

# Ultrastructure of Lipopolysaccharide Isolated from *Treponema pallidum*

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Received for publication 9 November 1972

A lipopolysaccharide (LPS) fraction was extracted from Nichols, nonpathogenic *Treponema pallidum* by the hot, phenol-water procedure. The LPS was freed of nucleic acids and water-soluble proteins by successive exposures to ribonuclease, deoxyribonuclease, and Pronase. Purified LPS responded positively in a colorimetric assay for lipopolysaccharide. Electron microscope examination of the LPS both before and after purification demonstrated a heterogeneous mixture of forms including spheres, doughnuts, and ribbons. The trilaminar nature of the ribbon forms was observed by both negative staining and thin sectioning. Lyophilization of the LPS caused an increase in the number and length of ribbon forms seen. Results suggest that the surface layers of treponemes are similar to those of gram-negative bacteria.

Lipopolysaccharide (LPS) has been extracted chemically from intact cells of the Reiter strain of *Treponema pallidum* (1-3, 6, 13, 14). This material has been analyzed for its general chemical composition (6) and has been used primarily to study the antigenic relationships between the Reiter and other strains of *T. pallidum* (1-3, 11, 13, 14).

The location of LPS in intact treponemes is not known. However, there are two indications that LPS extracted from *T. pallidum* is located in the surface layers of these cells. First, both the location and ultrastructure of the surface layers of treponemes, when viewed in thin section, are similar to those of gram-negative bacteria. Both types of cells are surrounded by an inner unit membrane representing a plasma membrane (PM), one or more intermediate layers representing the peptidoglycan (PG) structure of the wall, and an outer unit membrane (OM) (Fig. 1). In gram-negative bacteria all surface layers combined are called the cell envelope (ENV) (12), whereas in treponemes the outer membrane alone is called cell envelope. Since the OM of gram-negative cells represents the LPS-containing layer of the wall (12), it is likely that the outer membrane, or cell envelope, of the treponemes also contains LPS.

A second indication that LPS is located in the surface layers of treponemes is that the cell envelope is known to contain lipid, protein, and carbohydrate (Pillot, Ph.D. thesis, University

of Paris, 1965). A membrane with these components is compatible with an LPS-containing structure.

Our paper describes the ultrastructure of an LPS fraction chemically extracted from the Nichols, nonpathogenic strain of *T. pallidum*. We have compared the morphology of this LPS to that of *Salmonella* species and *Escherichia coli* to indicate further the probable location of treponemal LPS in the outer cell envelope.

## MATERIALS AND METHODS

**Organisms and growth conditions.** Nichols, nonpathogenic *T. pallidum* was grown in a complex broth medium, as described previously (7). Cells were harvested routinely after 5 days of incubation, when cultures had attained their maximum growth, as measured by nephelometry. Harvested cells were washed once in equivalent amounts of 0.0025 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.2), frozen, and lyophilized.

**Extraction of lipopolysaccharide.** The method used for extraction of LPS was that of Westphal and Jann (18). A weighed sample of lyophilized cells was extracted three times at 68 C with a 45% solution of phenol. After each extraction, the sample was cooled to 10 C and centrifuged at  $1,000 \times g$  for 45 min. The water phases from the three extractions were combined, washed three times with ether to remove phenol, and dialyzed for 45 h against six changes of 10 mM  $MgCl_2$  (5). The sample was cleared of debris by centrifugation at  $5,900 \times g$  for 15 min at 4 C. The LPS was sedimented by ultracentrifugation (Beckman Model L-2) at  $100,000 \times g$  for 3 h at 4 C. The clear

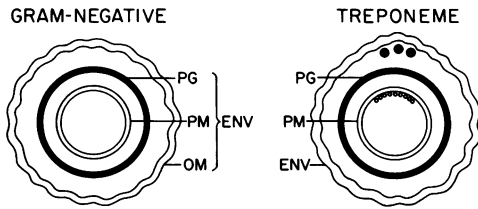


FIG. 1. Diagrammatic representation of a cross-section of both a gram-negative organism and a treponemal cell. Abbreviations: PG, peptidoglycan; PM, plasma membrane; OM, outer membrane; ENV, envelope.

pellets obtained, labeled "crude LPS," were suspended in a minimal amount of distilled water. A sample was removed for electron microscopy, and the rest of the fraction was lyophilized.

To purify the crude LPS, a modification of the method described by Romeo et al. (15) was used. LPS was treated successively with ribonuclease (RNase) and deoxyribonuclease (DNase) (Sigma Chemical Co.) to remove nucleic acids and then was treated with Pronase to remove water-soluble proteins. In addition to the pancreatic RNase used in the original method, we also used T1 RNase (Sigma Chemical Co.) in the same concentration and buffer as pancreatic RNase (Nutritional Biochemical Corp.). Enzymatic treatment was continued until there was no peak of absorbance at 260 nm. Purified LPS was lyophilized until needed for chemical, colorimetric, or electron microscope analyses.

**Colorimetric analysis of LPS.** For the colorimetric estimation of LPS, the method reported by Janda and Work (8) was used.

**Electron microscopy.** LPS dissolved in water was negatively stained with 2% phosphotungstate, pH 7.0, for 30 s or with 2% uranyl acetate, pH 4.5, for 8 min (10). All grids for negative staining had been previously treated with 0.01% bovine serum albumin to facilitate spreading of the samples.

For thin sectioning, lyophilized LPS was dissolved in water and was centrifuged at  $100,000 \times g$  for 3 h at 4 C to obtain a pellet. The pellet was prefixed overnight in 3% glutaraldehyde, (pH 7.2), washed with 1 M Veronal buffer (pH 6.0), and postfixed in 1% OsO<sub>4</sub>. At the end of 8 h the sample was washed with distilled water, stained with 1% uranyl acetate for 2 h, dehydrated in ethanol and propylene oxide, and embedded in Maraglas 655 (Marblette Corp.) Sections were cut with a diamond knife and were poststained with uranyl acetate and lead citrate. All samples were observed with a Siemens Elmiskop IA electron microscope equipped with a liquid nitrogen decontamination device.

## RESULTS

**Absence of nucleic acid in LPS.** Since the phenol-water procedure used for extraction of LPS also extracts nucleic acids into the water phase, the crude LPS was treated with RNase and DNase, followed by exposure to Pronase to

remove water-soluble proteins. The crude LPS absorbed strongly at 260 nm, even when tested in a 1:10 dilution (Fig. 2). After enzymatic treatment, however, no peak of absorbance remained in the undiluted LPS sample, indicating the presence of little or no nucleic acid.

**Colorimetric analysis of LPS.** The purified LPS fraction responded positively in the colorimetric assay for lipopolysaccharides. This assay is based on the ability of LPS to combine with a cationic carbocyanine dye, causing a shift in the absorbance maximum of the dye from 510 nm to a wavelength in the range of 468 to 478 nm. The LPS material from *T. pallidum* demonstrated a peak of absorbance at 470 nm after reacting with the dye reagent (Fig. 3). In addition, the amount of absorbance obtained at 470 nm was related in a linear fashion to the concentration of LPS (Zey and Jackson, manuscript in preparation).

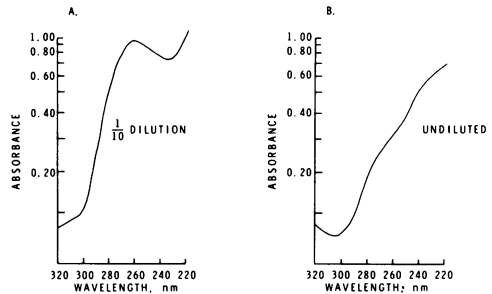


FIG. 2. Effect of treating treponemal LPS successively with ribonuclease, deoxyribonuclease, and Pronase as measured by the peak of absorbance at 260 nm. A, Crude LPS (1:10 dilution) before treatment with nucleases and Pronase; B, LPS (undiluted) after treatment with nucleases and Pronase.

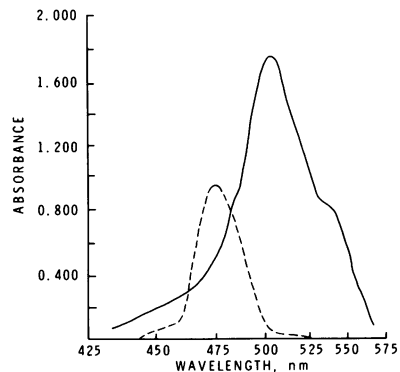


FIG. 3. Absorbance spectra of reagent containing carbocyanine dye and of LPS-reagent complex, showing a shift in absorbance maximum from 510 nm to 470 nm. Symbols: ----, 50 µg of LPS per ml from *T. pallidum* plus reagent; —, reagent alone.

**Ultrastructure of LPS.** The crude LPS fraction, when observed by negative staining prior to lyophilization, consisted of a heterogeneous mixture of morphological forms including spherical particles with an average diameter of 14 nm, ribbons of material with an average diameter of 10 to 12 nm, and occasional doughnut forms composed of a light rim with an average diameter of 11 to 12 nm and a dark center of approximately 17 nm (Fig. 4). After lyophilization of crude LPS, an increased number of ribbon forms was seen (Fig. 5, 6). In addition to an increase in quantity, ribbons also increased in their length, when compared to those seen in the LPS fraction before lyophilization (Fig. 4). Ribbons stained with uranyl acetate (UA) measured 16 nm in diameter, as opposed to 10 to 12 nm when stained with phosphotungstic acid (PTA). The staining of the ribbons was homogeneous with both PTA and UA. However, at occasional intervals, the ribbons stained with PTA exhibited small segments of parallel-line structures. An enlarged photograph of one of these segments is shown in Fig. 7. For the most part, there appeared to be no branching of the ribbons, although occasional fork-like structures were noted. The spherical forms displayed no evidence of any trilaminar structure (Fig. 4-7).

After purification of the crude LPS by exposure to nucleases and Pronase, no structural changes were noted. Negatively stained, purified material resembled the crude LPS fraction with respect to the types of morphological forms present and their staining characteristics (Fig. 8). Thin sections of purified LPS demonstrated many fragments with trilaminar appearance (Fig. 9). The average width of these parallel-line structures was 10 nm.

## DISCUSSION

Extraction of Nichols, nonpathogenic *T. pallidum* by the classical phenol-water procedure resulted in the isolation of LPS material that demonstrated a variety of shapes, including spherical particles, doughnut forms, and ribbons. Such a polydispersity of shapes is characteristic of bacterial lipopolysaccharides (16).

The extracted LPS, which shows a predominance of spherical particles measuring 14 nm in diameter before lyophilization, shows a predominance of ribbons in material observed after lyophilization. Increase in the quantity of ribbons as opposed to spheres probably is due to aggregation of smaller components during the freezing and lyophilization procedures. Such a change in physical appearance was reported for

the LPS from *Salmonella typhimurium*, which consisted of trilaminar structures prior to lyophilization and indented vesicles after lyophilization (17). In contrast to the highly branched ribbons of LPS isolated from *S. typhimurium* (17) and *E. coli* (10), ribbon forms of the LPS from *T. pallidum* only rarely show branching.

When stained with UA, the LPS ribbons of *T. pallidum* are about 5 nm wider than those stained with PTA. This difference in diameter might be explained by the fact that only a small amount of UA accumulates around the ribbons which then might become flattened due to surface tension. In contrast, those ribbons stained with PTA are much more deeply embedded in the staining matrix and are probably protected from such forces. Ribbons stained with both PTA and UA have a homogeneous appearance with the exception of infrequent segments of trilaminar structure in the PTA-stained preparations. Isolated areas showing a trilaminar appearance are similar to those seen in the LPS of *S. typhimurium* (17). Shands, Graham, and Nath (17) suggested that the entire LPS polymer is trilaminar, but, because of a tendency to lie on its widest surface, the trilaminar nature can be seen only when the ribbon twists, allowing the LPS ribbon to stand on its thin edge. It is possible, then, that areas of treponemal LPS having a triple-layered appearance also represent twists in the entire ribbon.

Isolation of lipopolysaccharide from *T. pallidum* that morphologically resembles LPS from gram-negative organisms indicates the gram-negative nature of treponemal cell walls. Such an analogy has been suggested previously (Pillot, Ph.D. thesis, University of Paris, 1965; references 7, 9) on the basis of similarities in thin sections between surface layers of treponemes and gram-negative cells. Limited chemical studies lend support to the analogy.

The intermediate layer of the treponemal surface, situated just external to the plasma membrane, has been shown to contain the peptidoglycan of the treponemal cell wall. First, both muramic acid and glucosamine have been found in intimate association with the cell bodies of treponemes that have been stripped of their cell envelope (Pillot, Ph.D. thesis, University of Paris, 1965). Second, these treponemes, devoid of their envelope, have been shown to retain their spiral shape (Pillot, Ph.D. thesis, University of Paris, 1965; reference 7). Maintenance of the shape of a cell is a function usually attributed to the rigid peptidoglycan layer of the wall. Third, treponemes, which normally

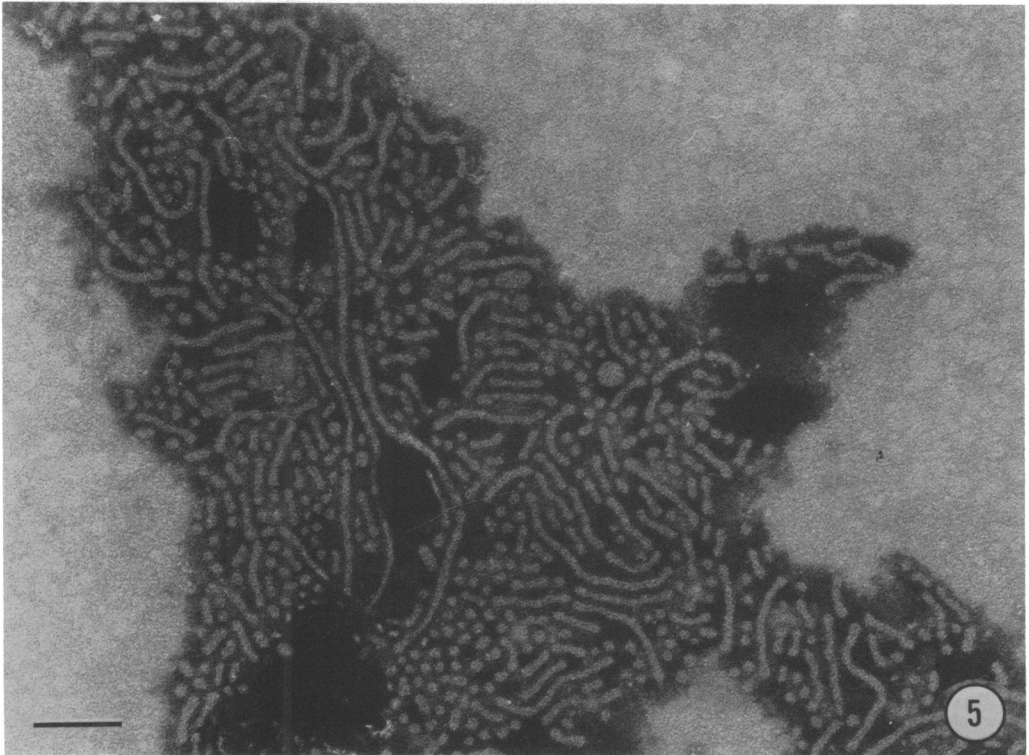
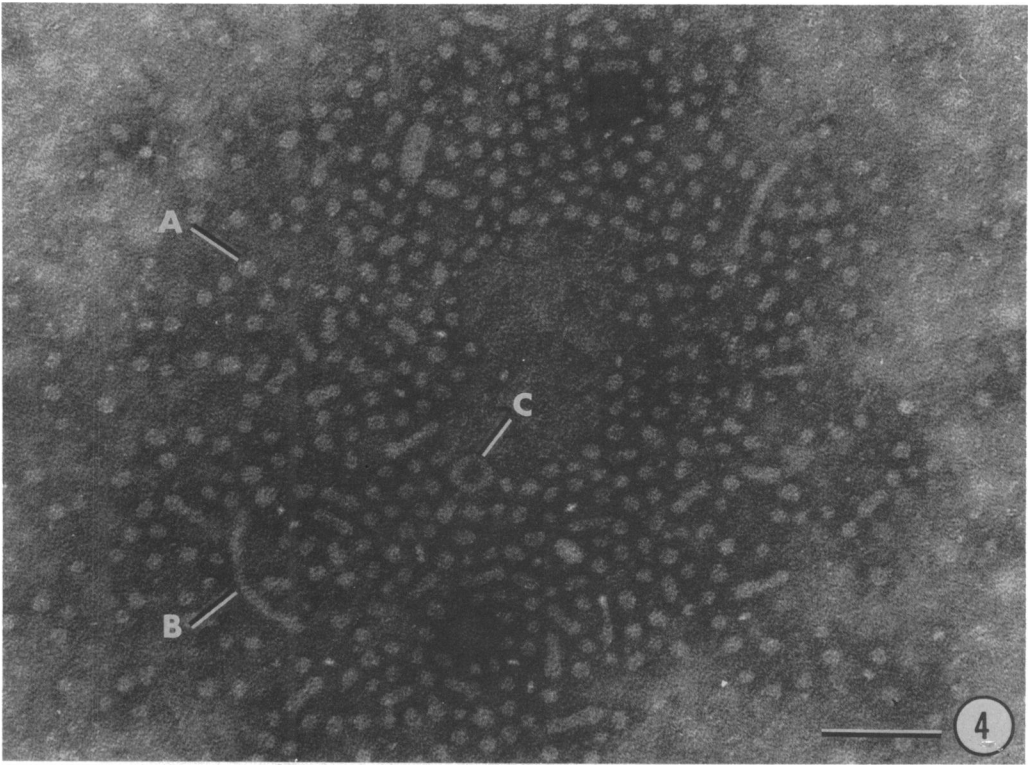


FIG. 4. Crude LPS fraction stained with 2% phosphotungstic acid (PTA), pH 7.0, for 30 s. Visible are the spheres (A), ribbons (B), and doughnut forms (C). The marker in all micrographs represents 100 nm.

FIG. 5. Crude LPS fraction after lyophilization stained with PTA. LPS fragments consist primarily of the ribbon forms; fewer spherical forms are seen than before lyophilization.

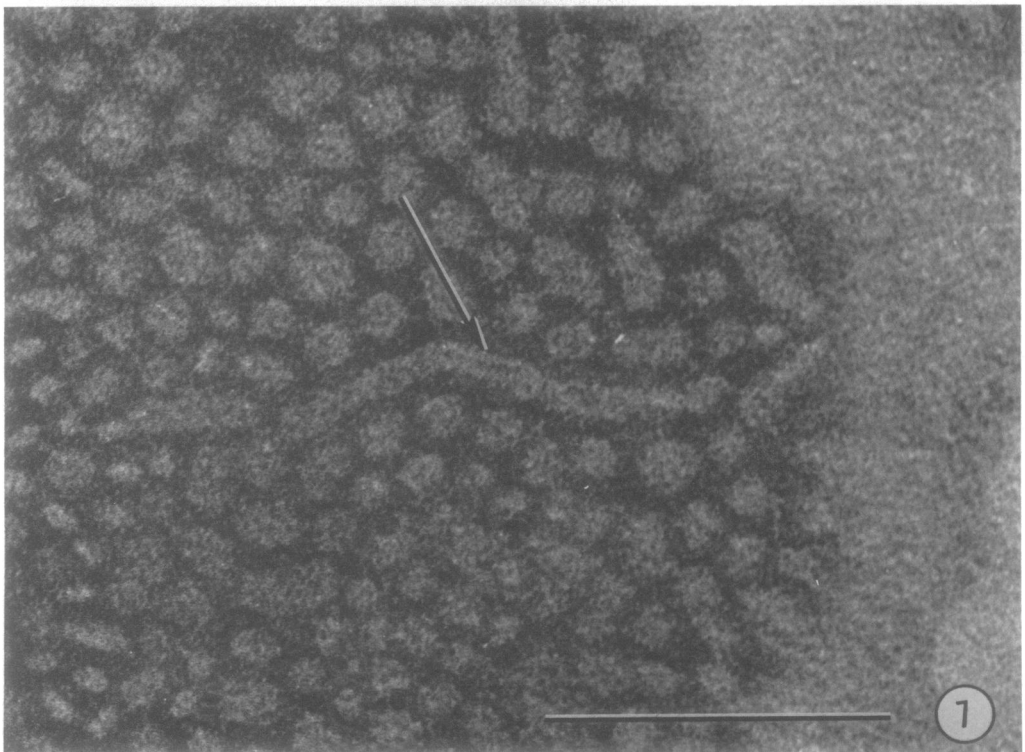
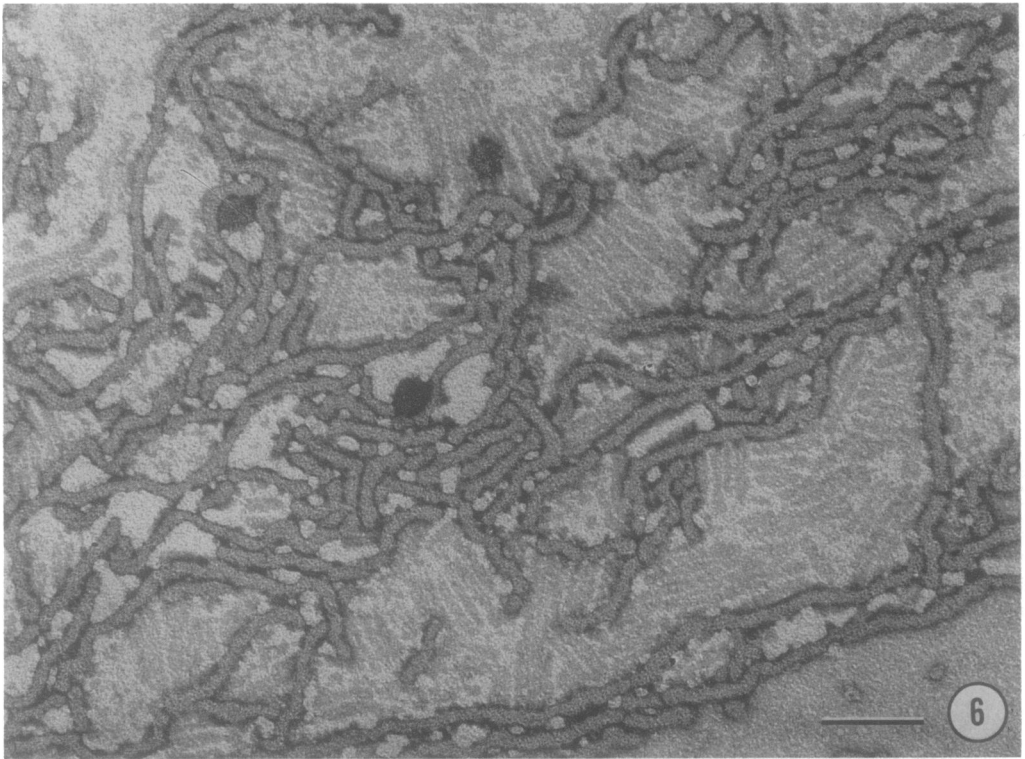


FIG. 6. Crude LPS fraction after lyophilization stained with 2% uranyl acetate (UA), pH 4.5, for 8 min. LPS fragments consist primarily of the ribbon forms; fewer spherical forms are seen than before lyophilization.

FIG. 7. An enlarged region of an LPS ribbon stained with PTA showing a trilaminar structure (arrow).

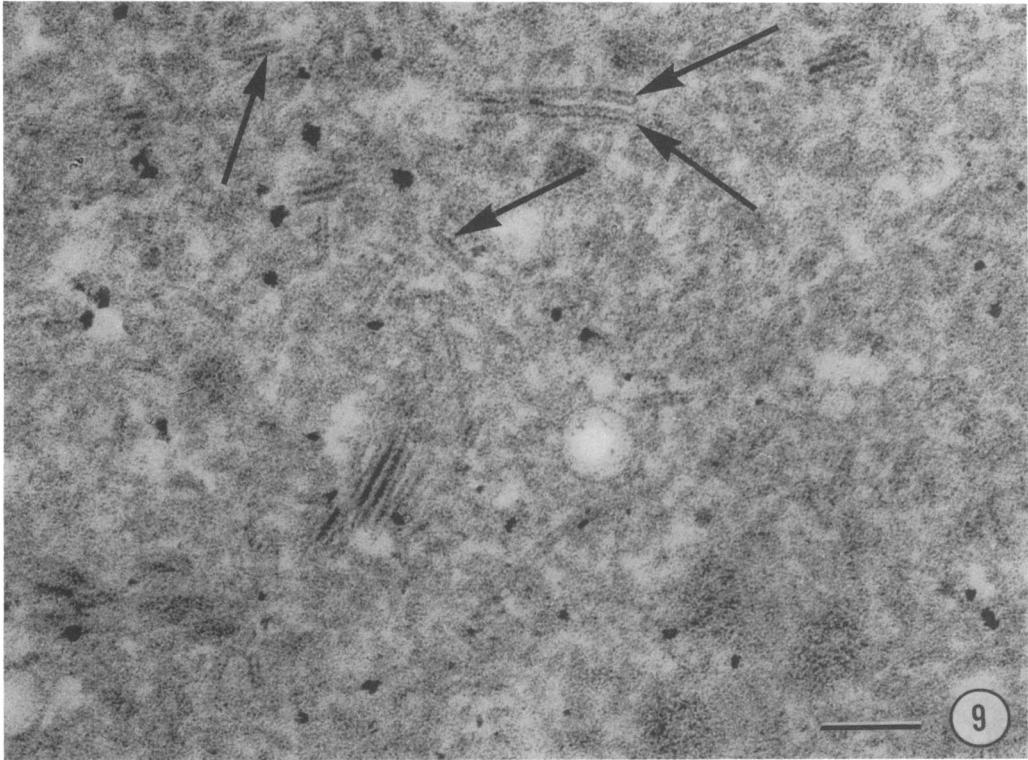
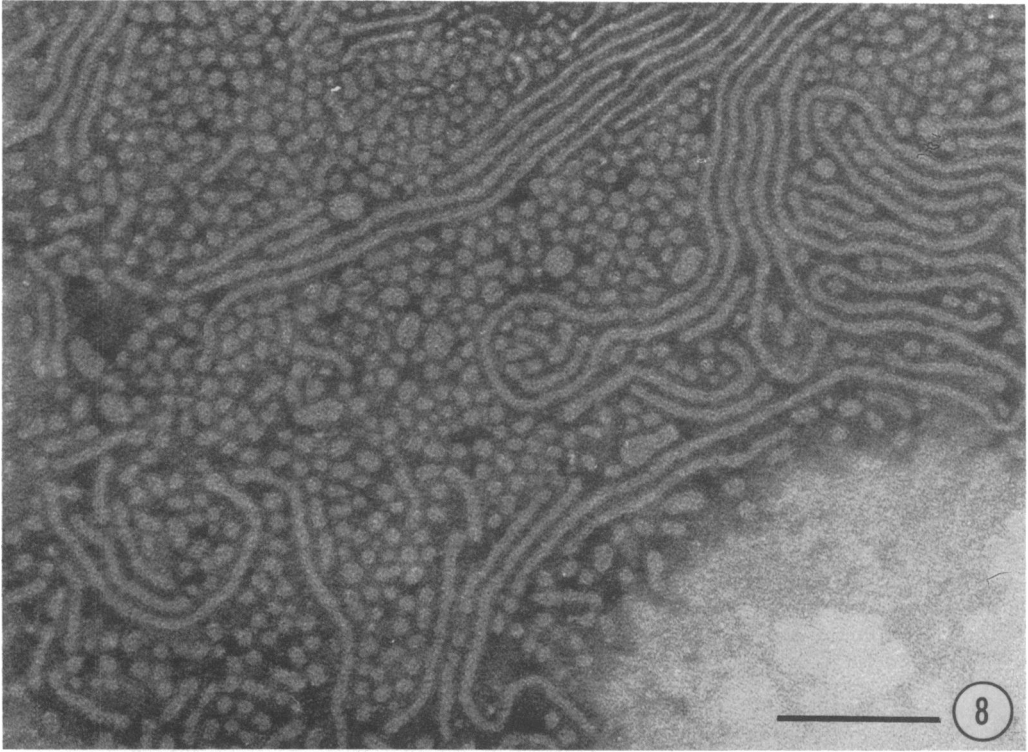


FIG. 8. LPS material after treatment with nucleases and Pronase and stained with PTA. Note the lack of structural alteration when compared to the crude LPS in Fig. 4.

FIG. 9. Thin section of crude LPS fraction showing the trilaminar appearance of LPS fragments (arrows).

are not sensitive to lysis by lysozyme, have been reported to be sensitive to such lysis if first stripped of their envelope (7).

In contrast to the more rigid intermediate layer of the treponemal surface, the outermost layer, or cell envelope, is flexible (7). Pillot (thesis, University of Paris, 1965) isolated the envelope from the Reiter strain of *T. pallidum* and reported that it contains polysaccharides bound to lipids but does not contain components characteristic of a peptidoglycan polymer. He reported that polysaccharides of the envelope are responsible for both immunological activity and specificity of treponemes; moreover, he suggested specificity of the envelope is analogous to the specificity by which species of *Salmonella* are serotyped. Species-specific polysaccharide antigens also have been detected in spirochetes other than treponemes (4); however, the location of these antigens in the cell has not been determined.

Chemical similarity between the LPS from treponemes and from gram-negative bacteria is indicated by the positive reaction of treponemal LPS in the colorimetric assay for lipopolysaccharide. A more detailed knowledge of the chemical composition will make the comparison more substantial.

#### ACKNOWLEDGMENTS

This investigation was supported by U.S. Public Health Service grant AI 10767 from the National Institute of Allergy and Infectious Diseases and, in part, by a grant to Dr. John M. Knox from the Hartford Foundation.

We thank Roberta Halloran for her excellent technical assistance.

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