Purification and Structural Determination of Nontoxic Lipid A Obtained from the Lipopolysaccharide of *Salmonella Typhimurium*.

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Endotoxin extracted from the heptose-less mutant of *Salmonella typhimurium* was hydrolyzed in 0.1 N HCl in methanol/water (1:1, v/v) at 105 °C to yield lipoid A, which was then fractionated on a Sephadex LH-20 column to yield a major monophosphoryl lipid A fraction. The monophosphoryl lipid A was further fractionated by preparative thin layer chromatography. This process yielded three major bands (TLC-1, -3, and -5) and two minor bands (TLC-7 and -9). The purity of these fractions was established by ion exchange and reverse phase high performance liquid chromatography.

The thin layer fractions were analyzed by fast atom bombardment mass spectrometry. TLC-1 and -3 gave molecular ions (M-H)- at m/e 1730 and 1716, respectively. Both of these fractions contained β-hydroxymyristic, lauric, and 3-myristoxymyristic acids in O-acyl linkages. The molecular formula and molecular ions (M-H)- at m/e 1280 and 1098, respectively. TLC-1 contained lauric and β-hydroxymyristic acids in the O-acyl linkages. TLC-7 contained lauric and β-hydroxymyristic acids in the O-acyl linkages. TLC-9 contained a single O-acylated β-hydroxymyristate group. TLC-1 and -3 were nontoxic in chick embryo lethality test and regressed established tumors in the syngeneic guinea pigs.

Ribi et al. (1) showed that when the crude endotoxin from the heptose-less mutant of *Salmonella typhimurium* was combined with trehalose dimycolate from mycobacteria in oil droplets and injected directly into established tumors (line 10 hepatocellular carcinoma) in syngeneic guinea pigs, over 90% of the animals were cured. Recently, Takayama et al. (2) isolated a nontoxic lipid A fraction that also has antitumor activity. These studies indicated that the next step to take is to determine the relationship of the structure of lipid A to both toxicity and tumor regression activity. However, structural determination requires highly purified samples and lipid A is acknowledged to be a complex mixture. A review of the literature suggested that the task of fractionating lipid A is extremely difficult (3).

Lipid A is a glucosamine disaccharide with a (β1) → 6 linkage to which are attached two phosphate groups at positions 1 and 4' (4, 5) (Structure 1). There are two N-acyl linkages at positions 2 and 2' with β-hydroxymyristoyl residues (R1), and three O-acyl linkages at positions 3, 4, and 6' to which 3-myristoxymyristoyl, β-hydroxymyristoyl, lauroyl, myristoyl, or palmitoyl residues (R2) can be linked. An examination of this structure shows that the variable factors in the biological activity-structure relationship might be: (a) the presence or absence of the two phosphate groups, (b) the number and kinds of O-acyl fatty acids, and (c) the presence or absence of 4-amino arabinose or pyrophosphorylethanolamine. Our previous studies had already shown that the removal of the acido-labile sugar 1-phosphate group reduces the toxicity of lipid A (2, 6). This problem called for the development of methods to completely separate lipid A and to analyze its purified fractions.

In our study, we were able to separate many structural analogs of lipid A from acid-hydrolyzed endotoxin to virtual homogeneity and to establish their exact sizes by FAB mass spectrometry. Two of these well characterized fractions were found to be nontoxic and retained tumor regression activity.

**Experimental Procedures**

**Materials**—Acetonitrile (distilled in glass), THF, chloroform, and methanol were purchased from Burdick and Jackson Laboratories, Muskegon, MI. Chemicals were purchased from the following companies: [1-14C]acetate, Amersham; TEA and myristoyl chloride, Aldrich Chemical Co.; tetrabutylammonium phosphate (Pic A), Eastman Kodak Co. and Waters Associates, Inc., Milford, MA; Silica Gel H thin layer plates, Analabs, North Haven, CT; and μPorasil (10-μm silica gel), Waters Associates.

**Growth of Bacteria**—Cells of the heptose-less mutant of *S. typhimurium* G30/C21 were grown in a New Brunswick 28-liter fermentor at 37 °C for 12-16 h in a modified ammonium medium of Anderson (2, 7). Radiolabeled cells of *S. typhimurium* were grown in 100-ml cultures containing 1 mCi of [1-14C]acetate (60 μCi/μmol). These cells were the source of [14C]-endotoxin, which contained the label in the fatty acids to the extent of 26.7%.

**Isolation of the Endotoxin**—The scheme for preparing and purifying lipid A from *S. typhimurium* G30/C21 is shown in Fig. 1. The endotoxin was extracted from 1-kg cells by the method of Galanos et al. (1). The abbreviations used are: FAB, fast atom bombardment; HPLC, high performance liquid chromatography; THF, tetrahydrofuran; TEA, triethylamine.

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Structure of Nontoxic Lipid A

**Analytical Procedures**—Total phosphorus and 2-keto-3-deoxy-octonate content were determined by the methods of Bartlett (11) and Osborn (12), respectively. Samples for the glucosamine assay were hydrolyzed in 3 N HCl for 4 h at 95 °C and analyzed by the method of Enghofer and Kress (13).

**Fatty Acid Analyses**—14C-labeled TLC-1, -3, and -5 (80,000 cpm each) were hydrolyzed in 0.5 ml of 4 N HCl at 100 °C for 5 h. β-Hydroxymyristic, lauric, myristic, and palmitic acids (350 μg) were added as carriers and the reaction mixture was extracted with 4 ml of petroleum ether three times. The extract was dried, methylated with diazomethane, and subjected to HPLC. A Radial Pak A cartridge (8 mm × 10 cm) was used with a solvent system of acetonitrile/water (1:2) (v/v) at a flow rate of 5 ml/min. The fatty acid content was quantitated by determining the radioactivity of the separated fatty acid esters. The recovery of radioactivity from the cartridge was quantitative.

**TEA Treatment**—Purified labeled TLC-3 (20 μg and 20,000 cpm) was suspended in 300 μl of water and 10 μl of TEA and heated at 100 °C for 0–10 min. One of the samples of TLC-3 (60,000 cpm) was suspended in water and heated to 100 °C. TEA was then added and the mixture was allowed to stand at 22 °C for 4 days. Samples were lyophilized and standard oleic and β-hydroxymyristic acids (10 μg each) were added. Samples were applied to Silica Gel H plates (250 μm) and developed in chloroform/methanol/water/concentrated ammonium hydroxide (50:25:4:2, v/v). Bands were visualized by either exposing the plates to iodine vapor or by spraying with dichromate-sulfuric acid reagent and charring very lightly. The visualized bands were scraped into vials and counted in a scintillation spectrometer. The reaction mixture, incubated at 22 °C, was analyzed by both TLC and reverse phase HPLC.

**Chemical Synthesis of Myristoxyxymyristic Acid—β-Hydroxymyristic acid (26.8 mg) and myristoyl chloride (50 μl) were added to 2 ml of anhydrous toluene and heated at 100 °C for 30 min. Water (2 ml) was added to the reaction mixture. The mixture was heated for 15 min to hydrolyze excess acyl chloride and extracted three times with 3-ml portions of chloroform/methanol (2:1, v/v), and the extract was dried with a stream of N₂. The synthesized 3-myristoxyxymyristic acid was purified by TLC using Silica Gel H (250 μm) plates and the solvent system of chloroform/methanol/water/concentrated ammonium hydroxide (50:25:4:2, v/v). The following Rf values were obtained: 0.61 for 3-myristoxyxymyristic acid, 0.50 for oleic acid, and 0.41 for the β-hydroxymyristic acid.

**HPLC Fractionation**—HPLC was performed with two Waters 6000A solvent delivery systems, a Waters 660 solvent programmer, a Waters U6K universal liquid chromatographic injector, a variable-wavelength detector (Model LC-55, Perkins-Elmer Corp., Analytical Instruments), and a radial compression module (Model HCM-100, Waters Associates). A Radial Pak A cartridge (8 mm × 10 cm) (C-18-bonded silica, Waters Associates) was used at a flow rate of 3 ml/min. For the fractionation of labeled monophosphoryl lipid A, a linear gradient of 0-1 μl ammonium acetate in methanol/water (90:5, v/v) over a period of 80 min at a flow rate of 1 ml/min. Fractions (2 ml) were collected in counting vials and their radioactivity was determined.

**Results** of a representative fractionation which included TLC of 3.4 g of acid-hydrolyzed lipid A was suspended in 700 ml of 0.1 N HCl in methanol/water (1:1, v/v), refluxed for 45 min, and centrifuged at 10,000 rpm. The resulting precipitate was filtered through a sintered glass funnel and air-dried. The yield was 6.7 g of endotoxin/204 g of cells, dry weight.

**Preparation of Monophosphoryl Lipid A**—The endotoxin (4.9 g) was suspended in 700 ml of 0.1 N HCl in methanol/water (1:1, v/v), refluxed for 45 min, and centrifuged at 10,000 × g for 5 min. The residue was suspended in 120 ml of chloroform/methanol (2:1, v/v), washed with 48 ml of water, filtered, and dried. The yield of crude lipid A was 3.4 g. The lipid A (680 mg) was dissolved in 3 ml of chloroform/methanol (4:1, v/v) by warming, applied to a Sephadex LH-20 column (2 × 144 cm), and eluted with chloroform/methanol (4:1, v/v). Mass peaks were located by total phosphorus analysis of the column fractions or by charring aliquots on a Silica Gel G plate. Results of a representative fractionation which included 4 × 10⁶ cpm of 14C-lipid A are shown in Fig. 2. A total of 3.4 g of acid-hydrolyzed endotoxin was fractionated on the Sephadex LH-20 column to yield 1.66 g of monophosphoryl lipid A.

The purified monophosphoryl lipid A was applied to Silica Gel H plates (500 μm) (20 × 20 cm) as a streak at 6–7 mg/plate and developed in chloroform/methanol/water/concentrated ammonium hydroxide (30:5:25:4:2, v/v) (9). The sample separated into three major (TLC-1, -3, and -5) and two minor (TLC-7 and -9) bands, as shown in Fig. 3A.2

**Chemical Composition**—The neutral constituents of the lipid A (3.4 g) were hydrolyzed in 3 N HCl for 4 h at 95 °C and analyzed by the method of Enghofer and Kress (13).

**Fatty Acid Analyses**—14C-labeled TLC-1, -3, and -5 (80,000 cpm each) were hydrolyzed in 0.5 ml of 4 N HCl at 100 °C for 5 h. β-Hydroxymyristic, lauric, myristic, and palmitic acids (350 μg) were added as carriers and the reaction mixture was extracted with 4 ml of petroleum ether three times. The extract was dried, methylated with diazomethane, and subjected to HPLC. A Radial Pak A cartridge (8 mm × 10 cm) was used with a solvent system of acetonitrile/water (1:2) (v/v) at a flow rate of 5 ml/min. The fatty acid content was quantitated by determining the radioactivity of the separated fatty acid esters. The recovery of radioactivity from the cartridge was quantitative.

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2 TLC-9 was obtained in greater amounts when the endotoxin was hydrolyzed with aqueous 0.1 N HCl at 100 °C for 40 min.

**Fig. 1.** Scheme for preparing and purifying monophosphoryl lipid A from S. typhimurium C30/C21.
were purified by TLC as previously described. A-Monophosphoryl lipid A (30 mg) was suspended in 6 ml of water (1:1, v/v). However, when the endotoxin was hydrolyzed in aqueous 0.1 N HCl, the amount of TLC-1 produced was small (or nil), whereas TLC-3, -5, -7, and -9 were present.

TLC-1 through -7 were analyzed for purity by reverse phase HPLC (Fig. 4). TLC-1 and -3 resolved into one major peak which represented about 85% of the total radioactivity (Fig. 4, A and B). Both of these fractions had an identical elution time of 41 min, indicating similarity of molecular weight. TLC-5 showed one major (72%) and one minor (18%) peaks with elution times of 35 and 38 min, respectively (Fig. 4C). Most of the minor component in TLC-5 was present in TLC-4, an intermediate TLC area that contained a mixture of TLC-3 and -5. TLC-4 resolved into four major peaks which eluted between 28 and 45 min (data not presented). TLC-7 resolved into one major (48%) and two minor (21 and 16%) peaks, eluting at 25, 28, and 35 min, respectively (Fig. 4D). The peak eluting at 35 min was thought to be an overlapping contaminant from TLC-5. Based on the retention time values on reverse phase HPLC, the size differences could be predicted as follows (major components only): TLC-1 = TLC-3 > TLC-5 > TLC-7. Although TLC-1 and -3 could not be separated by reverse phase HPLC, these two fractions were separated by ion exchange HPLC using a DEAE-Sepharose column. As shown in Fig. 5, the TLC-1 peak eluted at 45 min, while the TLC-3 peak eluted at 52 min. This suggested that TLC-1 has a lower net negative charge than TLC-3.

Chemical Analyses—The chemical compositions of various TLC fractions of monophosphoryl lipid A are shown in Table I. The glucosamine/phosphate ratio of these purified samples ranged from 1.98-2.15. These purified samples were devoid of 2-keto-3-deoxyoctonate. Fatty acid analysis was carried out on 13C-monophosphoryl lipid A using reverse phase HPLC (Fig. 6). Both TLC-1 and -3 showed a 4:1 ratio of β-hydroxyamyrinic acid/lauric acid/myristic acid. This ratio was 4:1:0 for TLC-5. Only qualitative fatty acid analysis was done for TLC-7 and -9. The results were consistent with the gas-liquid chromatographic analysis performed as previously described (2) (data not presented).

Identification of Myristoxymyristic Acid in Monophosphoryl Lipid A—The 3-myristoxymyristic acid was isolated
from TEA-treated monophosphoryl lipid A and purified by TLC. The chemically synthesized 3-myristoxymyristic acid and the fatty acid isolated from the lipid A were methylated and subjected to electron impact mass spectrometry (Fig. 7).

Molecular ion (M) at m/e 468 and M-31 at m/e 437 were present. The cleavage of the internal ester bond yielded prominent fragments at m/e 211, 240, and 257. This fragmentation pattern was consistent with the structure of the methyl ester of 3-myristoxymyristic acid.

**TEA Hydrolysis of Purified Monophosphoryl Lipid A**

Incubation of 14C-TLC-3 in aqueous TEA for 1, 2, 5, and 10 min at 100 °C followed by the TLC analysis of the hydrolysis products showed that several new deacylated products were formed (Fig. 8). In this experiment, oleic and β-hydroxymyristic acid standards were added to the samples before TLC was performed. All of the visualized bands were scraped into counting vials and assayed for radioactivity. The time course of degradation of 14C-TLC-3 by TEA treatment was then determined (Fig. 9). Fig. 9A shows a rapid release of hydroxy fatty acid and a corresponding appearance of the first product, designated Prod-1 (Fig. 9B). The rates of release of lauric and 3-hydroxymyristyic acids were similar, corresponding to the rise in the second and third products (Prod-2 and Prod-3).

TEA hydrolysis carried out at 99 °C for 4 days showed the formation of only Prod-1 (Fig. 8) and the accumulation of only 3-[14C]hydroxymyristic acid. A portion of this reaction mixture was analyzed by reverse phase HPLC (Fig. 10). TLC-3, which has an elution time of 42 min, was degraded to yield a product with an elution time of 35 min. This deacylated TLC-3 was shown to give a molecular ion (M-H) at m/e 1490 by FAB mass spectrometry (Table II). This corresponded to a loss of a β-hydroxymyristic acid from TLC-3 (m/e 1716 - hydroxy-myristic acid + H2O). This suggests that one of three O-acyl positions (position 3, 4, or 6) on the glucosamine disaccharide is very labile to hydrolysis and that this position is occupied by a β-hydroxymyristic acid.

**FAB Mass Spectral Analysis**—The results of the FAB mass spectral analysis of TLC-1 through -9 are summarized in Table II. Representative spectra of TLC-1 and -3 are shown in Fig. 11.

<table>
<thead>
<tr>
<th>Table I</th>
<th>Chemical analyses of the structural series of purified monophosphoryl lipid A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Content</td>
<td>TLC-1</td>
</tr>
<tr>
<td>Phosphate (µmol/mg)</td>
<td>0.54</td>
</tr>
<tr>
<td>Glucosamine (µmol/mg)</td>
<td>1.07</td>
</tr>
<tr>
<td>2-Keto-3-deoxyoctonate</td>
<td>0.0</td>
</tr>
<tr>
<td>(µmol/mg)</td>
<td></td>
</tr>
<tr>
<td>Glucosamine/phosphate (molar ratio)</td>
<td>1.98</td>
</tr>
<tr>
<td>Fatty acid (cpm)</td>
<td>8,276</td>
</tr>
<tr>
<td>n-Fatty acids</td>
<td>16,282</td>
</tr>
<tr>
<td>Hydroxy fatty acids</td>
<td>1.97</td>
</tr>
<tr>
<td>Hydroxy/normal fatty acids (molar ratio)</td>
<td></td>
</tr>
</tbody>
</table>

* Analyzed by reverse phase HPLC of 14C-labeled samples.

**Fig. 5. DEAE-silica HPLC of purified 14C-monophosphoryl lipid A**

A, TLC-1; B, TLC-3. The mobile phase was a linear gradient of 0-0.1 mM ammonium acetate in methanol/water (95:5, v/v) in 80 min at a flow rate of 1 ml/min. Fractions were collected at 30-s intervals and assayed for radioactivity.
TLC-1 gave a major molecular ion (M-H)− at m/e 1730 which was identified as a monophosphoryl lipid A containing β-hydroxymyristic, lauric, and 3-myristoxymyristic acids in O-acyl linkages. The molecule also contained a methyl group. The assignment was consistent with chemical and chromatographic analysis as well as degradation studies performed on the purified sample. Since there were two N-acyl-β-hydroxymyristates and one phosphate group in the glucosamine disaccharide, the molecular formula is \( \text{C}_{94}\text{H}_{171}\text{O}_{22}\text{N}_{2}\text{P} \) and the \( M_r = 1731.16 \) for the free acid. The minor ions observed in this fraction were at m/e 1716, identified as m/e 1730 − methanol + H₂O; at m/e 1520, m/e 1730 − myristic acid + H₂O; and at m/e 1294, m/e 1730 − 3-myristoxymyristic acid + H₂O.⁵ We do not know whether these minor ions were products of degradation or fragmentation.

TLC-3 gave a major molecular ion at m/e 1716, identified as m/e 1730 − methanol + H₂O. The molecular formula is \( \text{C}_{94}\text{H}_{171}\text{O}_{22}\text{H}_2\text{O}_2\text{P} \) and the \( M_r = 1717.15 \) for the free acid. It contains β-hydroxymyristic, lauric, and β-myristoxymyristic acids.

⁵ Alternatively, it could be myristic and β-hydroxymyristic acids + 2H₂O. However, this is less likely since the normal fatty acid presumably attached directly to the sugar in our preparation is predominantly lauric acid.
acids in the O-acyl linkages. A minor ion appeared at m/e 1744, similar to one discussed above (m/e 1730), except that it contains a myristic acid in place of a lauric acid. Other minor ions were noted at m/e 1520 and 1294 which appeared to belong to the TLC-1 series. Minor ions were also observed at m/e 1506 (m/e 1716 - myristic acid + H2O) and m/e 1280 (m/e 1716 - 3-myristoxymyristic acid + H2O).

TLC-3 (treated with TEA) gave a major molecular ion at m/e 1490 which represented m/e 1716 - β-hydroxymyristic acid + H2O. The minor ions observed at m/e 1638 and 1462 were difficult to interpret. The ion at m/e 1262 might represent the dehydrated derivative of the m/e 1280 species. The ion at m/e 1054 represented m/e 1262 (m/e 1716 - 3-myristoxymyristic acid + H2O).

TLC-5 gave a molecular ion at m/e 1506 which was identified to contain two β-hydroxymyristic and one lauric acids in O-acyl linkages (or m/e 1716 - myristic acid + H2O). The molecular formula and M, of the major component are C9H16O2N2P and 1506.99, respectively. Other ions were observed at m/e 1534 (which might be m/e 1744 - myristic acid + H2O), 1520, and 1280.

TLC-7 gave a major molecular ion at m/e 1280, identified to contain two P-hydroxymyristic and one lauric acids in 0-acyl linkages (or m/e 1716 - myristic acid + H2O). The molecular formula and M, of the major component are C9H16O2N2P and 1506.99, respectively. Other ions were observed at m/e 1534 (which might be m/e 1744 - myristic acid + H2O), 1520, and 1280.

Table II
FAB mass spectral analysis of the structural series of purified monophosphoryl lipid A

<table>
<thead>
<tr>
<th>TLC fraction</th>
<th>Molecular ion (M–H)</th>
<th>Other ions (minor)</th>
</tr>
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<tbody>
<tr>
<td>TLC-1</td>
<td>1730</td>
<td>1716, 1520, 1294</td>
</tr>
<tr>
<td>TLC-3</td>
<td>1716</td>
<td>1520, 1506, 1294, 1280</td>
</tr>
<tr>
<td>TLC-3 (TEA-treated)</td>
<td>1490</td>
<td>1638, 1534, 1520, 1280</td>
</tr>
<tr>
<td>TLC-5</td>
<td>1506</td>
<td>1534, 1520, 1280</td>
</tr>
<tr>
<td>TLC-7</td>
<td>1280</td>
<td>1324, 1308, 1294</td>
</tr>
<tr>
<td>TLC-9</td>
<td>1098</td>
<td>None</td>
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</table>

Table III
Toxicity and tumor regression activity of endotoxin and purified derivatives from S. typhimurium G30/C21

<table>
<thead>
<tr>
<th>Material tested</th>
<th>Lethality (%)</th>
<th>No. of animals cured (%)</th>
<th>Animals cured</th>
<th>p value</th>
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</thead>
<tbody>
<tr>
<td>Endotoxin</td>
<td>0.008</td>
<td>5/8</td>
<td>63</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Diposphoryl lipid A</td>
<td>0.055</td>
<td>NT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monophosphoryl lipid A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLC-1</td>
<td>&gt;10</td>
<td>7/8</td>
<td>88</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TLC-3</td>
<td>&gt;10</td>
<td>6/8</td>
<td>75</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>TLC-5</td>
<td>0.199</td>
<td>8/8</td>
<td>100</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ACP</td>
<td>0/8</td>
<td>0</td>
<td>0</td>
<td>NT</td>
</tr>
<tr>
<td>Oil/Tween/saline</td>
<td>0/8</td>
<td>0</td>
<td>0</td>
<td>NT</td>
</tr>
</tbody>
</table>

* The test material was solubilized in pyrogen-free 0.15 M NaCl containing 0.5% TEA and diluted appropriately before intravenous injection into 11-day-old chick embryo.

* Each guinea pig received a single intratumor injection of 150 µg of the test material combined with 50 µg of trehalose dimycolate and 150 µg of ACP (a nontoxic acetone/chloroform precipitate side fraction of endotoxin that contains an ingredient needed for tumor regression in guinea pigs) in 0.4 ml of oil/Tween/0.9% NaCl. All fractions, when combined with trehalose dimycolate without ACP, were inactive in regressing tumors. All control animals given ACP plus trehalose dimycolate died within 60-90 days.

* As determined by χ² contingency table analysis for difference with guinea pigs given oil/Tween/saline.

* Data from Takayama et al. (2).

* NT, not tested.

* NS, not significant.

**Fig. 10.** Reverse phase HPLC of 14C-labeled TLC-3 hydrolyzed in TEA for 4 days at 22°C. The conditions of the chromatography were similar to those described in Fig. 4.

**Fig. 11.** FAB mass spectra of purified monophosphoryl lipid A. The samples analyzed were: A, TLC-3; B, TLC-1; and phosphazine standard (m/e 1706).
to contain one lauric and one β-hydroxymyristic acid in O-acyl linkages (or m/e 1716 – 3-myristoxyxymyristic acid + H₂O). The molecular formula and Mᵣ of the major component are C₆₉H₁₀₀O₅N₃P and 1280.63, respectively. The minor ions at m/e 1294 might represent m/e 1744 – 3-myristoxyxymyristic acid + H₂O, and at m/e 1308, m/e 1716 – β-hydroxymyristic and lauric acids + 2H₂O. We could not determine the origin of the ion at m/e 1324.

TLC-9 gave a single molecular ion at m/e 1988, identified to contain a single O-acylated β-hydroxymyristic acid residue. The molecular formula is C₆₉H₁₀₀O₅N₃P and the Mᵣ is 1988.69.

**Biological Activity**—The biological tests carried out on the purified monophosphoryl lipid A are shown in Table III. The chick embryo lethality test showed that both TLC-1 and -3 were nontoxic, whereas TLC-5 exhibited some toxicity. All three of the purified monophosphoryl lipid A fractions were active in the tumor regression assay.

**DISCUSSION**

We have extracted the endotoxin from the rough mutant of *S. typhimurium* by the method of Galanos et al. (8) and isolated monophosphoryl lipid A from acid-hydrolyzed endotoxin. The monophosphoryl lipid A was found to be amenable to fractionation by silica gel TLC, ion exchange HPLC, and reverse phase HPLC. These separations were achieved according to the degree of polarity and O-acylation. The purified samples were not only analyzed by the conventional chemical means but also by a new technique, FAB mass spectrometry, which yielded precise molecular ion (M-H)⁻ values.

We were able to sort out the complex mixture of the structural analogs present in our lipid A preparation. The distribution showed a wide range of different monophosphoryl lipid A(s) with varying number and kinds of O-acyl groups (summarized in Table IV). Our results were compared with the calculated O-acyl contents of isolated endotoxins reported by other investigators. TLC-1 and -3 represented the lipid A with the highest degree of O-acylation. It is notable that our preparations do not contain a significant amount of palmitic acid presumably attached directly to the sugar residue. The myristic acid in our preparation was predominantly associated with 3-myristoxyxymyristic acid.

Smaller lipid A(s) were present in our preparation. These included TLC-5, -7, and -9 with Mᵣ = 1507, 1281, and 1099, respectively. We suggest that the 3-myristoxyxymyristic acid in TLC-5 is absent, and in its place, there is a P-hydroxymyristic acid. We also suggest the absence of 3-myristoxyxymyristic acid in the lower analogs of monophosphoryl lipid A. It is presently difficult to determine whether the O-acyl contents of the lower analogs were naturally occurring or degradation products of TLC-1, -3, and -5. The O-acyl content of TLC-9 was similar to that of the lipid A precursor reported in the 2-keto-3-deoxyoctonate-defective mutant of *S. typhimurium* by Rick et al. (18).

We have developed a new reverse phase HPLC system for the fractionation of monophosphoryl lipid A. This fractionation on a C-18-bonded silica cartridge required the addition of tetrabutylammonium phosphate to the solvent system to serve as a paired-ion. High resolution was achieved based on the total chain length of the fatty acid residues. This system easily separated the lipid A(s) differing by only one fatty acid unit. Partial separation was indicated for lipid A(s) differing by only 28 atomic mass units (C-2). However, we could not follow the separation by changes in absorbance at 210 nm since highly purified THF with low ultraviolet absorbance was needed. To our knowledge, this is the first report which describes the successful HPLC fractionation of a large and amphiphatic molecule such as lipid A.

The presence of 3-myristoxyxymyristic acid in lipid A prepared from the endotoxin of Re mutant of *S. typhimurium* was confirmed by isolating the acid after TEA hydrolysis at 100 °C and comparing its mass spectrum to that of the chemically synthesized acid. Myristoxyxymyristic acid was initially shown to be present in *Salmonella minnesota* R595 by Retschel et al. (17).

TLC-1 and -3 had identical fatty acid composition. However, the two compounds differed by 14 atomic mass units, suggesting the presence of an additional methyl group in TLC-1. Since no methoxy fatty acid was detected on fatty acid analysis, the methyl group could not be on the fatty acid.

Because of the large difference in the elution time of TLC-1 and TLC-3 on DEAE-pMorsa1 column chromatography (also on TLC), suggesting a difference in charge, the methyl group might be on the phosphate residue. We do not know whether a TLC-1-type structure occurs naturally or is a product of the acidic methanol/water hydrolysis of endotoxin.

Since monophosphoryl lipid A(s) are nonvolatile, they are not amenable to analysis by the usual electron impact mass spectrometry. We showed that a newly developed technique of FAB mass spectrometry (21, 22) is eminently suited for lipid A analysis. By this method, we were able to obtain the molecular ion (M-H)⁻ values with an accuracy of ±1.0 atomic mass units. The presence of the phosphate group appeared to contribute to the strong signals received for the molecular ion in the negative mode of operation.

When TLC-3 was treated with TEA at 22 °C, β-hydroxy-myristic acid was selectively released, probably from the 6’

<table>
<thead>
<tr>
<th>Source of lipid A/endotoxin</th>
<th>O-Acyl fatty acids*</th>
<th>Mᵣ</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. typhimurium</em> G30/C21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLC-1</td>
<td>1 1</td>
<td>1731</td>
<td>Present study</td>
</tr>
<tr>
<td>TLC-3</td>
<td>1 1</td>
<td>1717</td>
<td>Present study</td>
</tr>
<tr>
<td>TLC-3 (TEA)</td>
<td>1 1</td>
<td>1491</td>
<td>Present study</td>
</tr>
<tr>
<td>TLC-5</td>
<td>2 1</td>
<td>1507</td>
<td>Present study</td>
</tr>
<tr>
<td>TLC-7</td>
<td>1 1</td>
<td>1281</td>
<td>Present study</td>
</tr>
<tr>
<td>TLC-9</td>
<td>1</td>
<td>1099</td>
<td>Present study</td>
</tr>
<tr>
<td><em>S. minnesota</em> R595</td>
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<tr>
<td><em>S. typhimurium</em> PRX20</td>
<td>1 1</td>
<td>1729*</td>
<td>Retschel et al. (17)</td>
</tr>
<tr>
<td>E. coli K12</td>
<td>1 1</td>
<td>1099*</td>
<td>Rick et al. (18)</td>
</tr>
<tr>
<td>E. coli K12</td>
<td>1 1</td>
<td>1265*</td>
<td>Boman and Monner (19)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1491*</td>
<td>Rosner et al. (20)</td>
</tr>
</tbody>
</table>

*OH-C₆, β-hydroxymyristic acid; MM, 3-myristoxyxymyristic acid.
*Calculated for the monophosphoryl derivative of lipid A.
*The diposphoryl lipid A is believed to be a precursor of lipid A.
position. The product of this hydrolysis was a compound with an $M_1 = 1491$. The O-acyl fatty acid composition of this product is similar to that suggested for the endotoxin from *Escherichia coli* K12 by Rosner et al. (20).

The suggested structure of the monophosphoryl lipid A with the highest degree of O-acylation (major component of TLC-3) is shown by Structure 2, where we have placed either lauroyl or 3-myristoxymyristoyl residue or hydrogen ($R_1$) at positions 3 and 4 and a $\beta$-hydroxymyristoyl residue ($R_2$) at position 6'. If there is a free hydroxyl group at position 3 or 4, the lauric acid could be associated with the nitrogen-linked hydroxy fatty acid. This should reflect the O-acyl composition of the native endotoxin from *S. typhimurium*. The higher homolog of TLC-3 might have one of the $R_2$ equal to a myristoyl instead of a lauroyl residue where the molecular ion (M-H)$^- \times \text{unity} \approx 1744$.

We have described a simple and effective method for the purification and characterization of monophosphoryl lipid A. TLC-1 and TLC-3 were found to be nontoxic in the chick embryo lethality test and to regress established tumors in syngeneic guinea pigs. These results show that the monophosphoryl lipid A with the highest degree of O-acylation (four O-acyl groups) is nontoxic. We shall now test these preparations for other biological activities, i.e. proliferation of B-cells and activation of macrophages.

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**REFERENCES**