Lipopolysaccharides of Neisseria gonorrhoeae can be defined in vitro by their susceptibility to the bactericidal action of normal human sera (1-4). Serum-sensitive strains are killed by complement-mediated immune lysis involving antibody directed against the components located on the surface of the outer membrane of the bacteria (1, 2). This action prevents the dissemination of Neisseria beyond the initial site of colonization in the mucous membrane of the host (5-10). Recent studies by Schneider and associates (4, 11-13) showed that serum resistance results from the absence of natural antibody directed against the LPS1 determinants that are the lytic loci on the bacterial surface. Study of the chemical structure of gonococcal LPS from the serum-sensitive and serum-resistant organisms is important to our understanding of their role in natural human immunity and their potential as vaccines.

LPS are amphipathic macromolecules found on the outer surface of the outer membrane of Gram-negative bacteria and are composed of three regions of differing chemical and biological properties (14). The polysaccharide carries the main serological specificity. It is linked to the core polysaccharide that is common to Gram-negative bacteria. The core is linked through KDO to the lipophilic lipid A component, which is responsible for most of the biological activities of LPS. The polysaccharide region of the LPS of N. gonorrhoeae differs from that of the Salmonella strains in that it is smaller (M, 3100-7100) (11) and apparently lacking in the repeating subunits. It appears to have multicomponents in which each component expresses different antigenic specificity (12). The structure of the polysaccharide region of N. gonorrhoeae is presently under intense investigation. The lipid A region of the LPS might also be important in influencing the lytic action of the host's immune system. However, very little is known about its structure.

The complete structures of lipid A's obtained from the LPS of Salmonella strains by the presence of shorter-chain fatty acids and by the normal fatty acid distribution in the reducing and distal subunits.

Monophosphoryl lipid A (MLA) obtained from the lipopolysaccharides of serum-sensitive strains of Neisseria gonorrhoeae was fractionated on a silicic acid column to yield the hexaacyl and pentaacyl MLAs. The dimethyl derivative of the hexaacyl MLA was analyzed by proton nuclear magnetic resonance spectroscopy. The dimethyl esters of hexaacyl and pentaacyl MLAs were further purified by reverse-phase high performance liquid chromatography, and all of the peaks were analyzed by laser desorption mass spectrometry. Considerable structural information was obtained by laser desorption mass spectrometry due to three kinds of specific fragmentations of the sugar at the reducing end. Two major fractions were also analyzed by positive ion fast atom bombardment mass spectrometry. High performance liquid chromatography was able to separate the dimethyl MLA according to number, nature, and position of the fatty acyl groups. Since almost no structural information is available, the mass spectra of the samples were interpreted on the basis of the established structure of a model lipid A (hexaacyl MLA derived from Salmonella minnesota).

Thirteen different structures of dimethyl MLA were identified. The four prominent dimethyl MLAs found in the fractionated samples were M1 (Mw = 1463), M2 (Mw = 1479), M3 (Mw = 1861), and M4 (Mw = 1677). These MLAs appear to have a 1' → 6 linked glucosamine disaccharide backbone. The most prominent hexaacyl MLA was M3. We propose that it contains hydroxyaurate at the 3- and 3'-positions in ester linkage and lauroxymyristate at the 2- and 2'-positions in amide linkage of the glucosamine disaccharide. The most abundant pentacyl MLA was M2. We propose that it contains hydroxyaurate at the 3- and 3'-positions in ester linkage, lauroxymyristate at the 2'-position in amide linkage, and hydroxymyristate at the 2-position in amide linkage of the disaccharide. The lipid A of N. gonorrhoeae appeared to differ from that of the Salmonella minnesota.
of Salmonella typhimurium and Salmonella minnesota were recently established (15-18). In these studies, the dimethyl derivatives of the MLA were first purified by HPLC and analyzed by modern instrumental techniques; i.e. FAB-mass spectrometry and two-dimensional NMR spectroscopy. We have now purified the dimethyl MLAs obtained from LPS of N. gonorrhoeae by HPLC and have analyzed the major fractions by both LD- and FAB-mass spectrometry. This is the first time that mass spectrometry has been used to closely follow the extent of purification of lipid A by HPLC fractionation. We obtained four prominent and structurally different "free" lipid A's from the LPS of a serum-sensitive F62 strain of N. gonorrhoeae.

EXPERIMENTAL PROCEDURES

Materials—HPLC-grade chloroform, methanol, acetonitrile, and isopropyl alcohol were purchased from Burdick & Jackson Laboratories, Inc., Muskegon, Mi. Bio-Sil HA (~325 mesh) was purchased from Bio-Rad; silica gel H thin-layer plates (250 μm) were purchased from Analtech, Wilmington, DE. Bio-Sil gel H (250 μm) and the solvent system of chloroform/methanol/water-concentrated ammonium hydroxide (50:25:4.2, v/v) were used. Spots were visualized by spraying the plate with dichromate-sulfuric acid reagent and charring. Identification of samples (20–30 μg): A, crude MLA mixture before fractionation; B, silicic acid column fraction I; C, silicic acid column fraction II; D, silicic acid column fraction III.Each sample and allowed to stand at 22 °C for 2 min. The samples were immediately dried with a stream of nitrogen in a warm water bath; they represented the dimethyl derivatives of the hexaacyl and pentaacyl MLAs.

HPLC Fractionation—HPLC was performed with two Waters 660 solvent programmers (Waters Associates Inc., Milford, MA), a Waters 150 universal liquid chromatograph injector, a variable wavelength detector (model LC-85B, Perkin-Elmer), and a radial compression module (model RCM-100, Waters Associates, Inc.). A Radial Pak cartridge (8 mm × 10 cm) (C18-bonded 10-μm silica, Waters Associates, Inc.) was used at a flow rate of 3 ml/min. For the fractionation of dimethyl MLA, a linear gradient of 20–80% isopropyl alcohol in acetonitrile was used over a period of 60 min (18). The wavelength of the detector was set at 210 nm.

LD-Mass Spectrometry—LD-mass spectra were obtained on a CVC-2000 (Rochester, NY) time-of-flight mass spectrometer equipped with a Tachisto (Needham, MA) model 215G pulsed carbon dioxide laser (24). The laser wavelength was 10.6 μm. Pulse width was 40 ns, and the power density of each pulse was approximately 106 W/cm2. The repetition rate was approximately 1 Hz. Twenty to 30 laser shots were used for each mass spectrum. The ions produced from each laser shot were gated and accelerated to 3 KeV into a 1-m drift region. They were then postaccelerated to 12 KeV and detected using dual Galileo channel plate detectors (Sturbridge, MA). The analogue signal was recorded as 8 K channels on a LeCroy (Spring Valley, NY) 3500SA signal averaging system with a 100-MHz analogue-to-digital waveform recorder. Mass assignments were made by determining the time-of-flight (channel) centroid of each peak and comparing them with the centroids of Na+ and K+ ions that appeared in each spectrum. Mass accuracy was approximately ±1 atomic mass units at 1000 atomic mass units. KCl was added to each sample to promote production of MK* molecular ions.

Positive FAB-Mass Spectrometry—Positive ion FAB-mass spectra were acquired using a KRATOS MS-50 (Manchester, UK) mass spectrometer equipped with a 23-KG magnet and postacceleration detector. The instrument was operated at an accelerating voltage of 8 keV and 3000 resolution. A KRATOS DS-55 data system interfaced to the mass spectrometer was used to acquire data and to average spectra. Mass calibration was achieved using cesium iodide. Samples

FIG. 1. Analytical TLC of silicic acid column purified MLA fractions. The source was LPS of F62 strain of N. gonorrhoeae. Silica gel H and a solvent system of chloroform/methanol/water-concentrated ammonium hydroxide (50:25:4.2, v/v) were used. Spots were visualized by spraying the plate with dichromate-sulfuric acid reagent and charring. Identification of samples (20–30 μg): A, crude MLA mixture before fractionation; B, silicic acid column fraction I; C, silicic acid column fraction II; D, silicic acid column fraction III.

Fractionation of MLA on Silicic Acid Column.—About 70 mg of MLA from strain F62 (as the free acid) were applied to a 2 × 25-cm silicic acid (Bio-Sil HA) column packed as a slurry in chloroform. The column was washed with 50 ml of chloroform. The MLA was eluted from the column with a linear gradient of 0–25% methanol in chloroform (700 ml). Six-mI fractions were collected and analyzed by spot-charring on a silica gel thin-layer plate. Char-positi
were dissolved in chloroform/methanol (4:1, v/v) and placed on a copper probe tip. Monothioglycerol was used as the sample matrix. The samples were bombarded with an 8-kV xenon atom beam generated by a saddle field source (Ion Tech, Middlesex, UK).

Proton NMR Analysis—TLC-purified dimethyl hexaacyl MLA (methylated fraction 1, 5.9 mg) was dissolved in 0.5 ml of benzene-d6-dimethyl sulfoxide-d6 (9:1, v/v) and analyzed on an XL-300 MHz Varian spectrometer. Spectra were recorded at an operating temperature of 33 ± 0.5 °C.

RESULTS

Preliminary Studies of LPS and Lipid A Obtained from N. gonorrhoeae—The LPS obtained from strains F62 and WR213 of N. gonorrhoeae were hydrolyzed in 0.1 N HCl at 100 °C for 20 min to yield the MLA. Analytical TLC of such a sample from the two strains gave identical results. A representative TLC showed that two major bands co-migrated with authentic hexaacyl MLA and pentaacyl MLA obtained from the LPS of S. minnesota (18) (Fig. 1). The following bands were identified: hexaacyl MLA, Rf = 0.32; pentaacyl MLA, Rf = 0.26; tetraacyl MLA, Rf = 0.23; diphasphoryl lipid A, Rf = 0.12; unhydrolyzed LPS, Rf = 0.07. Only the hexaacyl and pentaacyl MLA were further studied.

Chemical analysis of the LPS showed the presence of KDO, glucosamine, and phosphate, whereas the KDO content in the crude MLA preparation was reduced to 0.01 μmol/mg to give a phosphate-to-KDO ratio of 1.00:0.01. The crude MLA contained lauric acid, hydroxylauric acid, and hydroxymyristic acid in a ratio of 1.00:0.65:0.85. Traces of palmitic acid were also detected. Based on chemical analysis and analytical TLC, the MLAs prepared from LPS of the F62 and WR213 strains were nearly identical.

Purification of Dimethyl MLA—MLA was prepared from the LPS obtained from strain F62 and fractionated on a silicic acid column by the method of Qureshi et al. (18). The column fractions (I—III) of MLA were methylated with diazomethane and subjected to preparative HPLC using a reverse-phase C18-bonded silica cartridge, as previously described (18). Subsequent mass spectral analysis revealed major components designated as M1 = 1463 atomic mass units, M2 = 1479 atomic mass units (pentaacyl MLA), M3 = 1661 atomic mass units, and M4 = 1677 atomic mass units (hexaacyl MLA), as well as several minor components.

Mass Spectral Analysis—Fraction I was separated by HPLC into four peaks, all of which were subjected to mass spectral analysis using LD (Fig. 2). The two major HPLC peaks (1-2 and 1-3) gave molecular ions (M + K+) at m/z 1716 and 1700, respectively (Fig. 2, c and d), corresponding to the two major hexaacyl components with molecular weights of 1677 (M1) and 1661 (M3). The mass spectrum of HPLC peak 1-3 also revealed a small amount of the heavier hexaacyl component (m/z = 1716) due to incomplete chromatographic separation.

The molecular weight of HPLC peak 1-3 was confirmed by FAB-mass spectrometry (Fig. 3), which showed an MH+ ion at m/z = 1662, MH+H2O at 1644, and MNa+ at 1684. The cluster of peaks around 1678 and 1680 in the FAB mass spectrum may be a mixture of M + NH4+ and the MH+ of the heavier component with molecular weight 1677. Fragmentation resulting in loss of hydroxyauric acid (OHC11) and lauric acid (nC11) gave peaks at 1446 and 1463 atomic mass units, respectively. The base peak of the spectrum at m/z = 876 corresponds to cleavage of the bond between the distal and reducing sugars. This suggested that the component with molecular weight 1661 has the structure shown in Fig. 4. The ester-linked hydroxyauric acids attached to the 3- and 3'-positions were easily cleaved and following H-transfer produced the loss of 216 atomic mass units observed in the spectrum. The acyl link between lauric acid and hydroxymyristic acid was also easily cleaved with H-transfer, showing loss of 200 atomic mass units in the spectrum. The amide-linked hydroxyauric acid was not cleaved.

The structure and arrangement of fatty acids in the dimethyl MLA were partially derived from FAB-mass spectrometry, which gave molecular ions, losses of 200 and 216 atomic mass units, and formation of oxonium ion at m/z 876. This structure was further supported by LD-mass spectrometry, which produced a far different fragmentation pattern. The LD-mass spectrum of the same HPLC peak (Fig. 2d) showed the same losses of hydroxyauric and lauric acids (at m/z 1484 and 1500) from the MK+ ion at m/z 1700. The oxonium ion was not observed; however, instead, as reported previously by Costes and Wilkens (25), LD produced prominent fragmentation within the sugar rings, and this fragmentation provided direct information on the identity and positions of the fatty acid chains.

We propose three major fragmentation pathways which are summarized in Scheme 1. Simultaneous cleavage of the C1-O bond and the C5-C6 bond of the reducing sugar results in the loss of a portion of the molecule which includes the amide-linked fatty acid. If the N-linked fatty acid (R4) is lauroylmyristate (C12:0/C14:0), then the loss of 467 atomic mass units produces the peak at m/z = 1233 observed in the spectrum (Fig. 2d). Analogously, hydroxylauroylmyristate or hydroxymyristate at that position would have produced losses of 483 and 285, atomic mass units, respectively. A second two-bond cleavage at C1-C5 and C3-C4 includes the loss of both the amide-linked (R4) and ester-linked (R5) fatty acids from positions 2 and 3 and can be used to distinguish between normal and hydroxyauric acid at the 3 position once R4 is known. The loss of 665 atomic mass units in the mass spectrum of HPLC peak 1-3 to give the peak at m/z = 1035 established R4 as hydroxyauric acid. A third fragmentation involves simultaneous cleavage of the C6-C7 and C7-0 bonds. The ion that results (see below) can be used to establish the identity of R4 and R5.

(distal subunit – 0 – CH2 – CH = 0) + K+

Fig. 2. A, HPLC of MLA fraction I, and LD-mass spectra of: B, peak 1-1; C, peak 1-2; D, peak 1-3; and E, peak 1-4.
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Fig. 3. FAB-mass spectrum of HPLC peak 1-3 from MLA fraction 1.

Fig. 4. Proposed structure of hexaacyl MLA (M₄) of M₄ = 1661. We have provisionally assigned the phosphate group to the 4'-position according to the model compound, the hexaacyl MLA derived from Salmonella minnesota.

Scheme 1

ence of a 1' → 6 glycosidic linkage in the dimethyl MLA disaccharide. If the linkage was 1' → 4, one might expect a fragment ion M-60 + K⁺. This was not observed.

Using this fragmentation scheme, we assigned structures to all of the other peaks from the HPLC separated fractions. The major component of fraction I (see Fig. 2c) showed a loss of 216 atomic mass units corresponding to hydroxyauric acid, but the loss of 200 atomic mass units was greatly reduced, suggesting that an additional hydroxyauric acid replaced one of the lauric acids. The peak at m/z = 1249 was a loss of 467 atomic mass units from the molecular ion, indicating that the extra hydroxyauric acid is not on the reducing end. The shift of 16 atomic mass units of the peaks at 990, 1050, and 1249 indicated that the distal lauric acid has been replaced by hydroxyauric acid. The two other peaks from the HPLC separation of fraction I were both mixtures and somewhat difficult to analyze. However, they were both minor components of fraction I. The first peak (Fig. 2b) had traces of the component in 1-2, but also showed several lower mass components. A molecular ion at 1688 atomic mass units, 28 mass units below 1716, suggested that one C₁₄ fatty acid has been replaced by a C₁₂ fatty acid. Other molecular ion peaks suggested differences in the number of hydroxy fatty acids. The multiplicity of fragment ion peaks suggested that this substitution is random. The final peak (1-4) appeared to be a mixture of (1-3) along with methyl and dimethyl derivatives. The peak at 1498 was the loss of OCH₁₄, while the peak at 1484 included the loss of methylated hydroxyauric acid (OMeC₁₂) from 1714 and the loss of OHC₁₄ from m/z = 1700. The peaks at 974, 988, and 1002 indicated that the distal portion of the molecule may be unmethylated, monomethylated, or dimethylated. The second methyl group must be on the sugar. The peaks at 1232 and 1246 suggested that additional methylation may occur in the reducing subunit. Therefore, we concluded that HPLC peak 1-4 contains a number of species with randomly methylated hydroxy fatty acids and/or sugar OH groups. It is not possible at this time to determine whether these methoxy fatty acids were naturally occurring or were generated by the diazomethane treatment. The structures are summarized in Table I and indicate, in general, that the number of hydroxylated fatty acids decreased with retention time.

Fractions II and III from the silicic acid column produced mainly pentaacyl MLA components (with some hexaacyl components at long retention times). The structure of one possible pentaacyl MLA with molecular weight 1479 is shown in Fig. 5.

Fraction II produced six peaks by HPLC separation (Fig. 6). Peak 2-1 had a molecular ion peak (M + K⁺) at 1534, corresponding to a molecular weight of 1495, or 16 atomic mass units more than the structure shown in Fig. 5. The loss of 216 atomic mass units produced the peak at 1318, indicative of hydroxyauric acid, but the absence of a loss of 200 atomic mass units suggested that there are no lauric acids present. The peak at 1249 was a loss of 285 atomic mass units from the molecular ion, indicating that the 2-position had hydroxyauric acid (OH₃C₁₄) rather than C₁₂OC₁₂. The peak at 989 (990) indicated that hydroxyauric acid is in the distal sugar as hydroxyauroxymyristate. This fully hydroxylated compo-
**Fatty acid distribution of HPLC-fractionated dimethyl MLA, fraction I**

<table>
<thead>
<tr>
<th>HPLC peak*</th>
<th>Retention time (min)</th>
<th>Fatty acid distribution*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>32.6</td>
<td>OHC$<em>{12}$, OHC$</em>{12}$OC$<em>{12}$, OHC$</em>{12}$, C$<em>{12}$OC$</em>{14}$, C$<em>{12}$OC$</em>{14}$, C$<em>{12}$OC$</em>{14}$</td>
</tr>
<tr>
<td>1-2</td>
<td>34.2</td>
<td>OHC$<em>{12}$, OHC$</em>{12}$OC$<em>{12}$, OHC$</em>{12}$, C$<em>{12}$OC$</em>{14}$, C$<em>{12}$OC$</em>{14}$, C$<em>{12}$OC$</em>{14}$</td>
</tr>
<tr>
<td>1-3</td>
<td>34.8</td>
<td>OHC$<em>{12}$, OHC$</em>{12}$OC$<em>{14}$, OHC$</em>{12}$, C$<em>{12}$OC$</em>{14}$, C$<em>{12}$OC$</em>{14}$, C$<em>{12}$OC$</em>{14}$</td>
</tr>
<tr>
<td>1-4</td>
<td>35.8</td>
<td>OHC$<em>{12}$, OHC$</em>{12}$OC$<em>{14}$, OMeC$</em>{12}$, C$<em>{12}$OC$</em>{14}$, C$<em>{12}$OC$</em>{14}$, C$<em>{12}$OC$</em>{14}$</td>
</tr>
</tbody>
</table>

* Silicic acid column fraction I was methylated and subjected to HPLC.

**Proposed structure of pentaacyl MLA (M$_2$) of M$_2$ = 1479.**

**Fig. 5.**

**Fig. 6.** A, HPLC of MLA fraction II, and LD-mass spectra of: B, peak 2-1; C, peak 2-2; D, peak 2-3; E, peak 2-4; F, peak 2-5; and G, peak 2-6.

Peak 2-4 (Fig. 6e) is a methylated derivative of a component with molecular weight 1479, since it showed losses of both hydroxyauric acid and methoxylauric acid. The peak at 1065 indicated that the lauric acid is attached to myristic acid on the reducing end.

Peak 2-5 (Fig. 6f) had a molecular ion (MK* at 1502) that corresponded to a molecular weight of 1463 and contained one less hydroxyl group than that shown in Fig. 5. The predominance of the loss of 200 atomic mass units suggested that there are fewer hydroxyauric acids. The peak at 974 indicated that this did not occur on the distal subunit. The loss of 467 atomic mass units at 1034 rather than 285 atomic mass units suggested that the reducing C$_{12}$OC$_{14}$ is intact.

Peak 2-6 was a hexaacyl MLA identical to the major component observed on peak 1-3 and appeared at a comparably long retention time. The structures of the components from fraction II are summarized in Table II.

The final fraction III was separated into four peaks and analyzed by LD-mass spectrometry (data not shown). The analysis showed that the components found in these peaks were virtually identical to those in the previous two fractions.

The four major components of MLA of *N. gonorrhoeae* with molecular weights of 1463, 1479 (pentaacyl MLA), 1661, and 1677 (hexaacyl MLA) are summarized in Table III. Additional peaks in the HPLC fractions were observed either as methylated derivatives of the major components or as several minor components. There was a small pentaacyl component in which the distal C$_{12}$OC$_{14}$ was replaced by an OHC$_{12}$OC$_{14}$ and had a molecular weight of 1495 (peak 2-1). There were also several minor hexaacyl components in which one of the N-linked hydroxyaurmyristic acids was replaced by hydroxylauric acid (peak 1-1). For both the major and minor components, one can derive several generalizations. For the pentaacyl MLA,
HPLC peak 2-2 from MLA fraction II.

TABLE II
Fatty acid distribution of HPLC-fractionated dimethyl MLA, fraction II

<table>
<thead>
<tr>
<th>HPLC peak*</th>
<th>Retention time</th>
<th>Fatty acid distribution†</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>25.0 min</td>
<td>OHC12, OHClzOC14, OHC12, OHC14</td>
</tr>
<tr>
<td>2-2</td>
<td>26.4 min</td>
<td>OHC12, C12OC14, OHC12, OHC14</td>
</tr>
<tr>
<td>2-3</td>
<td>27.5 min</td>
<td>OHC12, C12OC14, OHC12, OHC14, C20C14</td>
</tr>
<tr>
<td>2-4</td>
<td>29.4 min</td>
<td>OMeC12, OHC14, OHC12, C13OC14</td>
</tr>
<tr>
<td>2-5</td>
<td>30.6 min</td>
<td>OHClz, C12OC14, H, C13OC14</td>
</tr>
<tr>
<td>2-6</td>
<td>34.8 min</td>
<td>OHC12, C12OC14, OHC12, C13OC14</td>
</tr>
</tbody>
</table>

*Silicic acid column fraction II was methylated and subjected to HPLC.
†Refer to Figs. 4 and 5 for structures and Table I for abbreviations.

TABLE III
Fatty acid distribution of the major MLA components obtained from the LPS of Neisseria gonorrhoeae strain F62

<table>
<thead>
<tr>
<th>Dimethyl MLA</th>
<th>Monoisotopic mass</th>
<th>Fatty acid distribution*</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>1463.025</td>
<td>OHC12, C12OC14, H, C10OC14</td>
</tr>
<tr>
<td>M2</td>
<td>1479.020</td>
<td>OHC12, C12OC14, OHC12, OHC14</td>
</tr>
<tr>
<td>M3</td>
<td>1661.166</td>
<td>OHC12, C12OC14, OHC12, C13OC14</td>
</tr>
<tr>
<td>M4</td>
<td>1677.181</td>
<td>OHC12, OHC12, OHClzOC14, OHC12, C13OC14</td>
</tr>
</tbody>
</table>

*Refer to Figs. 4 and 5 for structures and Table I for abbreviations.

Lipid A Obtained from LPS of N. gonorrhoeae

The LPS of N. gonorrhoeae appears to be directly involved in natural human immunity and susceptibility to disseminated gonococcal infections. Since lipid A is the most biologically active part of the LPS molecule, it is conceivable that the structure of lipid A might influence the nature and magnitude of responses made by the host to infection or immunization. For example, the absence of a reducing-end phosphate group makes the lipid A (MLA) relatively nontoxic by several criteria (26, 27). The absence of a normal fatty acid in lipid A (as in precursor lipid A) also reduces the toxicity (26) and allows LPS nonresponder mice to become responder (28). Incorrect placement of the fatty acids into the glucosamine disaccharide in the organic synthesis of lipid A results in products with low biological activities (29, 30). These recent studies showed that subtle differences in the structure of lipid A can result in significant changes in their biological properties. For the gonococcus in particular, recent work indicates that lipid A is crucial to epitope expression by some components in a strain's LPS. The removal of acyl-linked fatty acids ablates or reduces the reactivity of such components with a monoclonal antibody. Covalent coupling of the LPS-derived oligosaccharide with long-chain aliphatic acids enhanced the antigenicity of the oligosaccharide. Knowledge of the fine structure of the lipid A moiety of gonococcal LPS is of primary importance in defining the role of LPS in the pathogenesis of and immunity to gonorrhea.

The LPS of N. gonorrhoeae is much less cytotoxic to macrophage than the LPS of other Gram-negative organisms. Peavy et al. (31) showed that 50 μg of LPS from N. gonorrhoeae reduced cell viability to 14%, whereas LPS from Escherichia coli 0111:B4, Salmonella enteritidis, and Serratia marcescens produced a similar degree of cytotoxicity at one-hundredth the concentration. This cytotoxicity is directly related to lipid A.

Knowledge of the structure of lipid A obtained from the LPS of N. gonorrhoeae is meager. Jennings et al. (32) determined the chemical composition of the LPS from a related organism, Neisseria meningitidis. Perry et al. (33), Stead et al. (34), and Schneider et al. (4) all determined the chemical composition of LPS and/or lipid A obtained from the LPS of N. gonorrhoeae. The results of these early studies showed the presence of KDO in their LPS preparations. The lipid A preparations were shown to contain phosphate, glucosamine, laurate, hydroxylaurate, and hydroxy-myristate.

Since almost no structural information is available, an assumption has been made that the basic structure of the MLA obtained from the LPS of N. gonorrhoeae is similar to that of MLA from other Gram-negative bacteria. This assumption is supported by the literature (35). The model lipid A chosen for comparison in the present study was the hexacetyl MLA derived from S. minnesota (18). Ester-linked fatty acyl...
groups are cleaved by FAB-mass spectrometry whereas amide-linked fatty acyl groups are not (16, 18). The normal fatty acids are generally involved in acyloxyacyl linkages (15-18). This information, along with the results obtained from the present study, was used to characterize the dimethyl MLAs derived from \textit{N. gonorrhoeae}.

We applied the fractionation procedures that had been used to successfully determine the complete structures of the MLAs derived from the \textit{Salmonella} strains (16-18). The MLAs were first fractionated on a silicic acid column to yield the crude hexaacyl and pentaacyl MLA, each fraction was then methylated, and reverse-phase HPLC was performed to yield a series of peak fractions. These peaks were analyzed by LD-mass spectrometry. FAB was used on two major fractions. A total of 13 different structures of dimethyl MLA were identified. From the results of these analyses, it became clear that the backbone structure of the MLA from \textit{N. gonorrhoeae} is a glucosamine disaccharide. We discovered that positive ion FAB and LD caused similar cleavage of the fatty acyl residues. Only the hydroxylauric and lauric acids were cleaved from the dimethyl MLA. There was no indication of the release of the two possible acyloxyacyl groups (lauroxylaurate or lauroxymyristate), demonstrating that the only ester-linked fatty acids were hydroxylauric and lauric acids. The hydroxymyristic acid must then be nitrogen linked. NMR spectrum of dimethyl hexaacyl MLA indicated that fatty acyl groups are esterified to the 3- and 3’-positions of the disaccharide, demonstrating that these are the sites of attachment of the hydroxylaurate.

The common oxonium ion at 876 seen in the FAB-mass spectrum of a hexaacyl (M8, peak 1-3) and pentaacyl (M9, peak 2-2) suggests that the distal subunit of M8 and M9 contain one each of glucosamine, phosphate, laurate, hydroxylaurate, and hydroxymyristate. The peak at 974 in the LD-mass spectrum of M8, M9, and M3 suggest that all three of these components have a similar composition in the distal subunit, while the peak at 990 in the mass spectra of M8 (peak 1-2) suggests an additional hydroxyl group in the distal subunit.

This study shows that LD-mass spectrometry complements the FAB-mass spectrometry in the structural analysis of dimethyl MLA. LD-mass spectrometry causes specific fragmentations of the reducing end sugar (Scheme 1) that allows one to establish the nature and location of the fatty acids at the reducing end.

Since there are only two available positions for the three fatty acyl groups in the distal subunit, one of these two positions must be occupied by an acyloxyacyl group. A similar situation appears to exist with the fatty acyl groups in the reducing-end subunit of M6. Our results support a structure of M6 (Fig. 4) where \( R_1 = R_2 = \text{hydroxylaurate}, \) and \( R_3 = R_4 = \text{lauroxymyristate} \). The suggested structure of M6 (Fig. 5) indicates \( R_1 = R_2 = \text{hydroxymyristate}, \) \( R_3 = \text{lauroxymyristate}, \) and \( R_4 = \text{hydroxyoxymyrustate} \).

There were four major structures of free lipid A generated by the acid hydrolysis of LPS from \textit{N. gonorrhoeae}. This can be related to the structural variations to be found in the lipid A moiety of LPS. The sizes of the lipid A(s) from this source were generally 84 mass units (or 6 CH2 groups) smaller than the corresponding lipid A(s) obtained from the \textit{Salmonella} strains. Moreover, the lauric acid (normal fatty acid) was singularly present in both reducing and distal subunits of the hexaacyl MLA. This compares with the presence of two normal fatty acids at the distal subunit of the hexaacyl MLA from the \textit{Salmonella} strain (16-18). The influence of this difference in the fatty acid distribution on biological activity is not known.

We examined the structural relationship among the four prominent components of the MLAs derived from the serum-sensitive strains of \textit{N. gonorrhoeae} (Table III). The pentaacyl MLAs (M1 and M2) appeared to differ by the presence of either a laurate or a hydroxylaurate group. The additional laurate in M1 is linked to hydroxyoxymyrustate to give an acyloxyacyl group.

The hexaacyl MLAs (M3 and M4) differed by the presence of an additional oxygen atom in M4, and we found that hydroxyoxymyrustate is replaced by a hydroxyoxymyristate in the distal subunit of M4. The presence of a hydroxyacyloxyacyl group in the lipid A of Gram-negative bacteria has been reported (36). This higher polarity of M4 over M3 could have led to the observed retention time that was slightly lower for M4 than for M3.

This study was undertaken to demonstrate that one can obtain much useful structural information on the lipid A by utilizing a combination of HPLC and mass spectrometry. We were able to obtain precise structural information on the components of MLAs found in \textit{N. gonorrhoeae} that might be useful for biological studies on the LPS. In this regard, it would now be interesting to compare the fine structure of MLAs obtained from a panel of LPS of the serum-sensitive and serum-resistant strains of \textit{N. gonorrhoeae}. Such a study is planned.

Although it is unlikely that further study of the MLAs of \textit{N. gonorrhoeae} will reveal new structural features, it is needed to unequivocally establish their complete structures. Although LD-mass spectrometry suggests 1' \( \rightarrow \) 5 glycosidic linkage of the glucosamine disaccharide, methylation analysis should be done to confirm this (37). Two-dimensional NMR spectroscopy should be done on HPLC-purified dimethyl MLA to establish the position of the phosphate group in the distal subunit and the anomeric configurations of the distal and reducing end sugars (18). These studies are presently in progress.

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REFERENCES
Lipid A Obtained from LPS of N. gonorrhoeae

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