Quantitation of Metal Cations Bound to Membranes and Extracted Lipopolysaccharide of *Escherichia coli*†

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ABSTRACT: Inductively coupled plasma emission spectroscopy was used to quantitate the metal cations bound to outer and cytoplasmic membranes and to extracted lipopolysaccharide from several *Escherichia coli* K12 strains. The outer membrane was found to be enriched in both calcium and magnesium relative to the cytoplasmic membrane. Both membranes contained significant levels of iron, aluminum, and zinc. The multivalent cation content of the lipopolysaccharide resembled that of the intact outer membrane. Lipopolysaccharide extracted from wild-type k12 strains contained higher levels of Mg than Ca regardless of the growth medium, but the medium used for growth did affect the relative amounts of bound Mg as well as the levels of the minor cations iron, aluminum, and zinc. In contrast, lipopolysaccharide isolated from a deep rough mutant strain, D21f2, contained more Ca than Mg. Electrodialysis of lipopolysaccharide from wild-type k12 strains removed 1 mol of Mg per mol of lipopolysaccharide but did not significantly affect the level of other bound metal ions. Dialysis of lipopolysaccharide against sodium (ethylenedinitrilo)tetraacetate removed most of the Mg and Ca, resulting in a sodium salt. The equimolar replacement of divalent cations with sodium in the sodium salt resulted in a net loss of counterion change. The sodium salt was dialyzed against either tris(hydroxymethyl)aminomethane hydrochloride, $CaCl_2$, $MgCl_2$, or $TbCl_3$, and the resulting lipopolysaccharide salts were analyzed for their ionic composition. It was shown that tris(hydroxymethyl)aminomethane and Ca can replace some but not all of the Na bound to the sodium salt, but all of the other multivalent cations tested replaced Na, resulting in uniform lipopolysaccharide salts. Lipopolysaccharide isolated from the deep rough mutant strain D21f2 was also converted into a sodium salt. Relative to the wild-type lipopolysaccharide, Na was able to neutralize the anionic charge to a greater extent in the mutant lipopolysaccharide. Our results suggest that the loss of specific groups in the core region of the lipopolysaccharide from the mutant strain results in a more open structure that allows the binding of larger cations and of more monovalent cations.

Divalent cations play an important role in the stabilization of the outer membrane of Gram-negative bacteria. In addition to reduction of charge repulsion between the highly anionic lipopolysaccharide (LPS)[1] molecules, divalent cations are thought to bridge adjacent LPS molecules and to link LPS with membrane proteins (van Alphen et al., 1978). The formation of complexes of outer membrane protein and LPS requires divalent cations such as magnesium (Nakamura & Mizushima, 1975). Chelating agents such as (ethylenedinitrilo)tetraacetic acid (EDTA) effect the release of up to 50% of the LPS from whole cells (Leive, 1974).

The physiochemical and pathophysiological properties of isolated LPS are also influenced by the ions bound. The morphology and aqueous solubility of LPS aggregates vary greatly depending on the salt form (Galanos & Lüderitz, 1975). As a result, the anticomplement activity of the soluble sodium salt of LPS from *Salmonella abortus* is much greater than that of the highly aggregated triethylamine salt (Galanos & Lüderitz, 1976). It has also been observed that mice which have been injected with iron salts prior to LPS administration are resistant to endotoxin stress (Snyder et al., 1977). Transition metal salts of LPS generally are only slightly soluble and have low toxicity (Prigal et al., 1973).

The tenacity with which LPS binds cations has been previously noted (Schindler & Osborn, 1979). Even after extensive dialysis, LPS remains complexed with a variety of cations. Although monovalent cations and several polyamines may be removed by electrodialysis, most of the tightly associated divalent cations remain bound (Galanos & Lüderitz, 1975; Coughlin et al., 1981). In this study, we have quantitated the levels of metal cations bound to isolated membranes and extracted LPS in an attempt to better characterize cation–LPS interactions.

Materials and Methods

Growth Conditions and Procedures for Membrane and LPS Isolation. Escherichia coli k12 strains W1485F⁻, D21, and D21f2 were grown at 37 °C either in M9 minimal medium plus 0.4% glucose or in nutrient broth (1% tryptone, 0.2% yeast extract, and 0.4% NaCl). Outer and cytoplasmic membranes were isolated (Janoff et al., 1979), and the purity of the isolates equalled or exceeded that described earlier. LPS was isolated by using either hot aqueous phenol (Westphal et al., 1952) or chloroform–petroleum ether (Galanos et al., 1969) extraction procedures. Extensive dialysis against double-distilled water yielded the native LPS (nLPS) product. Electrodialysis of the nLPS resulted in an acidic product which was analyzed in the acidic form (edLPS). The sodium salt of LPS, NaLPS, was obtained by dialysis of edLPS against three to five changes of 10 mM NaEDTA, pH 7.0 at 4 °C, followed by extensive

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[1] Abbreviations: LPS, lipopolysaccharide(s); EDTA, (ethylenedinitrilo)tetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; KDO, 3-deoxy-D-*manno*-octulosonic acid; nLPS, native LPS; edLPS, electrodialyzed LPS; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate.

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dialysis against double-distilled water. Other salt forms were obtained by dialysis of the sodium salt against 10 mM Tris-HCl, $CaCl_2$, $MgCl_2$, or $TbCl_3$, followed by extensive dialysis against double-distilled water. All LPS salts were shown to be neutral in pH.

Porin–LPS–peptidoglycan complexes were isolated from cells grown in nutrient broth and harvested as previously described (Janoff et al., 1979). Cells were washed in 10 mM NaHepes, pH 7.5, and lysed with a French pressure cell. Deoxyribonuclease I (Sigma Chemical Co.) was added at 20 μg/mL, and total membranes were pelleted and washed twice with distilled water. The porin–LPS–peptidoglycan complex was isolated by extracting the total membrane fraction with 1% sodium dodecyl sulfate ($NaDodSO_4$) twice and washing the complex extensively with distilled water.

Assays. 3-Deoxy-D-*manno*-octulosonic acid (KDO) was assayed by using the method of Dröge et al. (1970) to quantitate LPS recovery. Protein concentrations were determined by using the procedure of Lowry et al. (1954). Membrane proteins were characterized on $NaDodSO_4$–polyacrylamide slab gels as previously described (Janoff et al., 1979). Peptidoglycan was quantitated by assaying the levels of muramic acid (Hadzija, 1974).

Sample Ashing Procedures for Elemental Analysis. Wet ashing was accomplished by combining equal volumes of sample and concentrated nitric acid (Ultra R, Atomergic Chemicals Corp.) in a 15-mL Teflon screw-cap vial (Tuftainer, Pierce Chemical Co.). Samples were incubated at 70–75 °C for 24 h. Cobalt was added to give a final concentration of 1 μg/mL, and ashed samples were diluted to give a 15% (v/v) acid solution.

Dry ashing was accomplished by transferring the sample to a quartz crucible, lyophilizing, and then heating in a muffle furnace. The samples were ashed at 500 °C for 24 h. After dilution with water, the samples were filtered through acid-washed glass wool and analyzed with cobalt as an internal reference standard.

Samples of nLPS and edLPS were prepared for elemental analysis by both wet and dry ashing procedures. For convenience, most elemental analyses were carried out on wet ashed material, since analyses of dry material yielded virtually identical results.

Elemental Analysis. Quantitative elemental analysis was accomplished by plasma emission spectroscopy using a Jarrell-Ash Model 955 Atomcomp spectrometer with an $N + 1$ variable-wavelength accessory. The observation wavelengths (nanometers) for the specific elements were set as follows: Ca, 370.6; Mg, 279.0; P, 214.9; Na, 330.3; K, 766.5; Fe, 259.9; Al, 308.2; Zn, 213.8; Co, 228.6; Tb, 350.9. Potassium and terbium were quantitated on the $N + 1$ channel. The area around each emission line was examined for spectral interference and background effects by dynamic profiling. Profiles were obtained by manual micrometer adjustment of the entrance slit position. Added cobalt served as an internal standard to correct for changes in nebulizer spray characteristics due to variations in viscosity and/or dissolved solids. Samples were introduced into the nebulizer at a rate of 1.5 mL/min by a peristaltic pump. Elemental analysis results presented here are the average of measurements on three or more individual isolates unless indicated otherwise. Metal cation levels are expressed as molar ratios of the cation to phosphorus.

Results

Membrane Ionic Composition. Since multivalent cations are reported to be critical in stabilizing the structure of the outer membrane in Gram-negative bacteria, the levels of ions bound to outer and cytoplasmic membranes of *E. coli* were characterized and compared. The outer membrane contained 3 times the levels of divalent cations on a per phosphorus basis compared to the cytoplasmic membrane (Table I). Furthermore, both membranes were shown to contain small but significant levels of iron, aluminum and zinc. The high levels of divalent cations bound to outer membranes presumably result from the presence of anionic LPS in the outer monolayer and from the underlying anionic peptidoglycan. Note that the multivalent ion composition of nLPS was similar to that of the intact outer membrane (Table II).

A porin–LPS–peptidoglycan complex was purified and characterized to determine whether certain cations are involved in the interaction of outer membrane proteins with other membrane structures. This complex was shown to contain approximately 70% protein, 16% LPS, and between 7 and 15% peptidoglycan by weight. $NaDodSO_4$–polyacrylamide gels showed that the protein in the complex was comprised of approximately equal amounts of porins 1a and 1b with only small amounts of contaminating protein (data not shown). As indicated in Table III, this complex was enriched in divalent

Table I: Elemental Composition of Membranes from *E. coli* Strain W1485F⁻ Grown in Minimal Medium[a]

element/phosphorus (molar ratio)	cytoplasmic membrane	outer membrane
Ca/P	0.03 ± 0.01	0.09 ± 0.03
Mg/P	0.15 ± 0.04	0.45 ± 0.04
(Ca + Mg)/P	0.18 ± 0.05	0.54 ± 0.07
Fe/P	0.006 ± 0.003	0.009 ± 0.005
Al/P	0.006 ± 0.004	0.009 ± 0.002
Zn/P	0.0014 ± 0.0005	0.0006 ± 0.0002

[a] Average of four isolates ± SD.

Table II: Elemental Composition of LPS from *E. coli* Strain W1485F⁻ Grown in either Nutrient or Minimal Medium

element/ phosphorus (molar ratio)	nutrient medium		M9 glucose minimal medium	
	nLPS[a]	edLPS[a]	nLPS[b]	edLPS[a]
Ca/P	0.116 ± 0.003	0.135 ± 0.004	0.12	0.12 ± 0.02
Mg/P	0.33 ± 0.01	0.21 ± 0.03	0.43	0.30 ± 0.02
(Ca + Mg)/P	0.45 ± 0.01	0.35 ± 0.03	0.55	0.42 ± 0.04
Na/P	0.09 ± 0.05	0.04 ± 0.04	0.085	0.06 ± 0.02
Fe/P	0.04 ± 0.01	0.02 ± 0.01	0.01	0.02 ± 0.01
Al/P	0.01 ± 0.01	0.012 ± 0.001	0.02	0.01 ± 0.01
Zn/P	0.01 ± 0.00	0.012 ± 0.001	0.002	0.004 ± 0.001
+/P[c]	1.11	0.85	1.28	1.00
P/3KDO	7.3 ± 0.7	7.7 ± 0.4	ND[d]	ND

[a] Average of four isolates ± SD. [b] One isolate was analyzed. [c] Total positive charges per phosphorus. [d] Not determined.

Table III: Elemental Composition of LPS and of Porin–LPS–Peptidoglycan Complexes from *E. coli* Strain D21 Grown in Nutrient Broth[a]

element/phosphorus (molar ratio)	porin–LPS–peptidoglycan complex	native LPS
Ca/P	0.12 ± 0.01	0.116 ± 0.003
Mg/P	0.61 ± 0.04	0.33 ± 0.01
(Ca + Mg)/P	0.73 ± 0.05	0.45 ± 0.01
Na/P	0.65 ± 0.08	0.09 ± 0.05
Fe/P	0.07 ± 0.05	0.04 ± 0.01
Al/P	0.02 ± 0.01	0.01 ± 0.01
Zn/P	0.010 ± 0.004	0.01 ± 0.00

[a] Average of three isolates ± SD.

Table IV: Elemental Composition of Specific Salts of LPS from *E. coli* Strain D21 Grown in Nutrient Medium[a]

element/phosphorus (molar ratio)	NaLPS	Tris-LPS	CaLPS	MgLPS	TbLPS
Ca/P	0.0055 ± 0.0006	0.03 ± 0.01	0.56 ± 0.04	0.002 ± 0.001	0.0005 ± 0.0007
Mg/P	0.054 ± 0.008	0.05 ± 0.011	0.009 ± 0.005	0.78 ± 0.01	0.015 ± 0.004
(Ca + Mg)/P	0.060 ± 0.009	0.08 ± 0.02	0.57 ± 0.05	0.78 ± 0.01	0.015 ± 0.005
Na/P	0.54 ± 0.03	0.17 ± 0.05	0.2 ± 0.1	0.07 ± 0.04	0.06 ± 0.04
Fe/P	0.015 ± 0.003	0.05 ± 0.04	0.027 ± 0.006	0.003 ± 0.001	0.008 ± 0.004
Al/P	0.011 ± 0.005	0.01 ± 0.01	0.015 ± 0.005	0.002 ± 0.001	0.09 ± 0.02
Zn/P	0.003 ± 0.0003	0.001 ± 0.001	0.005 ± 0.003	0	0.001 ± 0.001
Tb/P	ND[b]	ND	ND	ND	0.28 ± 0.01
total +/P	0.74	0.52	1.50	1.66	1.24
P/3KDO	9.99 ± 1.11	ND	ND	8.7 ± 0.5	ND

[a] Average of three isolates ± SD. [b] Not determined.

and monovalent cations compared to nLPS. Specifically, the Mg content of the complex was nearly twice the amount found in the purified LPS and in the intact outer membrane. However, it could not be determined from these data whether the excess cationic charge was bound to protein, to the anionic peptidoglycan layer, or between the anionic groups within the complex.

LPS Ionic Composition. LPS isolated from *E. coli* strain W1485F⁻ by using either hot aqueous phenol or chloroform–petroleum ether methods resulted in nLPS preparations which had essentially identical ionic compositions. The nLPS isolated under these conditions contained between 7 and 8 mol of phosphorus per 3 mol of KDO (Table II) consistent with the findings of Jansson et al. (1979). Figure 1 indicates the reported position of the phosphate groups in the LPS structure. With the assumption of 7.5 phosphate groups per LPS molecule, elemental analysis of nLPS from cells grown in nutrient broth indicated that between four and five metal cations were bound to each LPS molecule with at least three of these being either calcium or magnesium. In an attempt to remove weakly bound cations including organic amines, the LPS was electrodialyzed, and the ionic composition of the acidic form (pH 3.5–4.0) is given in Table II. Electrodialysis consistently removed 1 mol of Mg per mol of LPS while the levels of all other metal cations were unaffected. The results suggest that the native form of LPS from *E. coli* W1485F⁻ grown in nutrient medium contains approximately 1 mol of calcium and between 2 and 3 mol of magnesium per mol of LPS, and one of the magnesium ions is removed with electrodialysis. Furthermore, this LPS contains 1 mol total of iron, aluminum, and zinc per 2 mol of LPS, and these ions are not removed with electrodialysis.

Effects of Growth Media on LPS Ionic Composition. Cells were also grown in M9 minimal medium containing 0.4% glucose to assess whether the growth medium influences the ionic content of LPS, and the LPS was extracted and analyzed. The levels of metal cations present in the extracted LPS from cells grown in the two media are shown in Table II. The differences in the ionic content of the two LPS isolates reflect to varying extents the differences in the ionic and phosphorus contents of the two media. From elemental analysis, the minimal medium was found to be higher in Mg, Na, K, and P content compared to the nutrient broth, whereas the Ca concentrations were similar (data not shown). Thus, LPS isolated from cells grown in minimal medium was higher in the level of total divalent cations bound per phosphorus compared to LPS isolated from cells grown in nutrient broth. The difference in bound divalent cations, mainly in Mg levels, may reflect the differences in phosphorus and magnesium levels in the two media. It has been reported that the level of phosphorylation of LPS is sensitive to the level of phosphate in the growth media (Rosner et al., 1979).

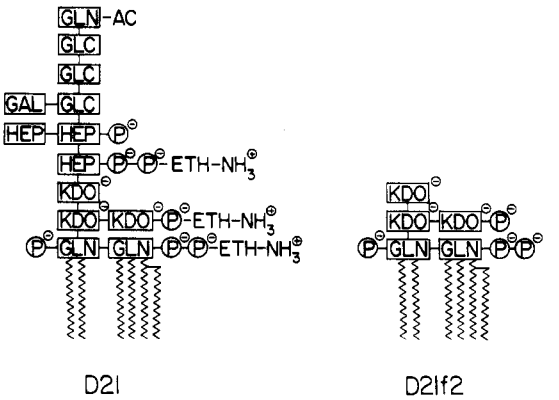

FIGURE 1: Proposed structure of lipopolysaccharide from *Escherichia coli* k12 strains D21 (rough) and D21f2 (deep rough). Abbreviations: GLC, glucose; GAL, galactose; HEP, L-*glycero*-D-*manno*-heptose; KDO, 3-deoxy-D-*manno*-octulosonic acid; GLN-AC, *N*-acetylglucosamine; ETH-NH_3, ethanolamine; P, phosphate. The structure of the polysaccharide core structure of LPS of strain D21 is taken from Jansson et al. (1981), and the phosphate substitution of the lipid A head group is inferred to be similar to that of *Salmonella typhimurium* (Mühlradt et al., 1977) except for the absence of arabinosamine on the 4′-phosphate (Lüderitz et al., 1978). The structure of LPS from strain D21f2 is inferred from composition analysis (Mayer et al., 1976), from ^{31}P NMR analysis (Rosner et al., 1979), and from our own analysis of KDO/P molar ratios (Table V) of LPS from *E. coli* heptoseless mutant strains.

LPS Salt Preparation and Characterization. Dialysis of electrodialyzed LPS against NaEDTA, pH 7.0, resulted in replacement of essentially all of the divalent cations with sodium (Table IV). Extensive dialysis of the sodium salt with 10 mM Tris-HCl, pH 7.0, decreased the level of bound Na from five Na per LPS to between one and two Na per LPS (mole per mole). Thus, it appears that the Tris cation was unable to replace all of the Na. The Na and Tris salts contained similar levels of Fe, Al, and Zn. They both formed clear pellets upon centrifugation, and the resuspended samples were very soluble and almost water clear.

The calcium and magnesium LPS salts appeared similar to nLPS. They were slightly opalescent in solution and upon sedimentation formed clear to opalescent pellets. Elemental analysis of the CaLPS indicated that although the calcium bound was equivalent in metal cationic charge to that in the nLPS, the CaLPS still contained more than 1 mol of sodium per mol of LPS even after extensive dialysis (Table IV). In contrast, Mg completely replaced sodium, and the total cationic charge bound as Mg was greater than that of the nLPS.

The TbLPS appeared as a white gel upon sedimentation but readily resuspended in water. As shown in Table IV, Tb replaced nearly all the Na as well as a significant amount of the Fe. The amount of cationic charge bound in the terbium

Table V: Elemental Composition of LPS from *E. coli* Strain D21f2 Grown in Nutrient Medium[a]

element/phosphorus (molar ratio)	edLPS, pH 4.0	NaLPS
Ca/P	0.46 ± 0.05	0.019 ± 0.004
Mg/P	0.18 ± 0.02	0.014 ± 0.001
(Ca + Mg)/P	0.64 ± 0.07	0.032 ± 0.005
Na/P	0.2 ± 0.1	1.5 ± 0.1
Fe/P	0.032 ± 0.003	0.021 ± 0.005
Al/P	0.03 ± 0.02	0.08 ± 0.02
Zn/P	0.02 ± 0.01	0.0007 ± 0.0002
total +/P	1.51	1.84
P/3KDO	ND	4.3 ± 0.2

[a] Average of three isolates ± SD.

salt was somewhat less than that bound to the MgLPS but similar to that detected in the nLPS.

Ionic Content of LPS from a Heptoseless Mutant. LPS was isolated from *E. coli* strain D21f2. This mutant strain produces LPS which lacks the carbohydrate groups distal to the KDO units (see Figure 1). Extraction of the LPS with chloroform–petroleum ether followed by electrodialysis of the sample resulted in an acidic edLPS which was analyzed for the levels of metal cations bound. As shown in Table V, the mutant LPS contained more Ca than Mg in the electrodialyzed form. Treatment of this heptoseless LPS with 10 mM Na-EDTA, pH 7.0, resulted in nearly total replacement of the divalent cations with Na. Analysis of the levels of KDO/P in the LPS of this mutant suggests that this molecule contained approximately four phosphate groups. These results imply that the sodium salt of the mutant LPS binds approximately 8 counterion charges, approximately 1.5 more cationic charges than were detected bound to the acidic electrodialyzed form. NaLPS from the mutant strain D21f2 also retained significant levels of iron, aluminum, and zinc.

Discussion

The outer and cytoplasmic membranes of Gram-negative bacteria differ significantly in their composition and function. The results presented here indicate that the two membranes also differ radically in their associated counterions. The outer membrane is 3-fold enriched in divalent cations compared to the cytoplasmic membrane (Table I). The anionic LPS on the outer monolayer of this membrane and the underlying anionic peptidoglycan layer provide multiple sites for cation interaction. Previous evidence suggests that porin proteins in the outer membrane associate with the peptidoglycan through divalent cation bridges (Sonntag et al., 1978). We found that the porin–LPS–peptidoglycan complex is enriched in Mg compared to LPS or to the intact outer membrane (Tables I and II). Presumably, either porin–LPS or porin–peptidoglycan complexes or the porin proteins themselves contain Mg binding sites.

Divalent cations also appear to be critical in stabilizing pure LPS domains within the outer membrane. The major metal cations detected in nLPS were Mg and Ca (Table II). The level of ions recovered in extracted LPS was dependent on the medium in which the cells were grown. The different levels of magnesium recovered in nLPS isolated from cells grown in the two media used in this study could be the result of differences in the LPS structure. Alternatively, the varying levels of bound metal cations may result from differences in the levels of these cations in the media or from differences in the amounts of organic polyamines bound to LPS. Polyamines such as spermidine and spermine may be binding to and neutralizing part of the anionic charge on nLPS, and the levels of polyamines present may be dependent on the growth medium.

Regardless of the growth media used, electrodialysis of the extracted nLPS resulted in the removal of approximately one Mg per LPS (Table II). This Mg binding site in the LPS may reside within the KDO groups in the core polysaccharide region (see Figure 1). Electrodialysis results in a drop of the pH of the sample to approximately 4. The partial neutralization of the carboxyl group of KDO would then allow release of the bound cation. Previous studies have shown that either Ca or Mg can readily bind to this site after the edLPS is neutralized to pH 7.0 with NaOH (Coughlin et al., 1981). Electrodialysis may also remove spermine and spermidine bound to nLPS (Galanos & Lüderitz, 1975) although the removal of polyamines cannot be detected directly by elemental analysis. In both native and electrodialyzed samples of LPS, we found significant levels of iron, aluminum, and zinc. The presence of these ions represents a major unreported component in LPS. We believe the presence of these ions is not the result of contamination of LPS during isolation since (1) both aqueous phenol and chloroform–petroleum ether extraction procedures gave essentially identical cation levels in the LPS isolates and (2) the isolated outer membrane contained similar cation levels.

The detection of iron bound to LPS may be of considerable importance, considering the profound influence iron has on the pathology of LPS. It has been proposed that for some Gram-negative organisms, the ability of cells to compete for iron within a host correlated with the virulence of the strain (Mickelson & Sparling, 1981). In their competition for ferric ions, bacteria such as *E. coli* produce and excrete several low molecular weight chelators called siderophores which can complex with Fe(III) ions. The iron–siderophore complex is then taken up by the cell by utilizing specific membrane receptor proteins (Neilands, 1976). Although these siderophores have very high affinities for ferric ions, there is evidence that a low-affinity, high-capacity iron uptake system exists (Hartman & Braun, 1981). Thus, even with such potent ferric iron uptake systems, there may be utility in the high levels of iron bound to LPS. It has been observed that when iron-starved cultures of *E. coli* were given LPS isolated from cells grown in complete medium, growth resumed, suggesting that iron was associated with the added LPS (Kochan et al., 1977). The ferric iron bound to LPS may thus serve as an iron reservoir accessible to the siderophore–receptor system.

The presence of aluminum in LPS and outer membranes may be fortuitous. However, at neutral pH, aluminum hydroxylate complexes present in the growth medium would contain a net positive charge. Such complexes bind tenaciously to negatively charged surfaces (Matijevic, 1973) and can dramatically alter membrane physical properties (Vierstra & Haug, 1978). Aluminum is potentially very toxic for the cell (Viola et al., 1980), and binding it to LPS on the cell surface may serve to sequester the toxic ion, preventing it from entering the cell.

The LPS binding capacity for zinc may also be critical in the pathology of this endotoxin. Zinc, like iron, has been shown to reduce the toxicity of endotoxin-challenged mice (Snyder & Walker, 1976; Sobocinski et al., 1977). Thus, the ability of LPS to bind these transition metals may be important in host–pathogen interactions as well as for the bacterial cell physiology. The only other metal reported to be bound to LPS is copper. Sourek & Tichy (1975) found that *Shigella dysenteriae* LPS isolated by using the hot aqueous phenol method was contaminated with copper. We have found no significant levels of copper or of manganese, molybdenum, arsenic, cad-

mium, chromium, mercury, lead, selenium, thallium, or cobalt in any of our LPS isolates.

We have shown that the divalent cations bound to LPS can be removed and defined salts can be formed. NaLPS contained lower cationic charges bound per phosphorus than were detected in nLPS (Tables II and IV). The lack of equivalent charge neutralization with Na likely results in an LPS complex with a higher net negative charge compared to nLPS. This conclusion is supported by studies of the LPS head-group mobility, as discussed in the following paper (Coughlin et al., 1983). Upon formation of the Tris salt, all but approximately one Na per LPS molecule was displaced. The number of Tris cations bound could not be determined in this study, but the overall anionic charge of the Tris salt of LPS is likely to be equal to or greater than that of the sodium salt. Presumably, the large Tris cation cannot associate with more anionic sites than can Na.

The calcium salt also contained between one and two Na. The reason that Ca was unable to completely displace Na in the LPS is not clear. The ionic radii of Ca^{2+} and Na^{+} are nearly the same. However, Mg, which is substantially smaller, was able to replace all of the Na following dialysis of NaLPS with $MgCl_2$ (Table IV). Calcium and magnesium may interact with the anionic sites differently since their coordination geometries are quite different. Both the magnesium and calcium salts of LPS from the k12 strain had higher levels of cationic charge bound than was detected in nLPS (Tables II and IV). This difference may result from the presence of undetected polyamines in nLPS which were removed upon formation of the defined salts. The trivalent terbium salt appeared unique in its properties. This salt of LPS contained bound cationic charge at levels significantly lower than that in the divalent salt forms (Table IV). The Tb^{3+} ion is close in ionic radius to Ca^{2+} (approximately 0.95 Å) and may be excluded from one or more sites which are specific for smaller ions.

Thus, the LPS from k12 wild-type strains preferentially binds cations of small ionic radius. Furthermore, at least some of the cation binding sites in this LPS are unable to accommodate two monovalent cations to neutralize the charge originally balanced by one divalent ion. In contrast, LPS from the heptoseless mutant strain, D21f2, appeared to have a more "open" structure which could accommodate the binding of larger cations. We have shown that the edLPS from this strain contains approximately two Ca and only one Mg per LPS. Furthermore, the Na salt of this mutant LPS appeared to neutralize more of its anionic charge with Na than was seen in the NaLPS from the parent D21 strain (Tables IV and V). Such difference in charge neutralization in the sodium salts of these two LPS molecules were also detected by differences in the head-group mobility as described in Coughlin et al. (1983). One structural difference in the mutant LPS which may account for the binding of larger cations and more monovalent cations is the absence of ethanolamine groups (Figure 1). The presence of covalently bound amines in the LPS of the wild-type k12 strain could allow cross bridging to anionic groups within and between LPS molecules, rigidifying and restricting other cationic interactions.

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Registry No. Calcium, 7440-70-2; magnesium, 7439-95-4; iron, 7439-89-6; aluminum, 7429-90-5; zinc, 7440-66-6; sodium, 7440-23-5.

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Physical Properties of Defined Lipopolysaccharide Salts†

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ABSTRACT: The electron spin resonance probes 5-doxylstearate and 4-(dodecyldimethylammonio)-1-oxy-2,2,6,6-tetramethylpiperidine bromide were used to characterize the fluidity of the acyl chain and head-group regions, respectively, of defined salts of lipopolysaccharide (LPS) from *Escherichia coli* K12. The removal of the weakly bound divalent cations from native LPS by electrodialysis and their replacement by sodium had little effect on the midpoint of the lipid-phase transition or on head-group mobility. In contrast, lipopolysaccharide acyl chain mobility increased following electrodialysis. The replacement of most of the remaining cations with sodium resulted in a further dramatic increase in mobility in both the polar and nonpolar regions of lipopolysaccharide. Head-group mobility of the sodium salt of LPS was shown to be reduced with the addition of divalent cations. Furthermore, evidence is presented which suggests that low magnesium concentrations may induce phase separations in the sodium salt. The magnesium salt of lipopolysaccharide closely resembled the native form in both head-group and acyl chain mobility although the cation charge to phosphorus ratio in the magnesium salt was greater than that detected in the native isolate. Analyses of other lipopolysaccharide salts support our hypothesis that many of the observed differences in the physical and pathological properties of lipopolysaccharide salts may simply be explained by the degree of charge neutralization.

The physical state of lipopolysaccharide (LPS)[1] has been reported to have a profound impact on its endotoxicity. A strong correlation seems to exist between the particle size of enteric LPS aggregates and their ability to inactivate serum complement (Galanos, 1975). LPS salts which have low particle size (e.g., triethylamine) do not interact with the complement system, whereas high particle size salts (e.g., sodium) are potent inactivators of complement. This pattern may not be common to all LPS. For example, the native and sodium salts of *Chromatia vinosum* LPS are equally effective in interacting with complement (Hurlbert & Hurlbert, 1977).

Another indication of the importance of the physical state of LPS on endotoxicity is the effect that mild alkaline treatment has on the ability of LPS to act as a mitogen (Goodman & Sultzer, 1977). Although high pH has been reported to cleave esterified fatty acyl chains from LPS, mild treatment simply lowers particle size without apparent chemical denaturation. In this case, alkaline-treated LPS of low particle size had enhanced B cell mitogenicity.

Attempts to characterize LPS aggregates by sedimentation coefficients or particle sizes can be complicated by gross changes in the morphology of the LPS aggregate. Furthermore, correlations between LPS aggregate size or shape and endotoxicity do not give attention to subtle differences in the intermolecular interactions between LPS within these aggregates.

We have attempted to characterize the differences between various LPS salts in their head-group and acyl chain mobilities. This was done in the hope that such differences may point toward a better understanding of the molecular interaction of cations with this complex anionic lipid and its involvement in bacterial pathology.

Materials and Methods

Cell growth and LPS isolation and electron spin resonance (ESR) probing were carried out as described earlier (Janoff et al., 1979; Coughlin et al., 1981, 1983). Defined LPS salts were prepared as described in the preceding paper (Coughlin et al., 1983). The temperature dependence of ESR spectral parameters was analyzed as described by Brunder et al. (1981). All temperature-dependent ESR parameters were reversible in the temperature range indicated. We observed that LPS solutions which had been frozen and thawed did not resuspend well. In addition, some samples showed age-dependent alterations in their physical properties. Consequently, all ESR experiments were performed on fresh or lyophilized and resuspended LPS samples stored in solution under nitrogen at 4 °C.

LPS samples were prepared for electron microscopic examination by negatively staining with 1% (w/v) sodium phosphotungstate, pH 7.0. The samples were visualized by using a Philips 300 transmission electron microscope operating at 80 kV.

Results

ESR Probing of D21-Defined LPS Salts. The proposed structure of the LPS from the two *Escherichia coli* strains used in this study is given in Figure 1 of the preceding paper (Couglin et al., 1983). Defined salts of strain D21 LPS were

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[1] Abbreviations: LPS, lipopolysaccharide(s); CAT_{12}, 4-(dodecyldimethylammonio)-1-oxy-2,2,6,6-tetramethylpiperidine bromide; 5DS, 5-doxylstearate; nLPS, native LPS; edLPS, electrodialyzed LPS; Tris, tris(hydroxymethyl)aminomethane.

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