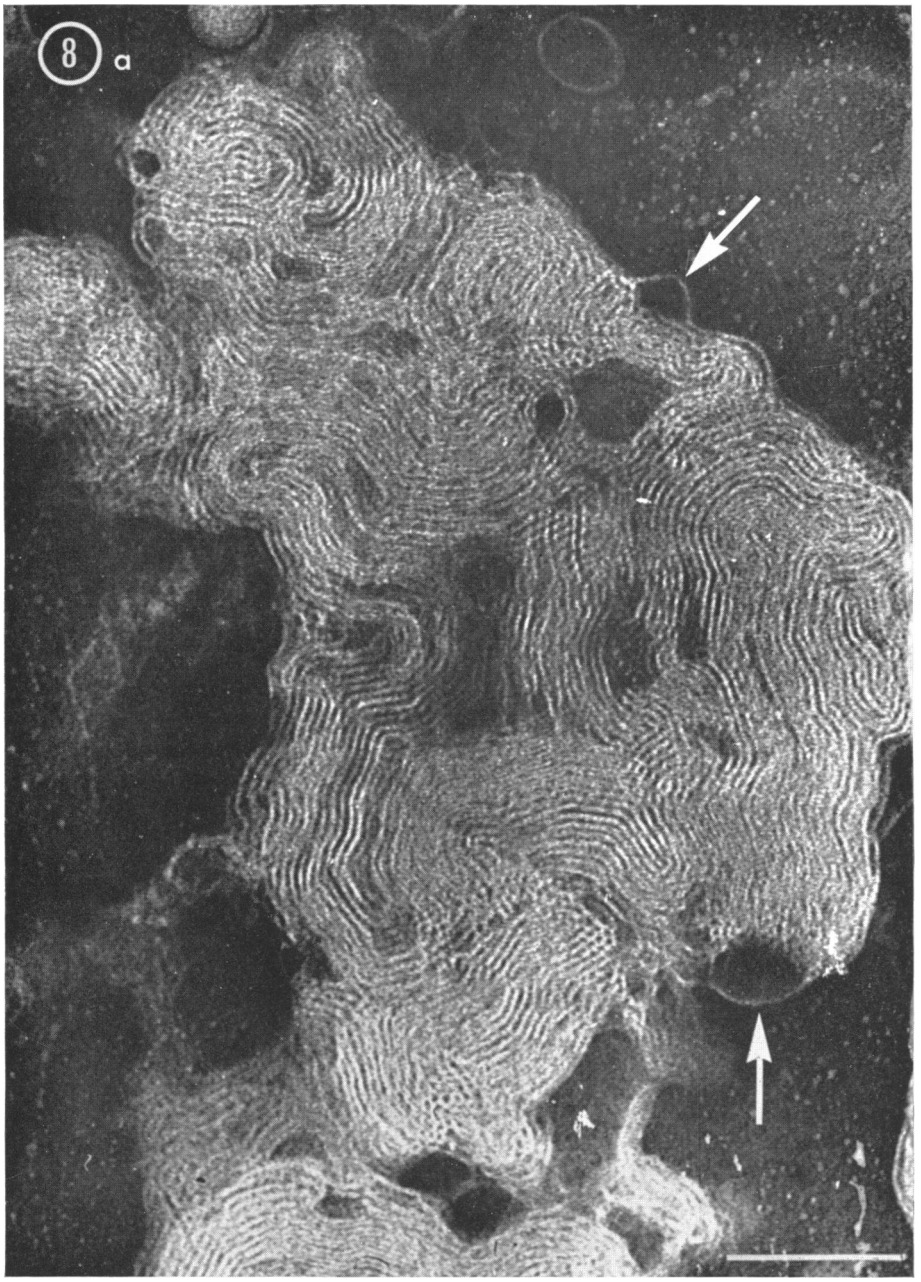
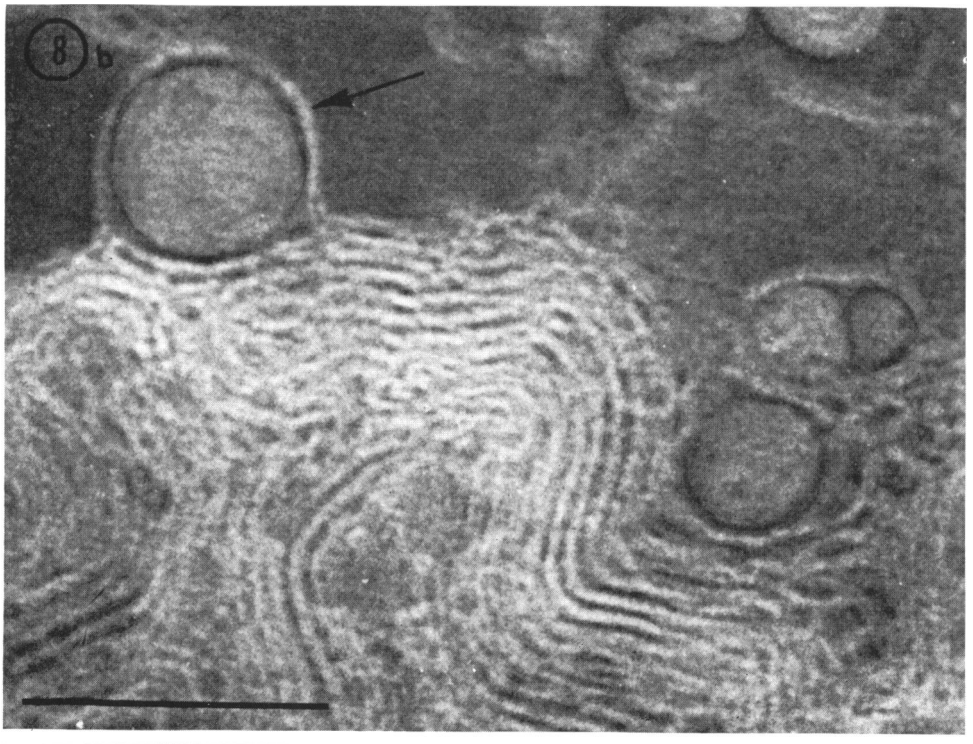


FIG. 8. *Electron micrographs of a mixture of lipopolysaccharide of Salmonella typhimurium G-30 and phosphatidyl ethanolamine, negatively stained with lithium tungstate. The markers indicate 1,000 A. The ratios of lipopolysaccharide to phosphatidyl ethanolamine (w/w) were: (a) 1.2 × 10^{-4}:1, (b) 1.2 × 10^{-1}:1, and (c) 3 × 10^{-2}:1. (a) The surface leaflets have formed elevated "blisters" (arrow) on the surface of a laminar area of phosphatidyl ethanolamine. (b) A similar "blister" is present, containing a second inner spheroid. (c) The leaflets of the phosphatidyl ethanolamine structure are spread apart, kinked, and distorted in many areas.*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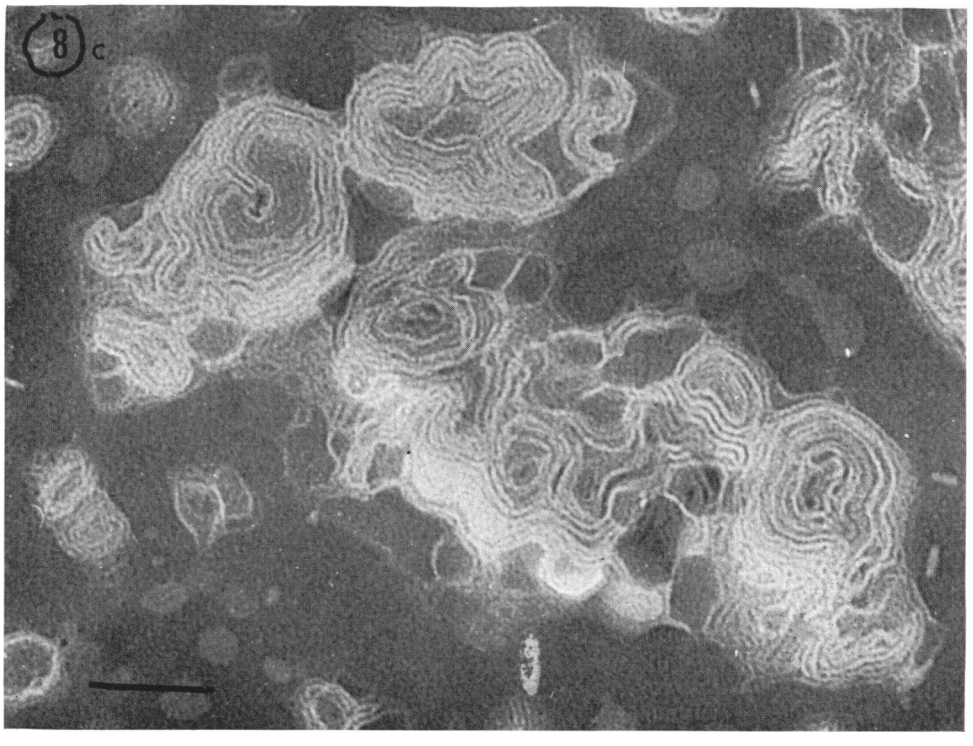
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structure, forming raised blisters on the surface of the phospholipid particles (Fig. 8a and b).

The same process also involved the deeper leaflets, and in the most profoundly affected regions the normally tightly packed structure of parallel leaflets disappeared and small islands with irregular lamellae became detached from the surface of the lipid particles (Fig. 8c).

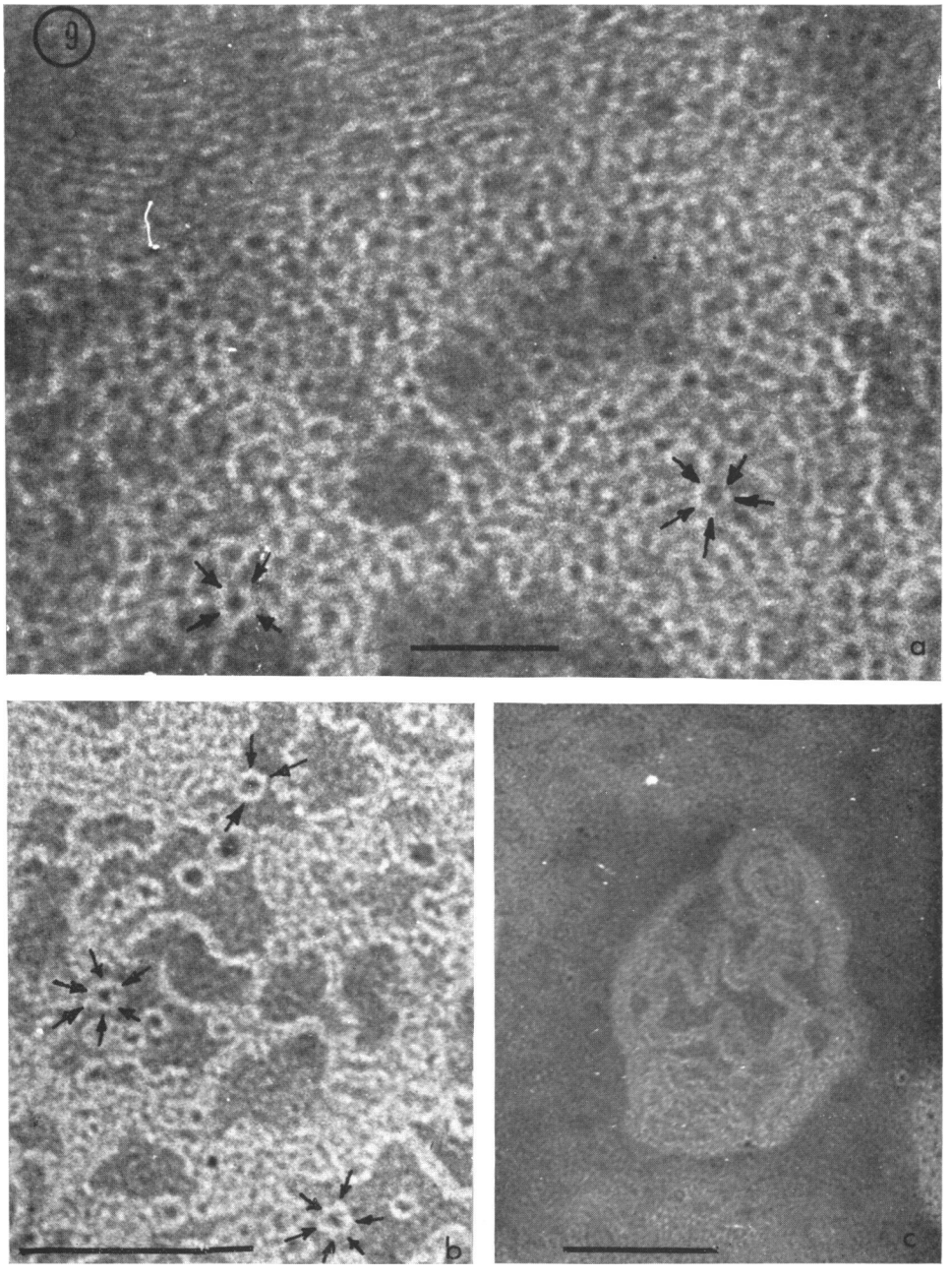

FIG. 9. *Electron micrographs of "saponin-like effect" seen with mixtures of lipopolysaccharide of Salmonella typhimurium G-30 and phosphatidyl ethanolamine in a ratio of 8 × 10^{-2}:1, negatively stained with lithium tungstate. The arrows indicate regions showing small, approximately spherical subunits arranged in groups. Many areas show groups of six subunits surrounding a central hole. The markers indicate 1,000 A; (a), (b), and (c) represent different areas of the same preparation.*

Saponin-like effect of lipopolysaccharide on phospholipid leaflets. At high ratios of lipopolysaccharide-phospholipid (see below), there were many areas in which leaflet structures were no longer seen. In their place were large numbers of small circular structures, containing a central cavity filled with negative stain surrounded by an array of approximately six spherical subunits (Fig. 9 and 10). The central canal was approximately 50 to 60 A in diameter when seen end-on, and the diameter of each globular subunit was approximately 35 A. In other areas, tortuous elongated figures were seen, each appearing to contain a central canal filled with negative stain (Fig. 9c). The appearance of perforations and the presence and size of the globular subunits were similar to the changes seen when saponin is mixed with lecithin-cholesterol suspensions, as described by Lucy and Glauert (15) and by Bangham and Horne (2).

Effect of lipopolysaccharide and phospholipid concentration and conditions of mixing. The ratio of lipopolysaccharide to phosphatidyl ethanolamine was varied from 6×10^{-4}:1 to 3:1 (w/w). At low ratios (below 6×10^{-3}:1), no free lipopolysaccharide spheroids were seen, but there were minor changes in phospholipid leaflet structure in several areas, including blistering of surface lamellae and irregularity of internal lamellar structure. With increasing amounts of lipopolysaccharide, there were increasing numbers of free spheroids which had not been incorporated into the phospholipid structure, and the disruption of phospholipid structure became more marked. The "saponin-like effect" was generally seen only with ratios above 10^{-2}:1. It was clear from the electron micrographs that lipopolysaccharide spheroids were not uniformly distributed, and "puddles" of unincorporated spheroids were seen in some regions.

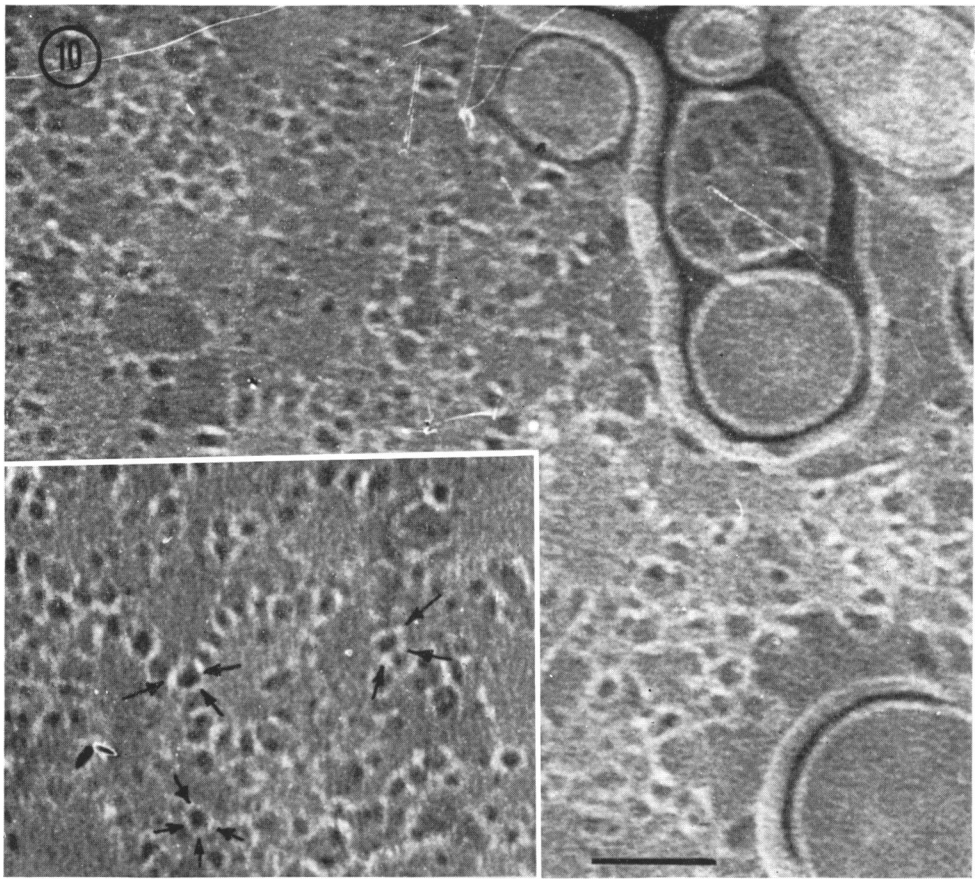

FIG. 10. *Electron micrographs of "saponin-like effect" seen with a mixture of lipopolysaccharide and phosphatidyl ethanolamine prepared as in Fig. 9. The marker indicates 1,000 A. In the inset, a selected area demonstrates a ribbon-like border, apparently composed of globular subunits (arrow) appearing to surround a central core of dense negative stain.*

Similarly, the extent of changes in phospholipid leaflet structure also varied in different regions of the specimen. The local ratios of lipopolysaccharide to phosphatidyl ethanolamine therefore differed considerably in different areas, and no absolute correlation can be made between concentration of lipopolysaccharide and specific effects on structure of the phospholipid leaflets. In previous studies of the transferase enzyme reaction, enzyme activity was proportional to concentration of lipopolysaccharide at lipopolysaccharide-phosphatidyl ethanolamine ratios between 10^{-2}:1 and 10^{-3}:1 (w/w).

The lipopolysaccharide-phospholipid mixture must be heated and slowly cooled to show maximal acceptor activity in the transferase reactions (21), and the effect of the heating-cooling procedure was therefore also studied by electron microscopy. When heating and slow-cooling was not performed, the changes in lipid structure were much less widespread at similar ratios of lipopolysaccharide to phosphatidyl ethanolamine, and many free lipopolysaccharide spheroids were seen even at ratios below 6×10^{-3}:1. At high concentrations of lipopolysaccharide, the heating-cooling procedure had no effect on the electron micrographic appearance. Similarly, appearance of acceptor activity in the enzymatic transferase reactions is also seen at high ratios of lipopolysaccharide to phospholipid, even in the absence of heating and slow-cooling.

Interaction of lipopolysaccharide with phosphatidyl choline. The disruptive effects of lipopolysaccharide on the leaflet structure of phosphatidyl ethanolamine were duplicated when mixtures of lipopolysaccharide and phosphatidyl choline were examined.

Interaction of wild-type lipopolysaccharide with phosphatidyl ethanolamine. Similar changes in the leaflet structure of phosphatidyl ethanolamine were seen when wild-type lipopolysaccharide was substituted for the galactose-deficient lipopolysaccharide used in the studies described above.

Monolayer experiments. Monolayer experiments have provided further evidence for direct penetration of lipopolysaccharide into a phosphatidyl ethanolamine leaflet structure (Fig. 11 and reference 25). A film of phosphatidyl ethanolamine was formed at an air-water interface, and lipopolysaccharide was introduced into the bulk aqueous phase below the monolayer. This resulted in a progressive increase in surface pressure (measured at constant area). There was a rough proportionality between rise in surface pressure and amount of lipopolysaccharide added. This evidence of penetration of the monolayer was

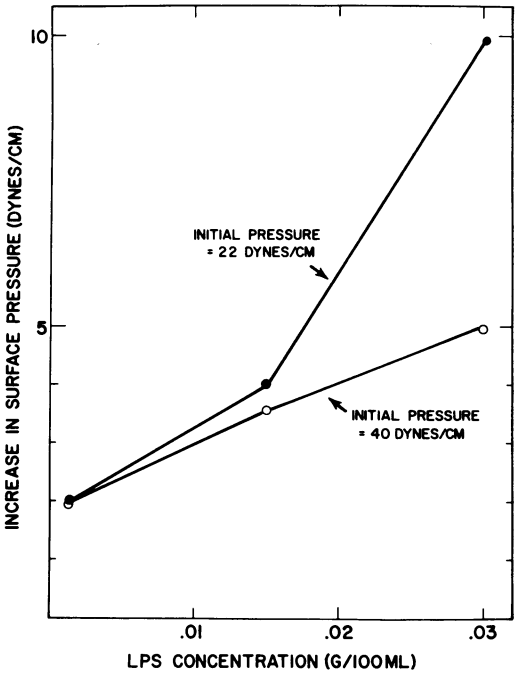

FIG. 11. *Penetration of lipid monolayer by lipopolysaccharide. A monolayer of phosphatidyl ethanolamine was formed at an air-water interface as described in Materials and Methods, and lipopolysaccharide was introduced into the aqueous phase. The initial surface pressures are indicated on the graph. When 0.03% lipopolysaccharide was added to the aqueous phase in the absence of a lipid film, a surface pressure of 18 dynes per cm was obtained.*

seen with initial monolayer pressures as high as 40 dynes per cm. The collapse pressure of phosphatidyl ethanolamine was 48 dynes per cm, defined as the point above which no further increase in pressure was seen despite further addition of phosphatidyl ethanolamine, and was reached after addition of 0.1 mg of the phospholipid.

When a phosphatidyl choline monolayer was studied in the same way, there was also a rise in surface pressure from 22 to 32.5 dynes per cm after introduction of 0.015% lipopolysaccharide into the aqueous phase.

Monolayer experiments were also performed with lipopolysaccharides from wild-type *S. minnesota* and from an R_{II} mutant of *S. minnesota*. At a lipopolysaccharide concentration of 0.015%, there were increases in surface pressure of a phosphatidyl ethanolamine film from 27 to 32 dynes per cm and from 28 to 34 dynes per cm, respectively.

DISCUSSION

The electron microscopic studies demonstrate that purified lipopolysaccharide of *S. typhimurium* in aqueous solution appears as distinctive hollow structures when examined by negative-staining techniques. Similar structures were described by Bladen and Mergenhagen (4) in crude phenol extracts of *Veillonella*, and have also been observed by Work, Knox, and Vesk (31) in both sectioned and negatively stained material, near the cell surface of *E. coli* strain 12408, under conditions in which large amounts of lipopolysaccharide are excreted into the medium. Knox, Cullen, and Work (*personal communication*) have also observed similar spheroids in negatively stained preparations of lipopolysaccharide purified from culture filtrates of this strain by phenol extraction and ethyl alcohol precipitation. The appearance of similar structures in material obtained from different sources and studied by fixation and sectioning techniques, as well as by negative staining, suggests that the spheroids may represent a true structure of lipopolysaccharide in aqueous solution. It should be noted that in certain cases lipopolysaccharides assume other structures when examined by negative-staining techniques. Thus, we have observed slender, tortuous structures, as well as spheroids, in lipopolysaccharides obtained by Otto Lüderitz by means of phenol extraction from wild-type and mutant strains of *S. minnesota*. The relation of lipopolysaccharide composition and method of purification to electron microscopic appearance and type of liquid crystalline structure is under investigation.

Each lipopolysaccharide spheroid is bounded by a continuous thin leaflet. As previously discussed (25), it is likely that the leaflet contains a large number of molecules in liquid crystalline structure. Several models are consistent with the formulation: (i) lipopolysaccharide molecules may exist in a continuous bimolecular leaflet; (ii) lipopolysaccharide molecules may be arranged to form a monomolecular leaflet with polar portions of individual molecules oriented to both inner and outer surface of the leaflet; (iii) despite the absence of detectable lipid impurities, the presence of a second amphipathic species could result in a mixed bimolecular leaflet. In all models, the molecules are arranged in a continuous sheet to exclude water from hydrophobic regions. The visible leaflet primarily represents the lipid portions of adjacent lipopolysaccharide molecules, with the hydrated polysaccharide side-chains and other polar portions of the molecule projecting into the aqueous phase around the spheroids and within their hollow interiors. More complex models, such as Lucy's suggestion of a planar array of micelles (14) arranged in a spherical leaflet, are also possible.

The 30-A thickness of the lipopolysaccharide leaflet corresponds to the length of approximately two fatty acid chains (29). It is similar to the thickness of the leaflets of phosphatidyl ethanolamine seen in the present study, and of the leaflets of other phospholipids revealed by electron microscopy and X-ray diffraction in other studies (2, 3, 9, 10, 15, 16, 28), assuming that the visible leaflets represent primarily the hydrocarbon portions of the leaflet structures.

In mixtures of lipopolysaccharide and phosphatidyl ethanolamine, selected electron micrographs appear to show continuity between recognizable lipopolysaccharide spheroids and phospholipid leaflets (Fig. 7), suggesting direct insertion of lipopolysaccharide into the phospholipid leaflet structure. The other observed alterations of phospholipid structure in the presence of lipopolysaccharide (Fig. 8) can also be explained by insertion of short segments of lipopolysaccharide leaflet or of individual lipopolysaccharide molecules directly into the phospholipid leaflet.

Monolayer experiments provided additional evidence that lipopolysaccharide can penetrate directly into a linear array of phospholipid molecules, and also suggested that the analogous observations by electron microscopy are not artifacts.

The most profound effect of lipopolysaccharide was complete disruption of organized phospholipid leaflets with formation of new structures differing in appearance from both lipopolysaccharide and phospholipid (Fig. 9 and 10). This final stage was seen with both phosphatidyl ethanolamine and phosphatidyl choline leaflets. It appears to be similar to the effect of saponin on lecithin-cholesterol leaflets (2, 15), and indicates a possible direct mechanism for disruption of cellular lipid membranes by exogenous lipopolysaccharide.

Previous studies of the enzymatic synthesis of lipopolysaccharide (21) indicated that a lipopolysaccharide-phospholipid complex exists in the native cell envelope and that a similar complex is formed by mixtures of the purified components in vitro. The present observations suggest that a common leaflet structure may be formed by lipopolysaccharide and phospholipid molecules in an aqueous environment under conditions similar to those of the enzyme studies. This suggests a tentative model, in which the

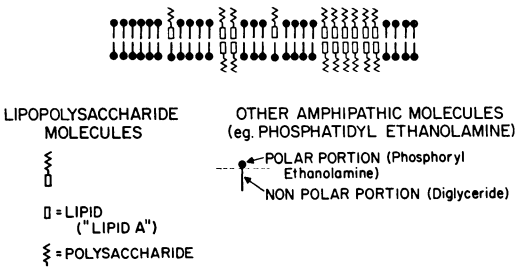

FIG. 12. *Proposed relationship of lipopolysaccharide and phospholipid molecules within the native cell envelope.*

native cell envelope contains lipopolysaccharide and phospholipid molecules in a common leaflet structure (Fig. 12). This structure is consistent with the widely accepted bilayer theory of Davson and Danielli (6) and the unit membrane model of Robertson (20), as well as with the electron microscopic studies of cell walls by Bladen and Mergenhagen (4). The present evidence does not indicate whether lipopolysaccharide is present as individual molecules or as short stretches of a lipopolysaccharide leaflet, and also does not exclude the presence of other molecular species in the leaflet structure. The figure shows the widely accepted bimolecular leaflet structure, but the observations are also compatible with other liquid crystalline structures forming a leaflet of similar dimensions (14).

This arrangement permits interactions between adjacent polar groups of phospholipid and lipopolysaccharide, and is therefore consistent with the previous demonstration of a specific role for the polar portion of phospholipids in the enzymatic transferase reactions. Although phosphatidyl choline is inactive in the transferase reactions (23), electron microscopy and monolayer experiments both demonstrated the ability of lipopolysaccharide to penetrate phosphatidyl choline leaflets. If the proposed model is correct, differences in activity of phosphatidyl choline and phosphatidyl ethanolamine are due to differences in polar group interactions within the membrane, and are not due to differences in ease of formation of a common lipopolysaccharide-phospholipid leaflet structure.

Before the role of a common leaflet structure can be accepted, however, it will be necessary to obtain direct evidence that the common leaflet participates in enzyme binding. Until this is done, and until such a common leaflet has been directly visualized within the cell envelope, the model must remain speculative.

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Portions of Fig. 4 and 7 were previously published in *Federation Proceedings* and are reprinted with permission.

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