Plasma desorption mass spectrometry has recently been used with success to characterize native, underivatized Re- to Re-type endotoxins in terms of their constituent lipopolysaccharides. The spectra give masses for the major molecular species of lipopolysaccharide present from which their probable compositions could be inferred using the overall composition determined by chemical analyses. Moreover, the relative intensities of the signals are roughly proportional to the abundance of their corresponding molecular species. Native Re-, Rb-, and Ra-type enterobacterial endotoxins with 5–10 core sugar units have been rendered amenable to plasma-desorption mass spectrometry analysis by improvements in their solubility and the use of cellobiose as an additive. The spectra of four *Salmonella* and *Escherichia* endotoxin preparations demonstrated heterogeneity in acylation and phosphorylation. Since these sources of heterogeneity are critical for many biological activities, the spectra underline the need to define the composition of each preparation of endotoxin used in structure-function studies.

Endotoxins play an important role during infections of animals and man by Gram-negative bacteria. They cause such symptoms as fever and endotoxic shock, and they also activate the immune system. Each endotoxin is an ensemble of related lipopolysaccharides (LPS) \(^1\) the structures of which depend on the bacterial source and its culture conditions (1–3). The LPS of most endotoxins contain a highly conserved lipid moiety called lipid A \(^4\), consisting of bisphosphorylated diglucosamine substituted by three to seven hydroxylated and nonhydroxylated fatty acids. The lipid moiety is attached via a Kdo bond linking it to the LPS core region. These signals testify generally to the heterogeneity that is due to variations in fatty acid composition \(^13\) as well as to substitutions on the lipid A phosphate groups. The two sets of signals (LPS molecular-ion and lipid A fragment-ion) often resemble each other in the number and relative spacing and intensities of the signals. All this information is important not only for structural analysis but also for structure-function studies since considerable variation exists among the results reported from different laboratories regarding endotoxin structure and function. It was therefore essential to adapt PDMS techniques to elucidate the structure of endotoxins having a complete core. New conditions of sample preparation and detailed analysis of the resulting spectra obtained with Rd1- to Ra-type endotoxins are presented here. The postulated structures of these endotoxic lipopolysaccharides are shown in Fig. 1.

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\(^1\) The abbreviations used are: LPS, lipopolysaccharide; PDMS, plasma desorption mass spectrometry; Kdo, 3-deoxy-d-manno-oct-2ulosonic acid; lipid A, the hydrophobic domain of endotoxic lipopolysaccharide; C12, dodecanoic acid; C14, tetradecanoic acid; C14OH, hydroxytetradecanoic acid; amu, atomic mass units.

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FIG. 1. Schematic structure of a *Salmonella*-type lipopolysaccharide.
**EXPERIMENTAL PROCEDURES**

Negative-ion spectra were obtained on a Deip 252\(^{252}\)Cf time-of-flight mass spectrometer (TOF 21, IPN, Orsay, France) with a drift distance of 46 cm and an accelerating voltage of 15 kV (14). Counting time varied from a few minutes to several hours. Masses were determined from peak centroid calculation.

Endotoxins were purified by extraction of phospholipids, treatment with proteases and nucleases, and centrifugation until thin layer chromatography (15) and UV spectra showed no detectable contaminants.

The original conditions of endotoxin preparation for PDMS (12) were, briefly: 12 \(\mu\)g of endotoxin was desalted with Dowex 50-Me\_2NH\(^{+}\) in 6 \(\mu\)l of chloroform/methanol/water (2:1:0.125) and deposited directly on Al-on-mylar discs previously sprayed with nitrocellulose (16).

**Desalting Procedures**—Endotoxins were desalted by 1) Chelex 100 (Bio-Rad) according to Rowatt (17), 2) electrodialysis, according to Galanos and Luderitz (18), 3) Dowex 50-Me\_2NH\(^{+}\), and 4) EDTA, according to Shands and Chun (4).

**Structured Bilayers**—Using a Langmuir-Blodgett apparatus as previously described (19), bilayers of a mixture of endotoxin and hexadecanoic acid (1:1) were deposited onto a target of gold foil 0.075-\(\mu\)m thick.

**Additives**—Several different classes of compounds were tried in cosolution with endotoxins: the detergents octyl-\(\beta\)-D-glucoside, octyl-\(\alpha\)-D-glucoside, and nonyl-\(\beta\)-D-glucoside; the amino acids cysteine and arginine, bovine serum albumin and especially the following sugars: glucose, glucuronic acid, glucosamine, N-acetylglucosamine, lactose, maltose, sucrose, cellulose, trehalose, raffinose, stachyose, and maltotriose. All of the latter were tried in proportions of 20:1 part endotoxin (by weight).

**Dissolution Aids**—To obtain clear solutions of endotoxins, the preparations were treated for various periods (a) in a Bransonic 220 ultrasonic bath at room temperature; (b) at 90-100 °C; or (c) in a Moulinex Microchef FM 1515 microwave oven.

The method adopted for sample preparation was the following: 100 \(\mu\)g of purified endotoxin was added to 25 \(\mu\)l of 4% aqueous cellulose solution and treated for 2 min in the microwave oven on position II. 25 \(\mu\)l of isopropyl alcohol-methanol (1:2) and a few grains of Dowex-50X Me\_N\+ were added and stirred. Then 40 \(\mu\)l of the supernatant were electrosprayed onto an Al-on-mylar target and put into the mass spectrometer.

Endotoxins were bought from Sigma: *Escherichia coli* F583, an R-type listed as Rd2 (Lot 57F-4071), *Salmonella typhimurium* TV119, an R-type (Lot 76F-4034), and *Shigella flexneri* Re-type (Lot 57F-4070); and from List Biological Labs (Campbell, CA): *Salmonella minnesota* R7, Rd1-type (Lot 3) and *S. minnesota* R345, Rb-type (Lot 4A).

**RESULTS AND DISCUSSION**

The solubility of LPS in the chloroform-methanol-water solvent used in our earlier work is inversely proportional to the length of their core domain. Therefore, with LPS having four or more core sugar units, the first step was to use a more polar solvent. A mixture of isopropyl alcohol/methanol/water (1:2:3), proved adequate and allowed the possibility of electrospraying the endotoxin sample onto the target disc.

The work of Deprun and Szabo (20) showed that salts were deleterious for PDM spectra of a lipid A analog. Since Galanos and Luderitz (21) had earlier established that many cations increase aggregation of LPS it was reasonable to think that optimally desalted LPS would give better yields of desorbed molecular ions. Using the Rc-type endotoxin of *E. coli* F583 as a model, several variations of each aspect of sample preparation, including salt removal, were tested, and the following results were obtained.

1) The resin treatment using Dowex 50-Me\_2NH\(^{+}\) gave the best results and was more convenient and rapid than other methods of desalting endotoxins (see "Experimental Procedures").

2) With the idea of reducing further the attraction between LPS molecules, binary solutions of endotoxin with a variety of other compounds were tried. Several of the additives, including octyl \(\beta\)-glucoside, bovine serum albumin, and lactose, improved the spectra somewhat but cellobiose proved to be the most promising.

3) The optimal proportions were established to be one part (by weight) of LPS to 10 parts of cellobiose.

4) LPS-cellobiose solutions treated briefly with microwave irradiation gave better spectra than those treated with heat or ultrasound which are frequently used to facilitate solution.

5) The optimal thickness of sample on a target was determined by comparing spectra from targets electrosprayed with 10-80 \(\mu\)l of sample preparation.

On the basis of spectral quality, sample preparation as described under "Experimental Procedures" was adopted. Fig. 2 shows the spectrum of *E. coli* F583 (Rc-type) endotoxin obtained under these conditions. The three major signals at the right (\(m/z\) 2347, 2573, 2784) come from molecular ions of tetra-, penta-, and hexa-acylated LPS (left to right). The three strongest weaker signals at the left are from the corresponding lipid A fragment ions. As has been noted before (12), the weak ketosidic bond of Kdo is the principal fragmentation point in endotoxins. It often gives rise to a pattern of lipid A signals (\(m/z\) 2000-2100) mimicking that of the molecular ions (\(m/z > 2100\)). In this case, the lipid A peaks are 987 amu (mass of the pentasaccharide core consisting of 2 Kdo, 2 heptose, and 1 glucose unit) downfield from their respective molecular counterparts. The \(m/z\) 1797 signal, which can be attributed to the hexacylated lipid A structure shown in Fig. 1, has been found in the spectra of all enterobacterial lipid A examined so far, as well as of some closely related genera (13).

The less-acylated LPS species giving signals at \(m/z\) 2347 and 2573 (Fig. 2) represent heterogeneity in the preparation and not fragmentation. This was determined from spectra of homogeneous LPS and synthetic lipid A preparations which give a single peak (not shown).

The improvement in the method can be seen by comparing the two spectra of the same endotoxin of *S. minnesota* R7 in Fig. 3. The upper spectrum (A) was obtained by the earlier method. It has few and poorly resolved molecular-ion signals (\(m/z > 2100\)) compared to the new spectrum (B) in which the signals \(m/z\) 2185, 2411.4, and 2622 correspond to tetra-, penta-, and hexa-acyl Rd1 LPS structures. The latter are analogous to those of the F583 endotoxin but without their terminal glucose unit (minus 162 amu).\(^{2}\)

The masses given for individual components of lipopolysaccharides represent their contribution to the total molecular mass, i.e. their mass minus 18 (the mass of H\(_2\)O).

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\(^{2}\) The masses given for individual components of lipopolysaccharides represent their contribution to the total molecular mass, i.e. their mass minus 18 (the mass of H\(_2\)O).
signal. This was attributed, as before (12), to a replacement of the tetracanoate unit (C14, 210 amu) with a fifth hydroxytetradecanoate (C14OH, 226 amu). Each of the four mentioned LPS signals (m/z 2185, 2411.4, 2622, and 2637.3) is followed by another, 80 amu higher, indicating the presence of molecular species having a third phosphate.

The major Rb2-type lipopolysaccharides of S. minnesota R345 endotoxin (Fig. 4) (m/z 3502, 3291, and 3067) differ from those of the Rc-type of E. coli FS83 by 718 atomic mass units. This is consistent with their having two hexoses (two galactoses in this case, 162 amu each), one heptose (192 amu), and a pyrophosphorylethanolamine (203 amu) more than the Rc-type. The hexa-acyl molecular species is most abundant in this preparation. The small signals at m/z 3720 and 3940 may represent molecular ions containing 3 and 4 Kdo units, respectively. It has been reported that the LPS of this strain contains 3 Kdo units and that those of other strains of S. minnesota contain nonstoichiometric amounts between 2 and 3 (22). We have analyzed the spectra of seven endotoxin preparations of other strains of this species, and this is the first evidence obtained by PDMS of LPS containing more than 2 Kdo units (12). Even though some fragmentation is expected at the Kdo ketosidic bonds it is not obvious, a priori, that it would be more frequent than that between Kdo and the glucosamine of lipid A. This may be another case of variability between different endotoxin preparations from the same bacterial strain (12). In any event, it would seem prudent to define each endotoxin on the basis of analyses of several different preparations.

The lipid A part of the spectrum (Fig. 4) contains several peaks that are unaccountable by the usual LPS analyses. The peaks in question do not appear in the spectrum of lipid A isolated from this endotoxin preparation (not shown). They are apparently lost during the mild hydrolysis and/or extractions involved in the preparation of lipid A. This suggests that they are contaminants. Thin layer chromatography of the endotoxin supported this idea by revealing the presence of several compounds that migrate faster than the LPS in a hydrophobic solvent.

Fig. 5 is the spectrum of an Ra-type (full core) endotoxin of S. typhimurium. The signals at m/z 4030, 3804, and 3595 correspond to the hexa-, penta-, and tetra-acylated lipid A with their 10-sugar-unit cores substituted with a pyrophos-
phorylethanolamine and 2 more phosphate units (unplaced). The tetra-acylated species dominate the two parts (lipid A fragment-ion and molecular-ion regions) of the spectrum. Since artificial mixtures of different homogeneous lipid A gave signals roughly proportional to their abundance in the sample† and, in this spectrum, the molecular-ion region resembles the lipid A fragment-ion region we conclude that the tetra-acylated LPS is most abundant in the preparation examined.

Table I sums up the masses of the principal LPS molecular species detected in the four spectra, with the respective contributions from their lipid and saccharide domains.

The less acylated species of LPS may represent incomplete synthesis or in vivo degradation of the endotoxin. If the former, the differences in mass values m/z tell us that, in strains R7 and R345 of S. minnesota and F583 of E. coli, the last and next-to-last fatty acid units added to the diglucosamine backbone are, respectively, tetradecanoic (210 amu) and hydroxytetradecanoic acid (226 amu), except, of course, when the LPS has no tetradecanoate (the case of some R7 LPS). In the S. typhimurium LPS examined, the order would be reversed since the penta-acylated molecular species (m/z 3804) contains tetradecanoate.

The new method gave completely satisfactory results when applied to lipid A and to the Re-type endotoxin of S. flexneri. However, since the previously described method is simpler and requires smaller quantities of material it is to be preferred for these more hydrophobic compounds.

The spectra indicated, and chemical analysis confirmed, that none of the preparations had appreciable amounts of hexadecanoic acid in their lipid domains despite the idea current among many immunologists that this fatty acid is characteristic of Salmonella LPS and distinguishes them from E. coli LPS.

To explain the effect of the cellobiose-plus-microwave treatment on LPS solutions, light scattering and buoyant density measurements are being carried out. Preliminary results concur with the idea that the treatment causes considerable disaggregation of the LPS.

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