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Formation of a Hexagonal Lattice Structure by an R-Form Lipopolysaccharide of *Klebsiella* sp.

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We extracted an R-form lipopolysaccharide (LPS) by the phenol-water method from *Klebsiella* sp. strain LEN-111 (O3−:K1−) and followed the changes in ultrastructure of the LPS during the extraction procedure. When the LPS was obtained from the water phase of an extract by addition of 2 volumes of 10 mM $MgCl_2$-ethanol, it consisted of membrane pieces with a hexagonal lattice structure with a lattice constant of 14 to 15 nm. The lattice structure of the LPS was disrupted into short rods with sodium dodecyl sulfate, but the same hexagonal lattice structure was again formed by precipitation with 2 volumes of 10 mM $MgCl_2$-ethanol. The LPS preparation after two cycles of treatment by the phenol-water method, which contained no detectable amounts of proteins, kept an unaltered ability to form the hexagonal lattice structure. Extensive treatment with pronase and extraction with chloroform did not impair the ability of the LPS preparation to form the lattice structure. When the other salts, NaCl, $CaCl_2$ or $Zn(CH_3COO)_2$, were used for precipitation of the LPS with ethanol in place of $MgCl_2$, the LPS did not form the hexagonal lattice structure. However, if the LPS precipitated with NaCl-ethanol was converted to the magnesium salt form after it was electrodialyzed, it formed the same hexagonal lattice structure as the LPS precipitated with $MgCl_2$-ethanol. From these results, it was concluded that the R-form LPS has the ability of in vitro self-assembly into a hexagonal lattice structure in the presence of Mg^{2+} without the help of other components such as proteins and free lipids from outer membrane.

The cell wall lipopolysaccharides (LPS) of smooth gram-negative bacteria consist of the O-specific polysaccharide, which is linked to the core polysaccharide, which in turn is linked to the lipid moiety termed lipid A. R mutants are blocked in the biosynthesis of the O-specific polysaccharide in various steps. We previously studied the ultrastructure of *Klebsiella* O3 LPS isolated from *Klebsiella* sp. strain LEN-1 (O3:K1−) and showed that it consists principally of flat ribbonlike structures branching freely (average width, 16 nm; average thickness, 7 nm) and of a small proportion of spheres (diameter, 20 to 50 nm), both structures covered with fine hairy structures of an average length of approximately 10 nm (5). When the polysaccharide of *Klebsiella* O3 LPS was stained by periodic acid-thiosemicarbazide-silver proteinate (11), silver granules were deposited on the ribbonlike structures and around the spheres, suggesting that the O-specific polysaccharide chain is located on their surface and that the fine hairy structures consist of the polysaccharide chain (5). Later we isolated a mutant strain LEN-111 (O3−:K1−) which produces an LPS lacking the O-specific polysaccharide chain from *Klebsiella* sp. strain LEN-1. We also examined the ultrastructure of the R-form LPS (R LPS) extracted from *Klebsiella* sp. strain LEN-111 to determine the difference between the S-form LPS (S LPS) and R LPS. As a consequence, we found that the R LPS possesses the ability to form a hexagonal lattice structure without the help of any other outer membrane components. In this report we describe the experimental results.

MATERIALS AND METHODS

LPS. The LPS was extracted from the bacterial cells of a mutant strain LEN-111 lacking the O-specific polysaccharide chain (O3−:K1−) derived from *Klebsiella* sp. strain LEN-1 (O3:K1−) (8). The bacteria grown overnight in Trypticase soy broth were harvested by centrifugation at 6,500 × *g* for 10 min, washed twice with distilled water, and treated with 45% phenol at 69 to 70°C for 15 min by the method of Westphal and Jann (12). The water phase was dialyzed overnight against tap water to remove phenol and concentrated by an evaporator. Then, 2 volumes of ethanol containing 10 mM $MgCl_2$ was added, and the mixture was cooled at −20°C overnight. The precipitate formed was washed twice with cold 70% ethanol by centrifugation at 3,500 rpm (2,000 × *g*) for 15 min, suspended in distilled water, and centrifuged at 100,000 × *g* for 2 h. The precipitate was suspended in a small amount of distilled water and lyophilized.

Electron microscopy. The LPS was difficult to dissolve in distilled water, but the turbidity of the LPS suspension was found to decrease at pH 8.0 or higher. Unless otherwise stated, therefore, the preparations to be tested were suspended in 50 mM Tris hydrochloride (Tris buffer) (pH 8.0). However, when the electrodialyzed preparation and uniform salt forms of the LPS were examined, they were suspended in distilled water. All the preparations were negatively stained with ammonium molybdate. A drop of the preparation to be tested was placed on a carbon-coated grid, and after 5 min the excess liquid was removed by filter paper, and the grid was air dried and washed quickly with a drop of water. Then, the grid was covered with a drop of 1% ammonium molybdate, and after 5 min the excess liquid was removed by filter paper, leaving a thin film that was air dried. All the materials were examined with a Hitachi H500 electron microscope operating at 100 kV.

Elemental analyses by an analytical electron microscope. Metals (Na, Mg, K, Ca, Fe, and Zn), phosphorus, and chlorine in thin specimens were analyzed with a Hitachi H800 electron microscope fitted with a Kevex 7000-Q dis-

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perse energy silicon detector. The preparations to be tested were suspended in distilled water at a concentration of 1 mg/ml. A drop of the preparation was placed on a copper grid, and after 5 min the excess was removed by filter paper and air dried. All analyses were carried out at 100 kV with a beam current of 0.3 to 0.5 nA.

Electrodialysis of LPS and conversion to defined uniform salt forms of LPS. Galanos and Lüderitz (3) have shown that the physical state of LPS is determined by the type of basic ions present in the salt form with acidic groups in the LPS molecules and that with the aid of electrodialysis it is possible to obtain LPS in defined uniform salt forms. Electrodialysis, which removes a large amount of basic ions in the LPS molecules, leads to acidic LPS preparations, often with reduced solubility, and the acidic LPS preparations are made soluble by neutralization with various kinds of alkali. Various uniform salt forms of LPS can be prepared by this procedure (3). The magnesium and sodium salt forms of the LPS from *Klebsiella* sp. strain LEN-111 were prepared by this method. LPS (10 mg) suspended in 5 ml of distilled water was subjected to electrodialysis with a constant current of 20 mA as previously described (6). The water outside the dialysis tubing was changed every 30 min. The release of basic materials from the tubing finished within 1 h, but electrodialysis was performed for 4 h to remove intensively basic materials. At the end of electrodialysis, the pH of the content of the tubing had fallen to 3.6 to 3.8. To prepare the sodium salt form of the LPS, a portion of the electrodialyzed LPS was neutralized with 0.1 N NaOH to pH 7.4. Galanos and Lüderitz (3) used $Mg(OH)_2$ to prepare the magnesium salt form of LPS. In our study, however, $Mg(OH)_2$, which is difficult to dissolve in water, could not neutralize the electrodialyzed LPS. Therefore, we used a filtrate of the mixture of 1 M $MgCl_2$ and 1/10 volume of 0.1 N NaOH in place of $Mg(OH)_2$ to neutralize the electrodialyzed LPS. The sodium and magnesium salt forms of the LPS thus prepared were dialyzed for 3 days against distilled water to remove excess ions.

Optical diffraction. Optical diffraction of the electron micrographs was carried out by basically the method described by Erickson et al. (2) with the meridional reflections obtained with an Olympus eyepiece ruled into 500-μm squares as the magnification standard.

Analytical methods. UV absorption spectra for an aqueous solution of LPS suspensions were recorded by a Hitachi automatic spectrophotometer model 333. Protein contents in LPS preparations were estimated by the method of Lowry et al. (7) with recrystallized bovine serum albumin as the standard. Lipid content was determined by weighing the chloroform-soluble material.

RESULTS

Properties of the LPS from *Klebsiella* sp. strain LEN-111. *Klebsiella* sp. strain LEN-111 was a leaky mutant. When LPS was extracted from bacterial cells cultivated for longer than 2 days, bands corresponding to the O-specific polysaccharide chain were faintly detectable by silver staining after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, but the amounts were small since the O3 antigen was not detectable serologically, and mannose, the sole monosaccharide of the O-specific polysaccharide of the O3 antigen (1, 4), also was not chemically detectable. However, in LPS extracted from the bacterial cells cultivated overnight, the O-specific polysaccharide chain was not detectable by SDS-polyacrylamide gel electrophoresis. The LPS was confirmed serologically to consist of the R-specific core and lipid A. Investigation about the chemical structure of the R core and the lipid A moiety is now under way and will be reported elsewhere.

Formation of a hexagonal lattice structure by the LPS precipitated by addition of 2 volumes of 10 mM $MgCl_2$-ethanol. The LPS in the water phase immediately after treatment of the bacterial cells with 45% phenol consisted of doughnutlike vesicles having diameters of 50 to 110 nm (Fig. 1A). After dialysis against tap water and concentration by an evaporator, the LPS consisted of a network of ribbonlike structures branching freely. The width of the ribbonlike structures averaged 16 nm but narrowed to 7 nm where they seemed to be twisted (Fig. 1B). The narrow parts were likely the lateral views of the ribbonlike structures, and they were considered as their thickness. Then, 2 volumes of 10 mM $MgCl_2$-ethanol was added, and the mixture was cooled at −20°C overnight. The precipitate formed was washed with 70% ethanol, suspended in distilled water, and centrifuged at 100,000 × *g* for 2 h. The precipitate was lyophilized. The electron micrographs of the preparations before and after ultracentrifugation and those after lyophilization were essentially the same. They consisted of various sizes of membrane pieces with a hexagonal lattice structure (Fig. 1C and D). Optical diffraction was performed with the electron micrographs of the lyophilized preparation suspended in 50 mM Tris buffer (pH 8.0) (Fig. 2). The optical diffractogram of the lattice showed that the lattice was hexagonal, and the lattice constant was estimated to be 13.7 nm. The value approximately corresponded to those estimated directly from the electron micrographs, 14.2 ± 0.2 (standard error) nm in both Fig. 1C and D.

As described above, the mixture of the LPS and 10 mM $MgCl_2$-ethanol was usually cooled at −20°C overnight. However, when the mixture was cooled at 4°C overnight, quite the same results were obtained.

Disintegration by SDS and reassembly of a hexagonal lattice structure of the LPS. When the lyophilized LPS was suspended in 50 mM Tris buffer (pH 8.0) containing 0.5% SDS and boiled for a few seconds, the LPS was disrupted into short rods, the length of which was 20 to 120 nm each (Fig. 3A). Then, 2 volumes of 10 mM $MgCl_2$-ethanol was added, and the mixture was cooled at −20°C overnight. The precipitate formed was washed twice with cold 70% ethanol and centrifuged at 100,000 × *g* for 2 h. At this point the short rods had reassembled into membrane pieces with a hexagonal lattice structure with a lattice constant of 14.7 ± 0.2 nm (Fig. 3).

Effects of other components contaminating the LPS preparation on the formation of a hexagonal lattice structure. Nucleic acids were undetectable by analysis of UV absorption spectra in the LPS preparation used in the experiments described so far. We obtained another LPS preparation containing definite amounts (about 50%) of RNA as a contaminant. This LPS preparation did not show such a hexagonal lattice structure as shown above but consisted of a network of ribbonlike structures (Fig. 4A). To remove RNA, the LPS preparation was treated with 20 μg of RNase (Sigma Chemical Co., St. Louis, Mo.) per ml at 37°C for 2 h. Then, the LPS was again treated by the phenol-water method to remove RNase. The LPS was precipitated by addition of 2 volumes of 10 mM $MgCl_2$-ethanol, washed twice with 70% ethanol, centrifuged at 100,000 × *g* for 2 h, and lyophilized in the same way as described above. After the treatment, the LPS formed a hexagonal lattice structure with a lattice constant of 14.6 ± 0.5 nm (Fig. 4B). These

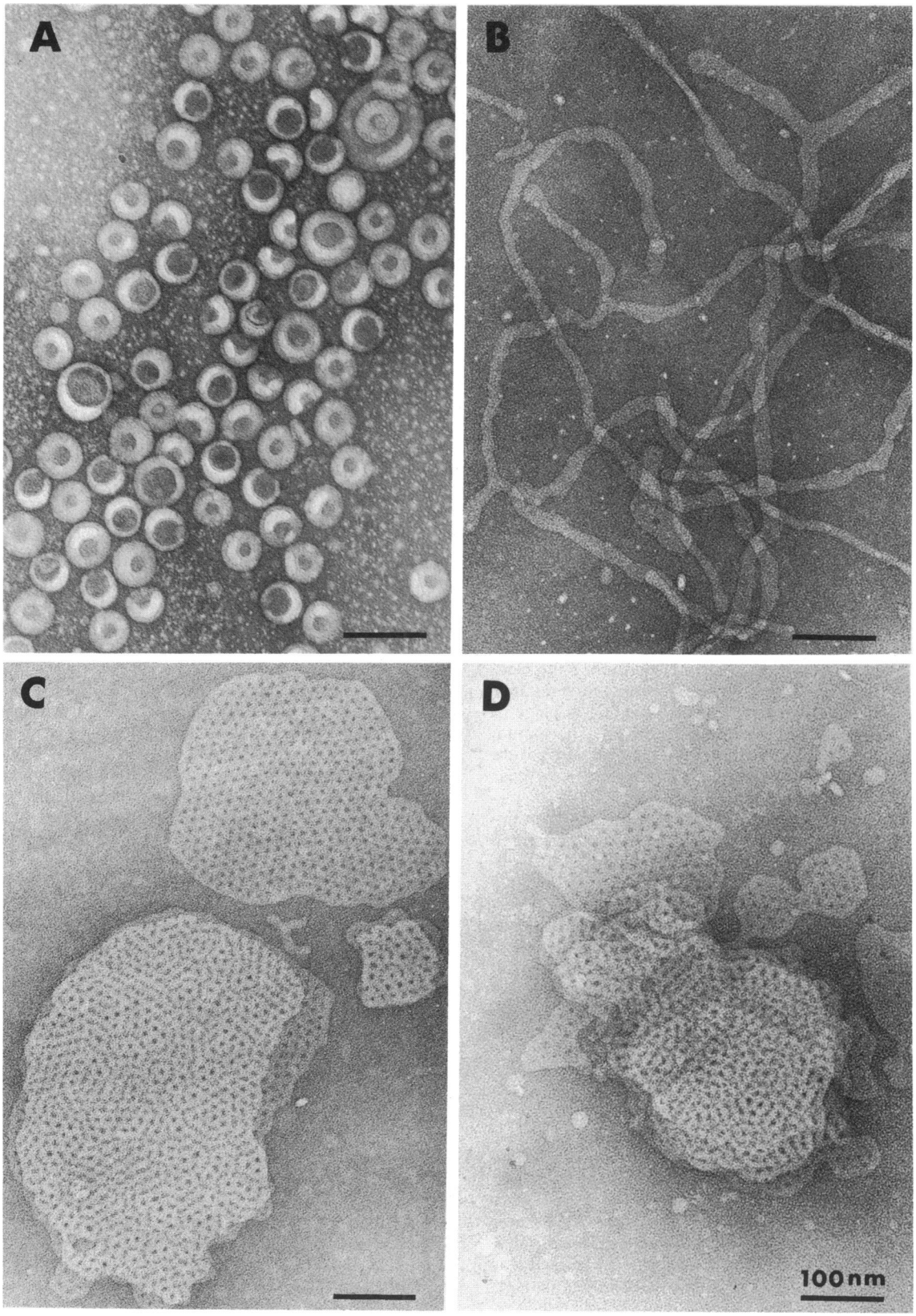

FIG. 1. The ultrastructures of the LPS from *Klebsiella* sp. strain LEN-111 at various steps of extraction by the phenol-water method. (A) The water phase immediately after extraction. (B) After dialysis and concentration by an evaporator. (C) After precipitation by addition of 2 volumes of 10 mM $MgCl_2$-ethanol, washing with 70% ethanol, and centrifugation at 100,000 × *g* for 2 h. (D) After lyophilization.

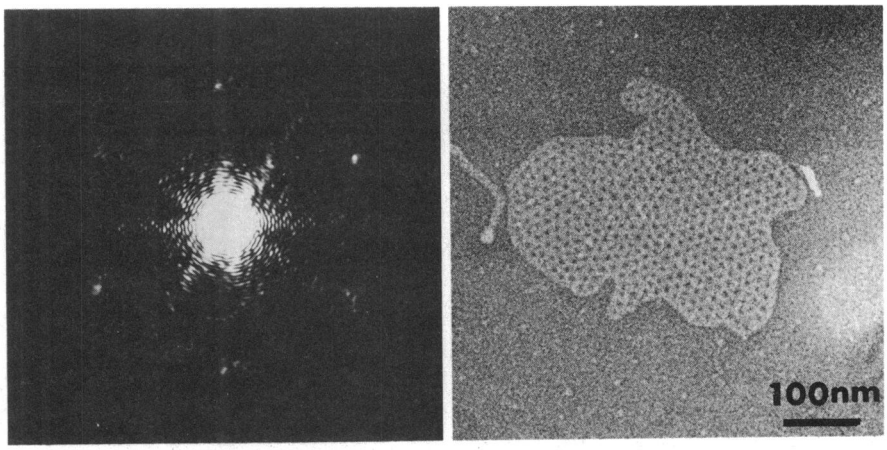

FIG. 2. The optical diffractogram of the LPS from *Klebsiella* sp. strain LEN-111 precipitated by addition of 2 volumes of 10 mM $MgCl_2$-ethanol. The same LPS preparation as shown in Fig. 1D was used. The area analyzed is shown in the right side.

results suggest that RNA contaminating the LPS preparation inhibits formation of the lattice structure.

The LPS preparation used in the experiment shown in Fig. 1 contained 1.1% proteins. However, the LPS preparation which was subjected to treatment with RNase and the second treatment by the phenol-water method no longer contained detectable amounts of proteins. On the other hand, in these two preparations there were no detectable contaminations with free lipids. The LPS preparation shown in Fig. 1D was subjected to two cycles of treatment with 0.1% pronase (Kaken Pharmaceutical Co., Ltd., Tokyo, Japan) at 37°C overnight followed by two cycles of treatment with chloroform, and its ability to form a hexagonal lattice structure was examined. After the treatment, the LPS preparation was first disrupted with 0.5% SDS, then precipitated by addition of 2 volumes of 10 mM $MgCl_2$-ethanol, washed

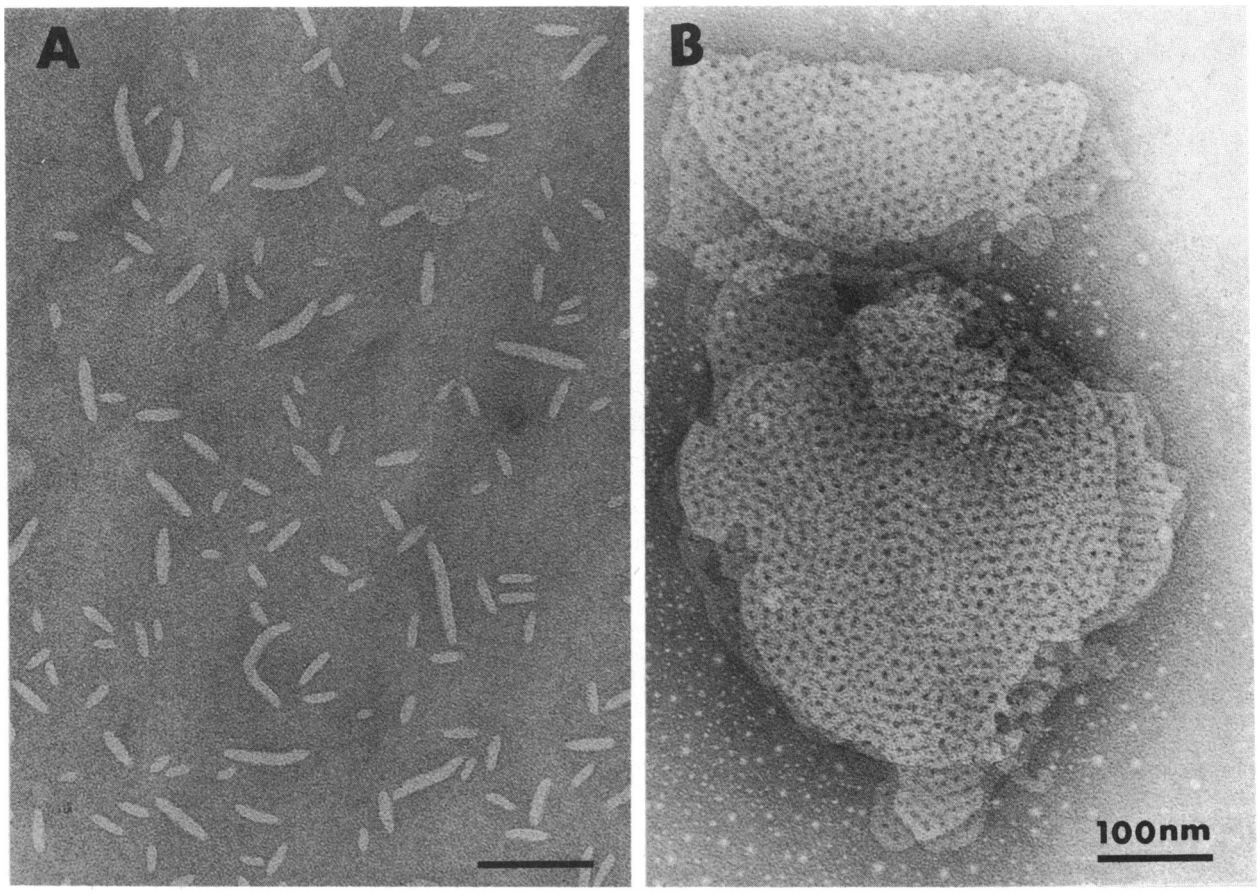

FIG. 3. Disintegration with SDS and reassembly of the LPS from *Klebsiella* sp. strain LEN-111 precipitated by addition of 2 volumes of 10 mM $MgCl_2$-ethanol. (A) After treatment with 0.5% SDS–50 mM Tris buffer (pH 8.0). (B) After precipitation by addition of 2 volumes of 10 mM $MgCl_2$-ethanol, washing with 70% ethanol, and centrifugation at 100,000 × *g* for 2 h.

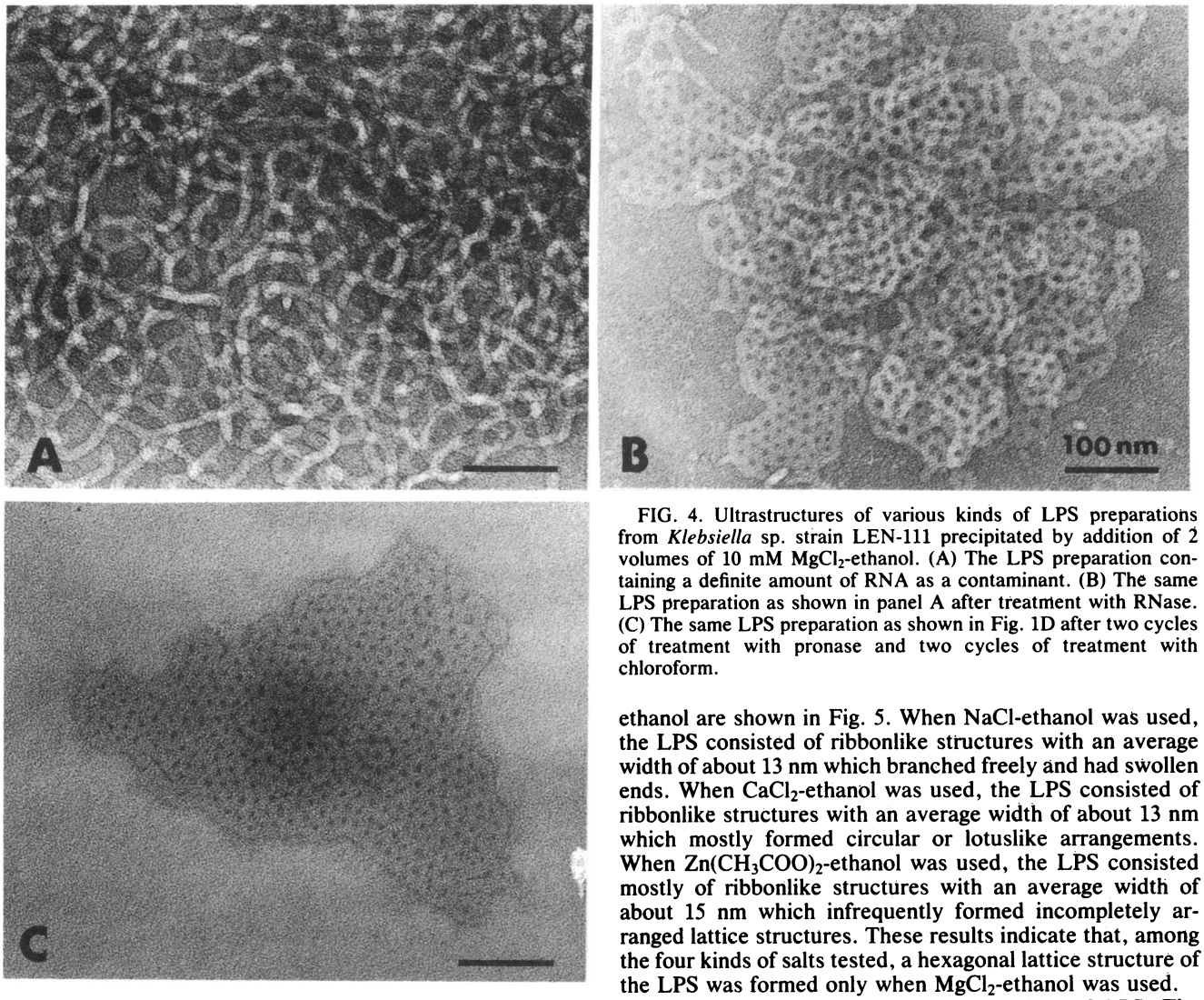

FIG. 4. Ultrastructures of various kinds of LPS preparations from *Klebsiella* sp. strain LEN-111 precipitated by addition of 2 volumes of 10 mM $MgCl_2$-ethanol. (A) The LPS preparation containing a definite amount of RNA as a contaminant. (B) The same LPS preparation as shown in panel A after treatment with RNase. (C) The same LPS preparation as shown in Fig. 1D after two cycles of treatment with pronase and two cycles of treatment with chloroform.

twice with 70% ethanol, centrifuged at 100,000 × *g* for 2 h, and lyophilized in the same way as described above. The LPS obtained was found to form a hexagonal lattice structure with a lattice constant of 15.1 ± 0.3 nm (Fig. 4C). It can therefore be concluded that proteins and free lipids which may contaminate the LPS preparation have no contribution to the formation of a hexagonal lattice structure by the LPS.

Effects of salts other than $MgCl_2$ on formation of hexagonal lattice structure of the LPS. To compare the effects of Mg^{2+} and other cations on formation of a hexagonal lattice structure, ethanol containing 10 mM NaCl, $CaCl_2$, or $Zn(CH_3COO)_2$ was used to precipitate the LPS from an extract in place of 10 mM $MgCl_2$-ethanol. The LPS was extracted by the phenol-water method, and the water phase was dialyzed overnight to remove phenol and treated with 20 μg of RNase per ml at 37°C for 2 h. The LPS was treated again by the phenol-water method to remove RNase. The LPS was precipitated by addition of 2 volumes of 10 mM NaCl-ethanol, $CaCl_2$-ethanol, or $Zn(CH_3COO)_2$-ethanol, washed twice with 70% ethanol, centrifuged at 100,000 × *g* for 2 h, and lyophilized in the same way as described above. The ultrastructures of the LPS preparations precipitated with 10 mM NaCl-ethanol, $CaCl_2$-ethanol, or $Zn(CH_3COO)_2$-ethanol are shown in Fig. 5. When NaCl-ethanol was used, the LPS consisted of ribbonlike structures with an average width of about 13 nm which branched freely and had swollen ends. When $CaCl_2$-ethanol was used, the LPS consisted of ribbonlike structures with an average width of about 13 nm which mostly formed circular or lotuslike arrangements. When $Zn(CH_3COO)_2$-ethanol was used, the LPS consisted mostly of ribbonlike structures with an average width of about 15 nm which infrequently formed incompletely arranged lattice structures. These results indicate that, among the four kinds of salts tested, a hexagonal lattice structure of the LPS was formed only when $MgCl_2$-ethanol was used.

Elemental analyses of various preparations of LPS. The elemental analyses were carried out with an analytical electron microscope with the various preparations of LPS tested for the ability to form a hexagonal lattice structure. Contents of metals, P, and Cl were measured (Fig. 6), and the atomic ratios of the elements to P were calculated (Table 1). The LPS precipitated by addition of 2 volumes of 10 mM $MgCl_2$-ethanol used for the elemental analyses was the same as that shown in Fig. 1C. Before use, however, it was treated with 20 μg of RNase per ml at 37°C for 2 h, then treated again by the phenol-water method to remove RNase, precipitated by addition of 2 volumes of $MgCl_2$-ethanol, washed twice with 70% ethanol, centrifuged at 100,000 × *g*, and lyophilized in the same way as described above. The LPS preparations precipitated by addition of 2 volumes of 10 mM NaCl-ethanol, $CaCl_2$-ethanol, or $Zn(CH_3COO)_2$-ethanol contained Mg, Na, Ca, or Zn, respectively, in the atomic ratios to P near to 1:1. The LPS precipitated with NaCl-ethanol also contained lesser amounts of Mg and Ca, and the LPS precipitated with $MgCl_2$-ethanol also contained a lesser amount of Ca. All the LPS preparations tested contained little amounts of Fe. These results indicate that the LPS combined in the greatest amount with the cation which was added into ethanol used for precipitation of the LPS.

Formation of a hexagonal lattice structure by the LPS which

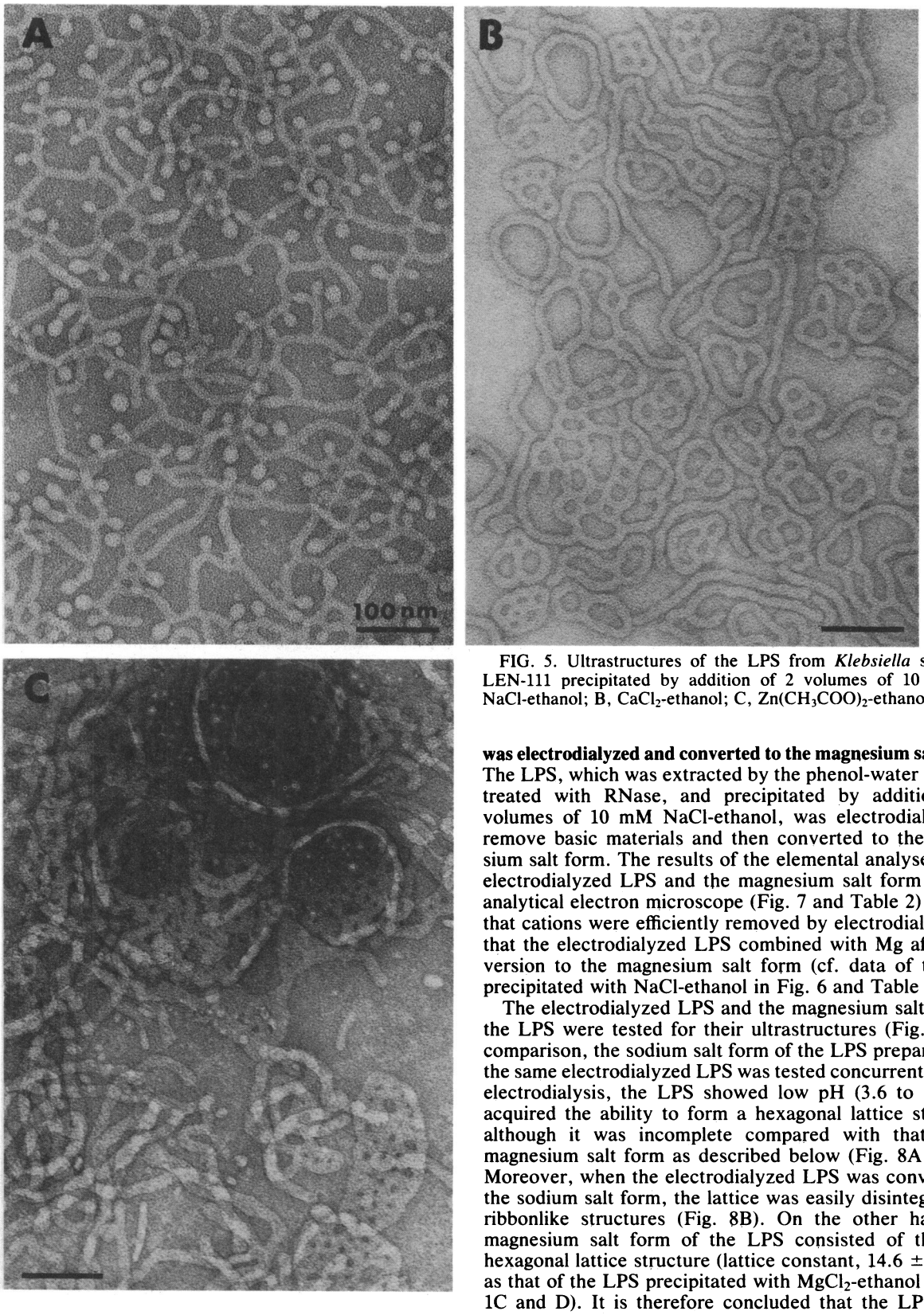

FIG. 5. Ultrastructures of the LPS from *Klebsiella* sp. strain LEN-111 precipitated by addition of 2 volumes of 10 mM: A, NaCl-ethanol; B, $CaCl_2$-ethanol; C, $Zn(CH_3COO)_2$-ethanol.

was electrodialyzed and converted to the magnesium salt form. The LPS, which was extracted by the phenol-water method, treated with RNase, and precipitated by addition of 2 volumes of 10 mM NaCl-ethanol, was electrodialyzed to remove basic materials and then converted to the magnesium salt form. The results of the elemental analyses of the electrodialyzed LPS and the magnesium salt form with an analytical electron microscope (Fig. 7 and Table 2) indicate that cations were efficiently removed by electrodialysis and that the electrodialyzed LPS combined with Mg after conversion to the magnesium salt form (cf. data of the LPS precipitated with NaCl-ethanol in Fig. 6 and Table 1).

The electrodialyzed LPS and the magnesium salt form of the LPS were tested for their ultrastructures (Fig. 8). For comparison, the sodium salt form of the LPS prepared from the same electrodialyzed LPS was tested concurrently. After electrodialysis, the LPS showed low pH (3.6 to 3.8) and acquired the ability to form a hexagonal lattice structure, although it was incomplete compared with that of the magnesium salt form as described below (Fig. 8A and C). Moreover, when the electrodialyzed LPS was converted to the sodium salt form, the lattice was easily disintegrated to ribbonlike structures (Fig. 8B). On the other hand, the magnesium salt form of the LPS consisted of the same hexagonal lattice structure (lattice constant, 14.6 ± 0.2 nm) as that of the LPS precipitated with $MgCl_2$-ethanol (cf. Fig. 1C and D). It is therefore concluded that the LPS, when converted to the magnesium salt form, forms a hexagonal lattice structure and that the treatment with ethanol is not an indispensable procedure for the formation of a hexagonal lattice structure of the LPS.

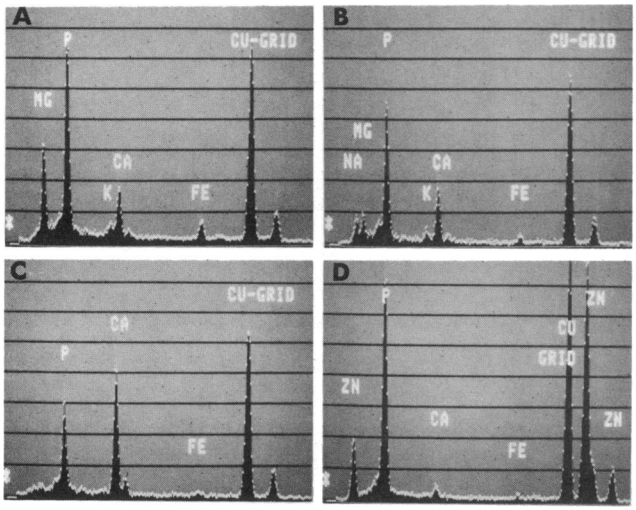

FIG. 6. Patterns of the elemental analyses by an analytical electron microscope with the LPS from *Klebsiella* sp. strain LEN-111 precipitated by addition of 2 volumes of 10 mM: A, $MgCl_2$-ethanol; B, NaCl-ethanol; C, $CaCl_2$-ethanol; D, $Zn(CH_3COO)_2$-ethanol. The preparation in panel A was the same as that shown in Fig. 1C, but it was treated with RNase before use for the analyses. The preparations shown in panels B, C, and D were the same as those shown in panels 5A, B, and C, respectively.

When the LPS precipitated with $Zn(CH_3COO)_2$-ethanol was used as the starting material in place of the LPS precipitated with NaCl-ethanol, essentially the same results were obtained (data not shown).

DISCUSSION

Ultrastructurally, the R LPS from *Klebsiella* sp. strain LEN-111 (O3−:K1−) immediately after extraction by the phenol-water method consists of doughnutlike small vesicles, and they are assembled into ribbonlike structures branching freely after removal of phenol by dialysis. Previously, we described the ultrastructure of the S LPS from the parent strain, *Klebsiella* sp. LEN-1 (O3:K1−) (5). The ribbonlike structures of the LPS at this step are very similar to those of the S LPS, although the latter is covered with fine hairy structures (average length, about 10 nm), which are considered to consist of the O-specific polysaccharide chains, but the former is not. The R LPS from *Klebsiella* sp. strain LEN-111 is assembled into a hexagonal lattice structure with a lattice constant of 14 to 15 nm either by precipitation with $MgCl_2$-ethanol or by conversion to the magnesium salt form after electrodialysis. The results of the present study clearly demonstrate that the R LPS has an ability of in vitro self-assembly into a hexagonal lattice structure in the presence of Mg without the help of the outer membrane components and that the formation of the lattice structure is reversible. As far as we are aware, the present paper is the first which reports this phenomenon.

TABLE 1. Contents of various elements in the LPS from *Klebsiella* sp. strain LEN-111 precipitated by addition of 2 volumes of 10 mM $MgCl_2$-ethanol, NaCl-ethanol, $CaCl_2$-ethanol, or $Zn(CH_3COO)_2$-ethanol[a]

Salt used for LPS precipitation	Contents (ratios)[b]							
	Na	Mg	P	Cl	K	Ca	Fe	Zn
$MgCl_2$	—[c]	0.89	1.00	—	<0.01	0.17	0.09	—
NaCl	0.78	0.47	1.00	—	0.05	0.31	0.07	—
$CaCl_2$	—	—	1.00	—	—	1.14	0.15	—
$Zn(CH_3COO)_2$	—	—	1.00	—	—	0.03	0.02	0.96

[a] The data were obtained from the results of the elemental analyses shown in Fig. 6.
[b] Contents of the elements are expressed as the atomic ratios to P.
[c] —, Undetectable.

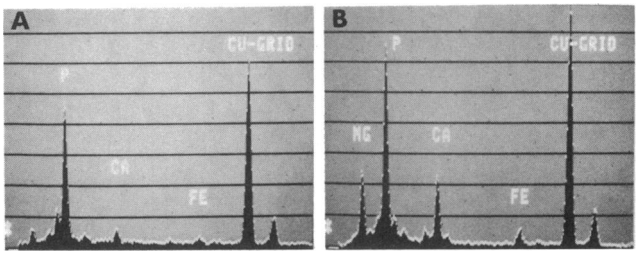

FIG. 7. Patterns of the elemental analyses by an analytical electron microscope with the LPS from *Klebsiella* sp. strain LEN-111 after electrodialysis and its magnesium salt form. The starting material used was the LPS which was precipitated by addition of 2 volumes of 10 mM NaCl-ethanol. (A) The electrodialyzed LPS. (B) The magnesium salt form.

The elemental analyses demonstrate that the LPS can combine with cations, such as Mg^{2+}, Ca^{2+}, Zn^{2+}, and Na^+, which are added into ethanol used for precipitation of the LPS. The LPS precipitated with $MgCl_2$-ethanol shows an atomic ratio between Mg and P similar to that of the LPS converted to the magnesium salt form after electrodialysis. These results suggest that precipitation of the LPS with ethanol containing the cations can also produce the respective salt forms of LPS. When NaCl-ethanol was used for precipitation of the LPS, the LPS combined with Na in the greatest amount, but it combined also with significant amounts of Mg and Ca. Mg and Ca which were combined with the LPS preparation were probably derived from the phenol extract of bacterial cells. It is suggested that the LPS combines with the divalent cations more easily than Na^+ because the concentrations of Mg^{2+} and Ca^{2+} present in the extract may be much lower than that of Na^+ added. In fact, the LPS precipitated with $CaCl_2$-ethanol contained no detectable amount of Mg, and the LPS precipitated with $Zn(CH_3COO)_2$-ethanol contained no detectable or very small amounts of Mg and Ca.

Rosenbusch (9) and Steven et al. (10) reported that one of the major proteins (matrix protein) constituted an orderly hexagonal lattice structure with a lattice constant of 7.7 nm on the peptidoglycan layer of *Escherichia coli* B and that the orderly structure was maintained in the absence of the peptidoglycan. Yamada and Mizushima (13) found that an orderly hexagonal lattice structure with a lattice constant of

TABLE 2. Contents of various elements in the LPS from *Klebsiella* sp. strain LEN-111 after electrodialysis and its magnesium salt form[a]

Material	Contents (ratios)[b]							
	Na	Mg	P	Cl	K	Ca	Fe	Zn
Electrodialyzed LPS	—[c]	—	1.00	—	—	0.06	0.07	—
Mg salt form	—	0.71	1.00	0.04	—	0.24	—	—

[a] The data were obtained from the results of the elemental analyses shown in Fig. 7.
[b] Contents of the elements are expressed as the atomic ratios to P.
[c] —, Undetectable.

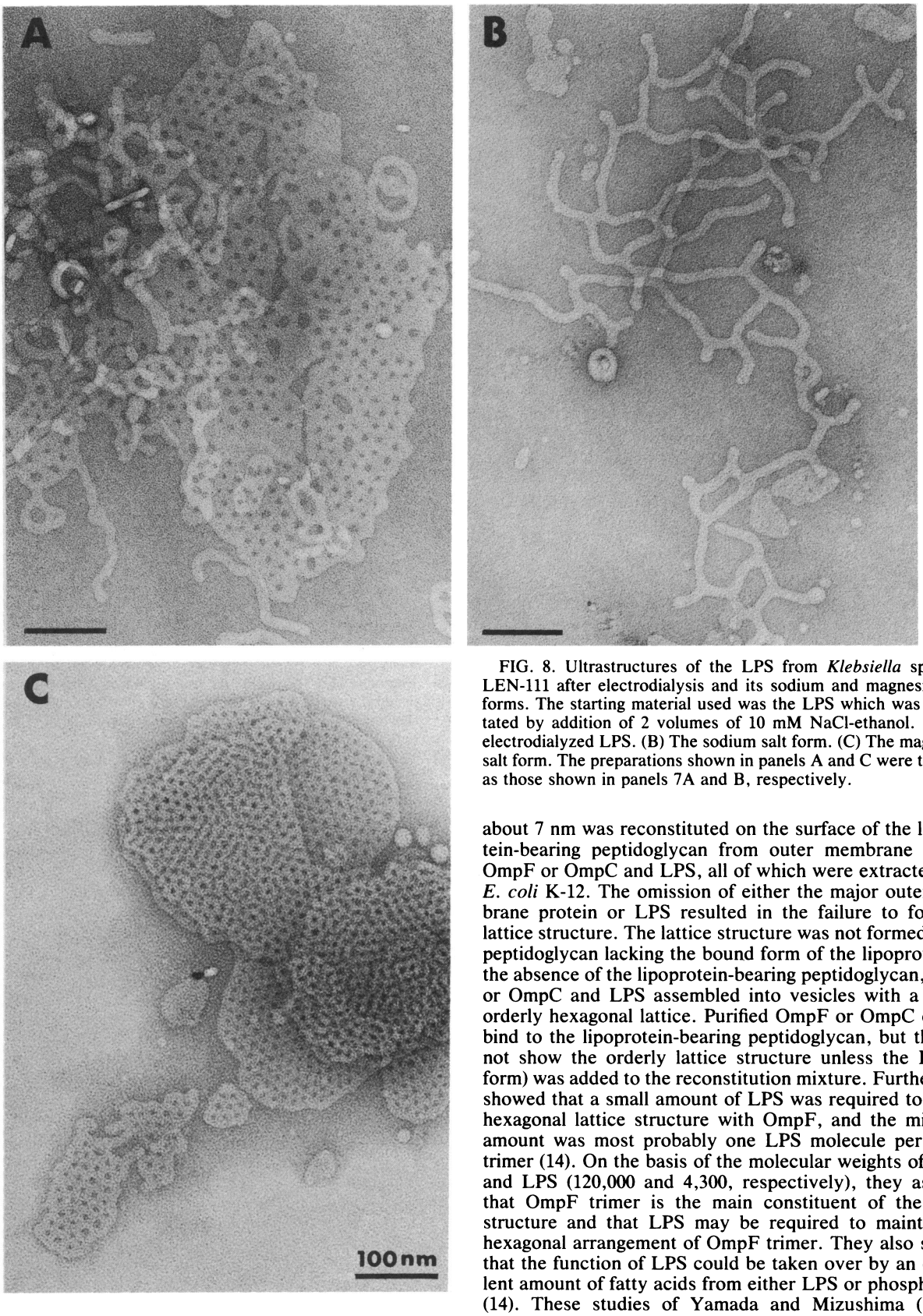

FIG. 8. Ultrastructures of the LPS from *Klebsiella* sp. strain LEN-111 after electrodialysis and its sodium and magnesium salt forms. The starting material used was the LPS which was precipitated by addition of 2 volumes of 10 mM NaCl-ethanol. (A) The electrodialyzed LPS. (B) The sodium salt form. (C) The magnesium salt form. The preparations shown in panels A and C were the same as those shown in panels 7A and B, respectively.

about 7 nm was reconstituted on the surface of the lipoprotein-bearing peptidoglycan from outer membrane protein OmpF or OmpC and LPS, all of which were extracted from *E. coli* K-12. The omission of either the major outer membrane protein or LPS resulted in the failure to form the lattice structure. The lattice structure was not formed on the peptidoglycan lacking the bound form of the lipoprotein. In the absence of the lipoprotein-bearing peptidoglycan, OmpF or OmpC and LPS assembled into vesicles with a similar orderly hexagonal lattice. Purified OmpF or OmpC or both bind to the lipoprotein-bearing peptidoglycan, but they did not show the orderly lattice structure unless the LPS (R form) was added to the reconstitution mixture. Further, they showed that a small amount of LPS was required to form a hexagonal lattice structure with OmpF, and the minimum amount was most probably one LPS molecule per OmpF trimer (14). On the basis of the molecular weights of OmpF and LPS (120,000 and 4,300, respectively), they assumed that OmpF trimer is the main constituent of the lattice structure and that LPS may be required to maintain the hexagonal arrangement of OmpF trimer. They also showed that the function of LPS could be taken over by an equivalent amount of fatty acids from either LPS or phospholipids (14). These studies of Yamada and Mizushima (13, 14) suggest that the major outer membrane proteins are the principal constituents of the basal framework of the cell surface of *E. coli*.

At present we cannot explain the relationship between the results of our present study and those of the reconstitution experiments by Yamada and Mizushima (13, 14). Our present results indicating that there can be a LPS from a bacterial species of *Enterobacteriaceae* which is capable of forming a hexagonal lattice structure without the help of any other outer membrane components may suggest an important role of LPS in the constitution of the basal framework of the outer membrane. To answer the question, we shall have to determine whether the hexagonal assembly of LPS is a natural arrangement in the outer membrane of gram-negative bacteria.

The S LPS from *Klebsiella* sp. strain LEN-1 (O3:Kl−) failed to form the hexagonal lattice structure even when it was extracted by the same procedure as used for the R LPS from *Klebsiella* sp. strain LEN-111 (O3−:K−) (5). Thus, the R-specific core seems to play an important role in the formation of such a hexagonal lattice structure of the R LPS in the reconstitution experiments in vitro. In *E. coli* and *Salmonella*, there are various kinds of mutants which are deficient in the synthesis of the R-specific core at different steps. Studies to clarify the relationship between the ability of LPS to form the hexagonal lattice structure and the structure of the R-specific core are in progress in this laboratory.

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LITERATURE CITED

1. **Curvall, M., B. Lindberg, J. Lönngren, and W. Nimmich.** 1973. Structural studies on the *Klebsiella* O group 3 lipopolysaccharide. Acta Chem. Scand. **27**:2645–2649.
2. **Erickson, H. P., W. A. Voter, and K. Leonard.** 1978. Image reconstruction in electron microscopy: enhancement of periodic structure by optical filtering. Methods Enzymol. **49**:39–66.
3. **Galanos, C., and O. Lüderitz.** 1975. Electrodialysis of lipopolysaccharides and their conversion to uniform salt forms. Eur. J. Biochem. **54**:603–610.
4. **Hasegawa, T., M. Ohta, M. Mori, I. Nakashima, and N. Kato.** 1983. The *Klebsiella* O3 lipopolysaccharide isolated from culture fluid: structure of the polysaccharide moiety. Microbiol. Immunol. **27**:683–694.
5. **Kato, N., M. Ohta, N. Kido, S. Naito, and T. Kuno.** 1984. Ultrastructure of *Klebsiella* O3 lipopolysaccharide isolated from culture supernatant: comparison with other lipopolysaccharides. Microbiol. Immunol. **28**:545–557.
6. **Kato, N., M. Ohta, N. Kido, S. Naito, and T. Kuno.** 1984. Ultrastructure of *Klebsiella* O3 lipopolysaccharide isolated from culture supernatant: structure of various uniform salt forms. Microbiol. Immunol. **28**:559–567.
7. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
8. **Ohta, M., M. Mori, T. Hasegawa, F. Nagase, I. Nakashima, S. Naito, and N. Kato.** 1981. Further studies of the polysaccharide of *Klebsiella pneumoniae* possessing strong adjuvanticity. I. Production of the adjuvant polysaccharide by noncapsulated mutant. Microbiol. Immunol. **25**:939–948.
9. **Rosenbusch, J. P.** 1974. Characterization of the major envelope protein from *Escherichia coli*. J. Biol. Chem. **249**:8019–8029.
10. **Steven, A. C., B. Ten Heggeler, R. Müller, J. Kietler, and J. P. Rosenbusch.** 1977. Ultrastructure of a periodic protein layer in the outer membrane of *Escherichia coli*. J. Cell Biol. **72**:292–301.
11. **Thiéry, J. P.** 1967. Mise en evidence des polysaccharides sur coupes fines en microscopie electronique. J. Microscop. (Paris) **6**:987–1018.
12. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides—extraction with phenol water and further application of the procedure, p. 83–91. *In* R. L. Whistler (ed.), Methods in carbohydrate chemistry, vol. 5. Academic Press, Inc., New York.
13. **Yamada, H., and S. Mizushima.** 1978. Reconstitution of an ordered structure from major outer membrane constituents and the lipoprotein-bearing peptidoglycan sacculus of *Escherichia coli*. J. Bacteriol. **135**:1024–1031.
14. **Yamada, H., and S. Mizushima.** 1980. Interaction between major outer membrane protein (O-8) and lipopolysaccharide in *Escherichia coli* K12. Eur. J. Biochem. **103**:209–218.