JOURNAL OF BACTERIOLOGY, Dec. 1967, p. 1824-1836
Vol. 94, No. 6

Endotoxic Glycolipid from a Heptoseless Mutant of *Salmonella minnesota*

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Received for publication 20 September 1967

The endotoxin of a heptoseless mutant of *Salmonella minnesota* R595 was extracted with phenol-water. Most of this material was found distributed in the insoluble fraction of the extract. The results showed that the R595 endotoxin behaved as a lipid rather than as a lipopolysaccharide (LPS). The preparation, although it does not contain O-specific polysaccharides, does contain 2-keto-3-deoxyoctonic acid (KDO), hexosamine, and several other unidentified compounds. Therefore, the term "glycolipid" is used in this paper instead of lipopolysaccharide. The crude glycolipid fraction, which was soluble in a mixture of chloroform-methanol (8:2), was purified by a procedure including fractionation with organic solvents and by different-column chromatographic methods. Although a chromatographic fraction of the glycolipid showed homogeneity in most systems investigated, the presence of contaminants could not be excluded. Chemical analysis of the glycolipids showed the absence of hexoses and heptoses. Constituents which were found were hexosamine, KDO, fatty acids, and phosphorus, which showed a relatively simple chemical composition. Partial acidic hydrolysis of the glycolipid yielded hexosamine-phosphates, as described in "Lipid A" fractions of smooth LPS preparations. Thin-layer chromatography of the partially hydrolyzed glycolipid showed a pattern similar to "Lipid A" fractions of other strains. The biological activity of the glycolipid was at the same level as that of other gram-negative endotoxins. Pyrogenicity, Shwartzman reactivity, and chick embryo LD_{50} values were as high or higher than those of purified *Serratia marcescens* endotoxin preparations, but mouse LD_{50} measurements gave significantly lower results.

Lüderitz and co-workers (10) found that the lipopolysaccharide (LPS) derived from a heptoseless rough mutant strain of *Salmonella minnesota* R595 consists mainly of 2-keto-3-deoxyoctonate (KDO) and "Lipid A" (used here as a blanket term for all fatty acid ester-containing firmly bound constituents of the LPS). Tripodi and Nowotny (26) found that the LPS still shows endotoxic activity, in spite of the fact that it lacks the O-specific polysaccharide and heptose. Later studies of the biological activity of such mutant LPS have been pursued by other investigators (9; Y. B. Kim and D. W. Watson, Bacteriol. Proc., p. 50, 1966), but contradictory results have been obtained.

The relative simplicity of the structure of this endotoxin preparation, judged from the few constituents present, makes this material ideal for studies of the relationship between structure and function. Therefore, we have attempted to isolate and purify the endotoxic material from a heptoseless mutant strain *S. minnesota* R595, and to investigate its chemical composition as well as its biological activities.

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MATERIALS AND METHODS

Bacterial strain. The heptoseless mutant strain, *S. minnesota* R595, used in this experiment was obtained through the courtesy of Otto Lüderitz.

Cultivation of bacteria. The bacterial cells of the R595 strain were cultivated in a broth culture medium consisting of 1.5% tryptone (Difco), 0.5% beef extract (Difco), 0.3% sodium chloride, 0.23% Na_2HPO_4, 0.5% yeast extract (Difco), and 0.3% glucose, which was described by Schlecht and Westphal (22).

A 10-liter portion of the culture medium, adjusted to *p*H 7.4, was transferred to a 15-liter bottle; it was inoculated with 500 ml of 18-hr broth culture of the R595 strain, and then incubated at 37 C for 6.5 hr. The mixtures in the bottles were stirred at 250 rev/min, and the aeration rate was 4 liters/min. A 1-liter portion of 5% phenol was added to the culture fluid after it had been cooled with tap water and allowed to stand overnight. The cells, harvested by centrifugation, were washed with saline and kept in a freezer. The yield of the wet cells was about 115 g/10 liters.

Antiserum. Rabbit antisera against the R595 cells were prepared according to the procedure described by Radvany, Neale, and Nowotny (18). Formalin-killed and heated R595 cells grown on an agar medium were used. The titer of the antisera was determined by bacterial slide agglutination; the serum showing the highest titer was used in immunodiffusion and immuno-electrophoretic analyses.

Phenol-water extraction of the R595 cells. The R595 cells were extracted with 45% phenol according to the procedure of Westphal, Lüderitz, and Bister (31). Packed cell sediment (230 g) was suspended in water. The total volume of the suspension was 1,350 ml. An equal volume of 90% freshly distilled phenol was added to the suspension at 65 to 68 C. The mixture was stirred continually for 15 min. The cold extract was centrifuged at 10,000 × *g* for 15 min and separated into an aqueous layer, an insoluble middle layer, a phenol layer, and an insoluble mucinous bottom fraction, as shown in Fig. 1.

The insoluble layers were re-extracted separately, using another 1,350 ml of distilled water, at 65 C, by mechanical stirring. The aqueous layers, combined with the aqueous solutions derived from this second extraction of the insoluble fractions, were dialyzed against distilled water, concentrated in vacuo to about 200 ml, and separated into sediment (AP) and supernatant fluid (AS) by centrifugation at 100,000 × *g* for 2 hr.

The residue of the insoluble middle layer was repeatedly extracted three times with distilled water, this time in a high-speed blender for 5 min. The homogenate was centrifuged at 10,000 × *g*, and the insoluble portion was designated as M-ins. The soluble fractions were subjected to high-speed centrifugation at 100,000 × *g* for 2 hr. The sediment obtained was called MP the supernatant fluid MS.

The precipitate, obtained from the phenol layer by the addition of three volumes of methanol and 10 g of sodium acetate dissolved in 30 ml of water, was resuspended in distilled water, dialyzed, and separated into the soluble (PhS) and the insoluble parts (Ph-ins).

The insoluble bottom fraction was treated in the same manner as described for the insoluble middle layer, and separated into sediment (BP), supernatant (BS), and insoluble part (B-ins). The yields of the fractions isolated are shown in Table 1.

Immunodiffusion analysis. A solution of 1% Noble Agar (Difco) in 0.1 M Veronal buffer (*p*H 7.8) was used in Ouchterlony's double-diffusion test on a microslide. Antigen fractions were dissolved with the same buffer at a concentration of 1 or 2 mg/ml. The aqueous solution of the glycolipid fraction was made by dispersion of the material in distilled water (adjusted to *p*H 7 with dilute NaOH), using a sonic oscillator (10 kc).

Pyrogenicity was estimated by a routine procedure (18). At least three rabbits were used for each dose.

Shwartzman reaction. Shwartzman skin tests were carried out according to the routine assay performed in this laboratory (18), using five rabbits for each sample. Either 20 μg of glycolipid or 20 μg of the partially purified trichloroacetic acid-extracted endotoxin derived from *Serratia marcescens* was used for a provocative injection.

Chick embryo lethality. This was measured by the method of Smith and Thomas (24), which entails inoculating endotoxin on the chorioallantoic membrane.

Chemical analysis. Hexosamine was determined by the Rondle and Morgan procedure (21), after hydrolysis of the sample with 5 N HCl for 6 hr in sealed tubes at 100 C. KDO was estimated by the method of Weissbach and Hurwitz (29) modified by Osborn (17). The spectrum of the colored product obtained with the thiobarbituric acid reagent was analyzed between 300 and 600 mμ. As a standard KDO source, LPS from *S. godesberg* was used, which contains 2.5% KDO. This preparation was generously donated by O. Lüderitz. Nitrogen content was determined by micro Dumas and micro Kjeldahl methods. Phosphorus

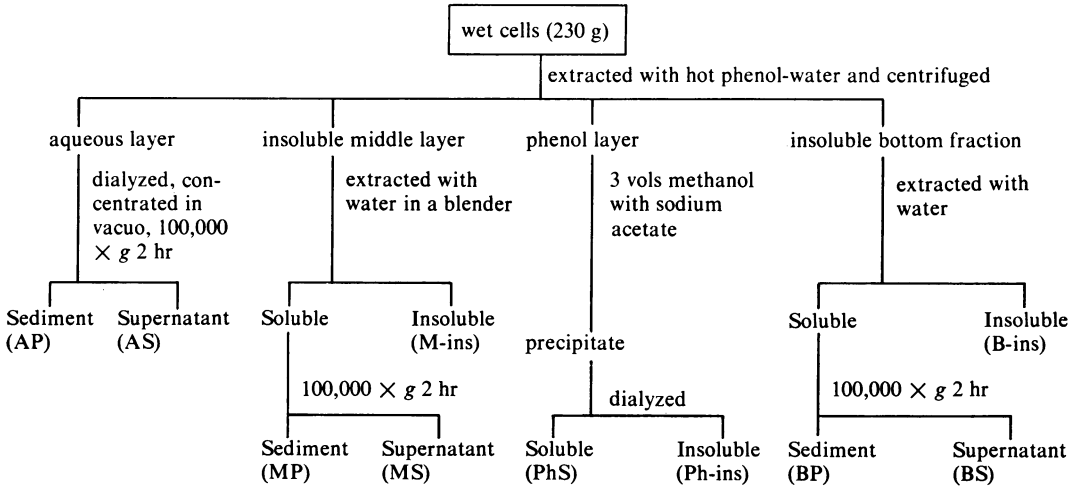

FIG. 1. *Phenol-water extraction and fractionation of the R595 cells.*

content was measured after digestion by the micromethod of Chen, Toribara, and Warner (2).

"Lipid A" content. The materials were hydrolyzed with 1 N HCl containing 15% NaCl at 100 C for 25 min. The precipitate was extracted with chloroform, and the amount of chloroform-soluble material obtained after this hydrolysis was measured as the "Lipid A" content. The lipid mixture thus obtained was also analyzed by thin-layer chromatography (TLC) after separation into acetone-soluble and acetone-insoluble portions.

Gel-filtration on Sephadex LD 20 column. Gel filtration of the glycolipid fraction was carried out by use of an alkylated Sephadex preparation in chloroform-methanol (8:2) and an organic solvent-resistant column, according to the directions of the manufacturer.

Silicic acid column chromatography. A silicic acid preparation for column chromatography, Bio-Sil A (200 to 325 μ), was used during this experiment. The column, 2.5 × 26 cm, was made from the slurry of the silicic acid suspended in a mixture of chloroform-methanol (8:2).

TLC. TLC of lipids was carried out according to the procedure recently described by Kasai et al. (6, 8).

Four silica gel preparations were used; Silica Gel G (Research Specialties Corp.), Kieselgel D-5 (Camag), Bio-Sil A (Bio-Rad), and Silica Rider (Dai-Ichi Chemicals, Tokyo). Three ion exchange cellulose preparations for TLC were also used; carboxymethyl (CM) cellulose, Ecteola cellulose, and diethylaminoethyl (DEAE) cellulose. Thin layers of all preparations except Silica Rider were made with distilled water.

The solvent systems used in these experiments were (A) chloroform-methanol-water (65:25:4), and (B) chloroform-methanol-water (65:35:5). The spray reagents used, in addition to 25% H_2SO_4, were 0.2% ninhydrin in acetone and molybdenum spray for phosphorus.

Infrared spectrum. The infrared spectrum of the glycolipid fraction was analyzed by a Perkin-Elmer and Hitachi EPIG II Infrared Spectrophotometer using KBr pellets.

RESULTS

Distribution of the endotoxic glycolipid. To find the fractions containing endotoxins, the preparations were analyzed for constituents which are characteristic for endotoxic LPS. Some of the results are shown in Table 1.

Preparation BP was derived from the insoluble bottom fraction. It showed typical endotoxic characteristics, with high contents of hexosamine, "Lipid A," and KDO. The Shwartzman reaction was very strong in the BP fraction. It also showed only the outermost precipitation line (slowest rate of diffusion), which was one of the three precipitation lines (outermost, intermediate, and innermost) which were observed using the isolated fraction.

However, the yield of this fraction was too low; it was therefore decided that the fraction M-ins should be subjected to further purification and analysis. This fraction, which was obtained in large amounts, also showed relatively high contents of hexosamine, KDO, and "Lipid A." Later analyses showed that this material, after further purification, had high biological activity.

TABLE 1. *Analyses of the R595 fractions*

Fraction	Yield	Hexosamine	Chloroform-soluble lipid after acid hydrolysis
	mg	%	%
AP	24	N.D.[a]	11.0
AS	939	0.55	0.8
MP	150	3.0	21.5
MS	171	0.5	1.0
M-ins	12,990	2.1	3.2
PhS	296	0.3	0.4
Ph-Ins	9,200	0.5	trace
BP	43	6.7	65.0
BS	89	0.5	1.3
B-ins	7,300	1.3	N.D.[a]

[a] N.D. = not determined.

Isolation and purification of the endotoxic glycolipids from the insoluble fractions. Isolation and purification of the endotoxin from the insoluble fractions were carried out according to the following two procedures. The first is shown in Fig. 2. Fraction M-ins was extracted twice with 150 ml of a mixture of chloroform-methanol (1:2) at about 55 C for 60 min. The defatted fraction (11 g) was repeatedly extracted with 200 ml of 2% sodium lauryl sulfate (SLS) in a Waring Blendor. It was then separated by centrifugation into supernatant fluid, brownish upper sediment, and white bottom sediment. The white sediment, suspended in distilled water, was dispersed by sonic oscillation (10 kc) for approximately 15 min under ice cooling. The opalescent colloidal solution obtained was dialyzed against distilled water and lyophilized. This material was called crude glycolipid fraction, CGF.

A 295-mg portion of the CGF was then dissolved in 50 ml of chloroform-methanol (8:2) at 50 C and filtered through a glass filter. The filtrate was concentrated to approximately 10 ml; five volumes of acetone were added, and the resultant precipitate was dried in vacuo.

TLC of the precipitated material showed that it was still contaminated with trace amounts of SLS. The material was applied to gel filtration using the alkylated Sephadex column to remove the contaminants. A 90-mg portion of the material was dissolved in 2 ml of chloroform-

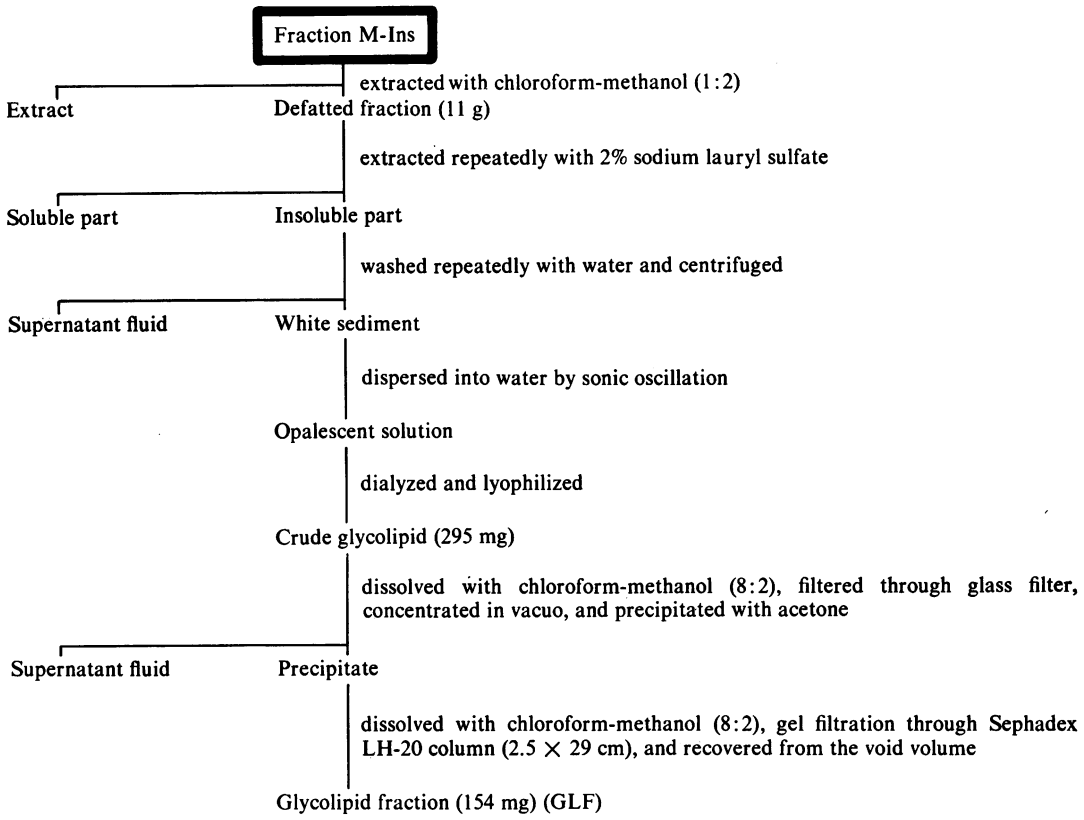

FIG. 2. *Purification of R595 glycolipid.*

methanol (8:2); this was applied on a column (2.5 × 29 cm), and then eluted with the same solvent mixture. The flow rate was 0.8 ml/min. Next, 2-ml fractions were collected, and the distribution of the material was analyzed by TLC. A fraction, referred to as glycolipid fraction (GLF), was recovered right after the void volume.

A total of 154 mg of the GLF was obtained from 295 mg of crude glycolipid through fractionation with organic solvents and by the gel filtration process. Presence of a ninhydrin-positive component in the GLF was detected by TLC on Bio-Sil A silicic acid. It was expected that the unidentified substance would be separated from the main GLF in view of the R_F values on the TLC. The GLF was therefore applied to silicic acid column chromatography. An example of the column chromatography is shown in Fig. 3. A 32-mg sample of the material dissolved in 2 ml of chloroform-methanol (8:2) was applied on a silicic acid column (2.5 × 26 cm); this was eluted first with 200 ml of chloroform-methanol (8:2) and then with chloroform-methanol (6:4). The flow rate was about 1.6 ml/min. Fractions (2 ml) of the effluent were collected and analyzed. The presence of organic material dissolved in the eluted fractions was checked by applying 10-μliter samples from each tube to TLC and by charring by heating with H_2SO_4. Another 0.1-ml sample from each tube was analyzed for KDO using the thiobarbituric acid reaction. The main fraction, which is referred to as chromatographic fraction I (CFI),

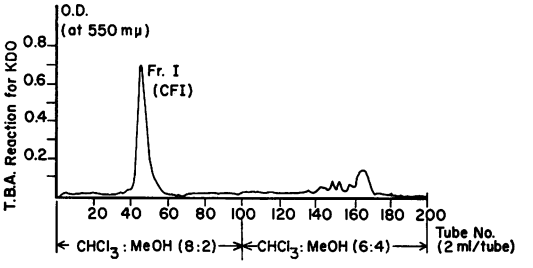

FIG. 3. *Silicic acid column chromatography of the glycolipid fraction (GLF). Column: Bio-Sil A (30 to 60 μ) 2.5 × 26 cm. Sample: 32 mg of GLF in 2 ml.*

was eluted with chloroform-methanol (8:2). The yield of CFI was about 80% of the starting GLF.

The second isolation procedure of the endotoxic glycolipid from the insoluble fractions was much simpler. It consisted mainly of the direct extraction of the BP-ins fraction with chloroform-methanol (8:2) and repeated precipitation with acetone or methanol. However, the TLC indicated that the CGF obtained by the second procedure was contaminated with phospholipid (probably phosphatidyl ethanolamine as judged from the R_F value of this component on TLC). It could be separated through gel filtration on the alkylated Sephadex column in the same manner as described previously. In this case the contaminating phospholipid was preceded by the glycolipid which was recovered after the void volume.

The gross yield of the GLF recovered from the insoluble fractions by the above two procedures was about 1% of the dry weight of the bacterial cells.

Homogeneity of the glycolipid fractions. The homogeneity of each GLF was examined by TLC, immunodiffusion analysis, and high-voltage paper electrophoresis.

The thin-layer chromatograms of the GLF on Silica Rider gave a diffused, mostly nonmigrating spot in both (A) and (B) chromatographic solvent systems; but this demonstrated that the GLF or CFI was free from any contaminants such as SLS, phosphatidyl ethanolamine, or fatty acids, which all have higher R_F values than the glycolipid (Fig. 4). TLC of the glycolipid using Bio-Sil A and solvent system (A) showed a somewhat better defined spot (Fig. 5). This migrated to a position near the solvent front. There was some difference between the R_F values of the GLF and the CFI. A weakly ninhydrin-positive substance was detected near the starting line in the case of the GLF, but not in the more purified CFI. On TLC of the GLF using Bio-Sil A and solvent system (B), in addition to the well-defined spot similar to that observed with solvent system (A), a more diffused spot was detected having a lower R_F value. By analyzing the more purified CFI on the same TLC system, the diffuse spot was much less visible. TLC of the GLF using ion exchange cellulose was not satisfactory in the two solvent systems used. The glycolipid migrated with the solvent front in CM and Ecteola cellulose layers and showed excessive tailing in the DEAE cellulose layer.

By use of immunodiffusion, the purified GLF showed only the slowest precipitation line (closest to the antigen well), which was quite similar to that of the fraction BP mentioned in the previous section. This line, however, showed some variations, being a sharp line or two lines or one diffused band, depending upon the individual variations of the R595 O-antiserum used in these tests. In immunoelectrophoresis, the glycolipid fraction showed two very close precipitation lines which migrated together from the antigen well towards the positive pole.

The CFI was free from contaminants which could be detected by ninhydrin and phosphoric acid spray reagents on TLC. Nevertheless, a small amount of ethanolaminelike substance was detected by high-voltage paper electrophoresis of the intact CFI without previous hydrolysis. About 2 mg of the sample dissolved in a small amount of chloroform-methanol (8:2) was applied on paper (Schleicher and Schuell No. 591 C), and electrophoresis at 1,000 volts for 30 min was carried out using pyridine-acetic acid-water (7:3:90) buffer at *p*H 5.3. The paper was

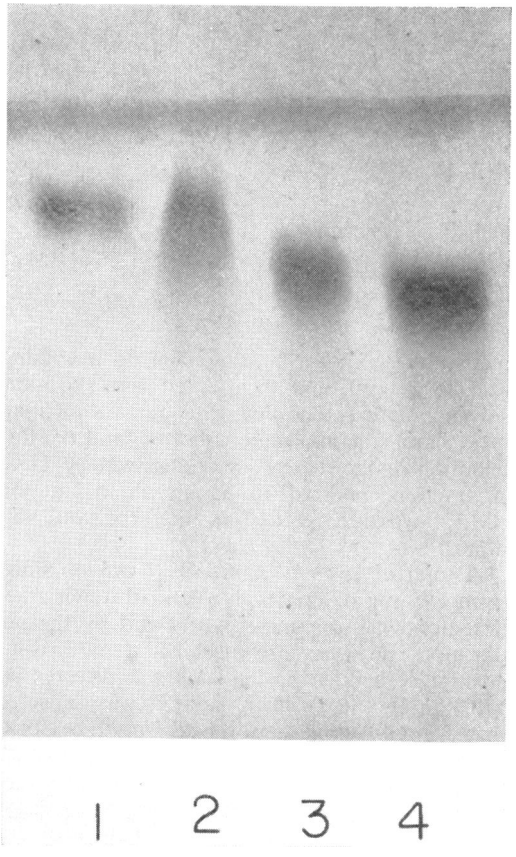

FIG. 4. *TLC of the glycolipid fractions using Silica Rider and solvent system A. The applied samples are: (1) β-hydroxymyristic acid; (2) a "Lipid A" fraction of Serratia marcescens; (3) glycolipid fraction (GLF); and (4) chromatographically pure glycolipid (CFI).*

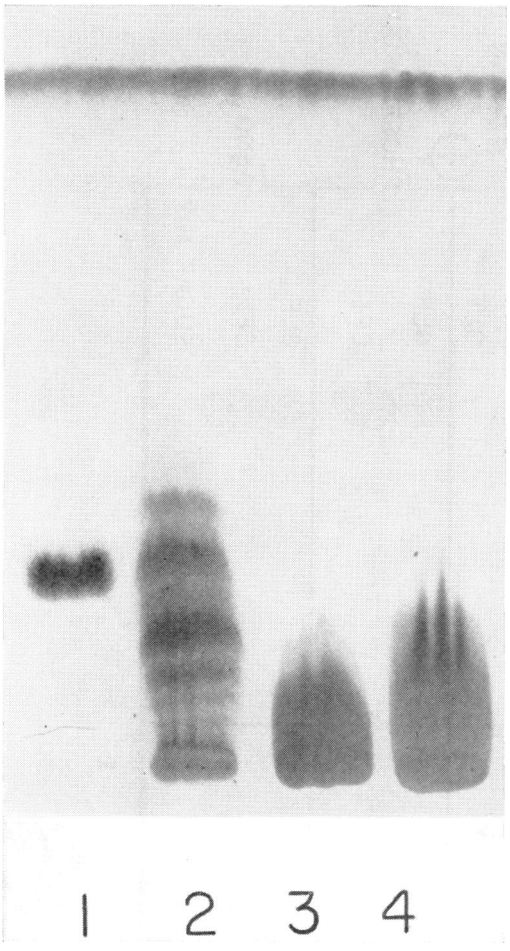

FIG. 5. *TLC of the glycolipid fractions using Bio-Sli A and solvent system A. The applied samples are: (1) β-hydroxymyristic acid; (2) a "Lipid A" fraction of Serratia marcescens; (3) glycolipid fraction GLF; and (4) chromatographically pure glycolipid CFI.*

dried and sprayed with 0.3% ninhydrin solution in butanol-lutidine (9:1). A weak ninhydrin-positive spot corresponding to the migration of a standard ethanolamine was observed, while the glycolipid remained at the starting line as a waxy, insoluble, ninhydrin-negative spot.

Solubility. By use of several organic solvents and solvent mixtures, it was found that the GLF and CFI were soluble only in chloroform and methanol mixtures; an especially good solvent mixture was chloroform-methanol (8:2). The GLF could be brought into solution in *p*H 7 distilled water, if the aqueous suspension of this preparation was treated with a sonic oscillator (10 kc) for a few minutes. The same material was barely soluble in water containing salts,

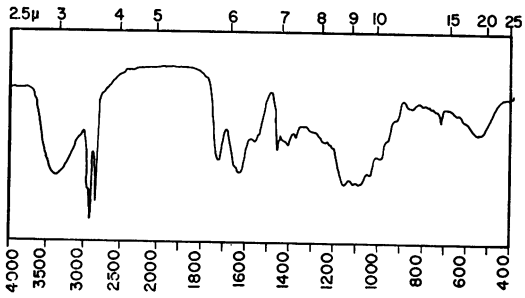

FIG. 6. *Infrared spectrum of the glycolipid fraction (GLF).*

such as NaCl, phosphate buffers, or weak acid (*p*H 4 to 5). The CFI was only sparingly soluble in *p*H 7 adjusted distilled water after sonic treatment.

Infrared spectrum. Figure 6 shows the infrared spectrum of the GLF. It appeared to be similar to the infrared spectrum of a partially purified component derived from the "Lipid A" mixture of *Escherichia coli* LPS (8).

The strong absorption at 3,400, 2,850, 1,720, 1,630, and 1,560 cm^{-1} would be assigned to OH, CH_3, COOR (R = alkyl), CO-NH-, and NH-, respectively. The absorption at about 1,240 cm^{-1} which is assigned to P = O stretching was rather weak.

Chemical composition. Water-soluble components of the acid hydrolysate were studied. The results of high-voltage paper electrophoresis of the water-soluble portion derived from the GLF and the CFI during strong acid hydrolysis in 5 N HCl for 6 hr on a boiling-water bath are shown in Fig. 7.

Trace amounts of aspartic and glutamic acids could be detected in the case of the GLF, but they were almost completely absent in the CFI. Bands corresponding to the R_F values of D-glucosamine-4-phosphate and D-glucosamine-6-phosphate, which have been described by Nowotny (16) in the hydrolysate of a "Lipid A" fraction, were also visible in the hydrolysate of the glycolipids. These migrated very slowly toward the positive pole. Strong spots corresponding to D-glucosamine and phosphoric acid and the weak spot of ethanolamine were detected by spraying the high-voltage electrophoretogram with the proper reagents.

The results of another high-voltage paper electrophoresis of the water-soluble portion derived from the GLF during weak acid hydrolysis in 0.1 N acetic acid at 100 C for 1 hr are shown in Fig. 8. Five ninhydrin-positive spots are visible, as explained on the figure. One strong and two weak spots were observed after spraying

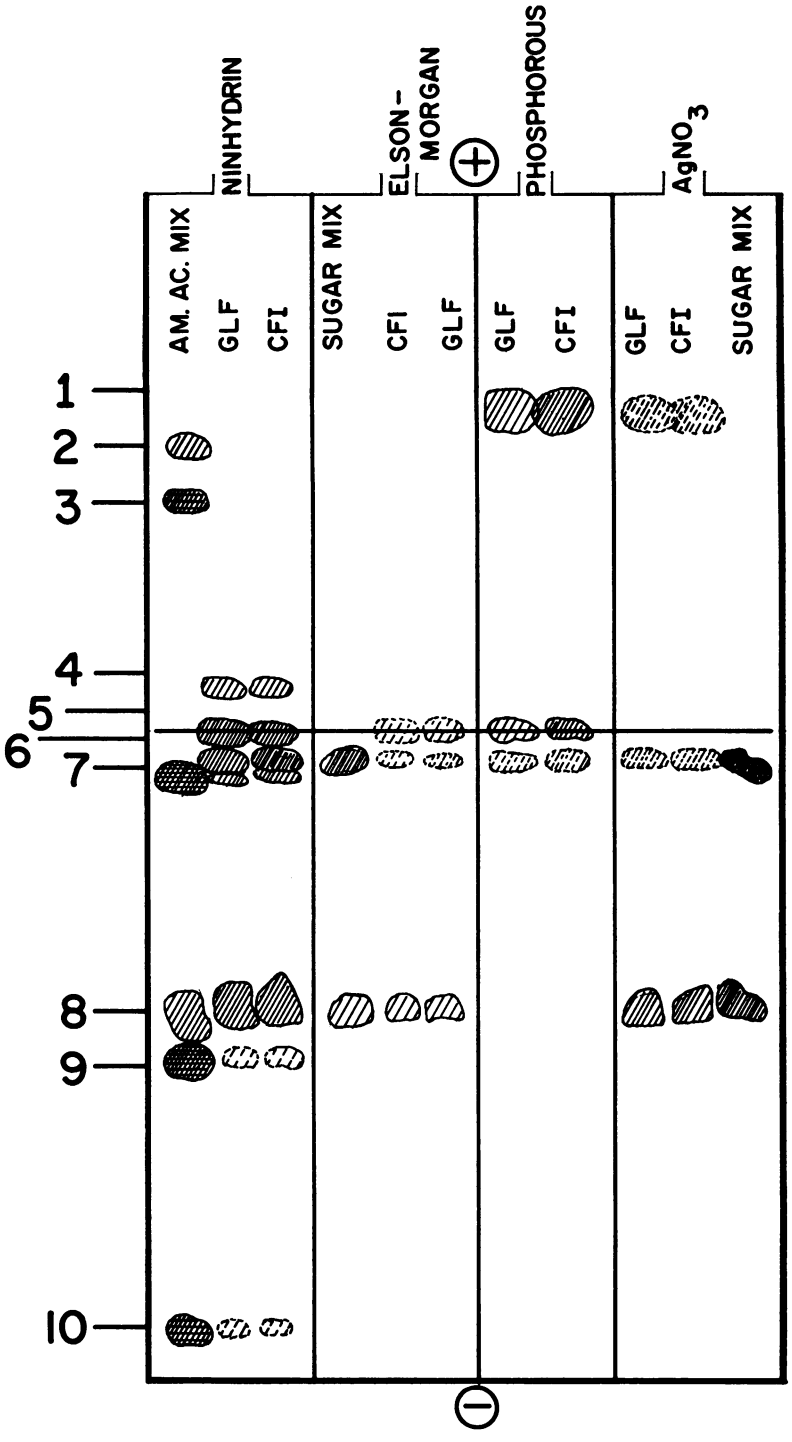

FIG. 7. *High voltage paper electrophoresis of the water-soluble components after hydrolysis with 5 N HCl. The strip was cut and sprayed with different reagents as indicated in the photograph. Components of the mixture (sugar) are D-glucosamine-6-phosphate and D-glucosamine. Explanation of the components found: (1) Phosphoric acid; (2) Aspartic acid; (3) Glutamic acid; (4) Peptide containing hexosamine-4-(?)-phosphate; (5) Hexosamine-4-(?)-phosphate; (6) Hexosamine-6-phosphate; (7) Mixture of neutral amino acids and carbohydrates; (8) Hexosamines; (9) Basic amino acids; and (10) Ethanolamine.*

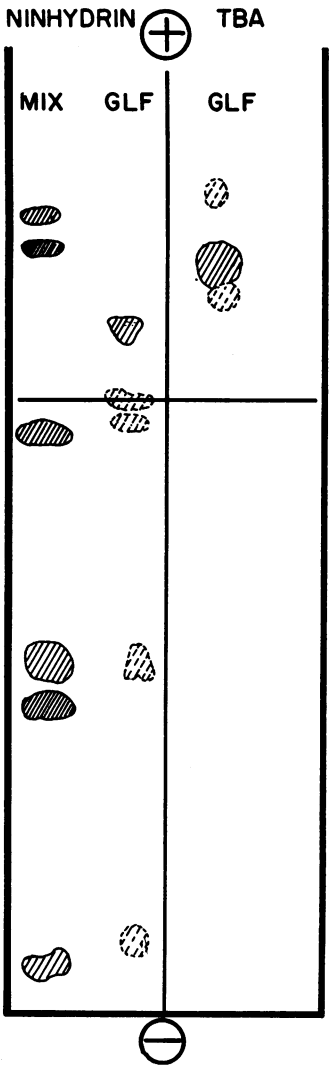

FIG. 8. *High-voltage paper electrophoresis of the water-soluble components after hydrolysis with 0.1 N acetic acid. Mixture is the same as in Fig. 7. The ninhydrin-positive components found in the glycolipid fraction (GLF) are, from the bottom up: ethanolamine; basic amino acids; hexosamine-6-phosphate; and hexosamine-4-(?)-phosphate. The spot towards the positive end is unidentified. The thiobarbituric acid (TBA)-positive components are most probably KDO derivatives.*

the paper with thiobarbituric acid reagent (28) to detect KDO derivatives.

The water-soluble portions derived from the GLF and the CFI by acid hydrolysis in 1 N HCl for 8 hr on a boiling-water bath were analyzed by paper chromatography using a butanol-pyridine-water (6:4:3) mixture or in a pyridine-ethyl acetate-acetic acid-water (5:5:1:3) solvent mixture. The chromatograms were developed with ammoniacal silver nitrate. No spots of galactose, glucose, or heptose were detectable. The same reagent developed two strong spots with bluish-gray color, corresponding to the R_F of NaCl and $CaCl_2$, indicating the presence of sodium and calcium in the glycolipid.

By the strong acid hydrolysis mentioned in the previous section, about 9.4 mg of the chloroform-soluble parts were recovered from 16.9 mg of the GLF. Lauric, myristic, palmitic, and β-hydroxymyristic acids were detected in the chloroform-soluble portion as the main long chain fatty acids, as well as four minor, unidentified fatty acids.

The "Lipid A" fraction isolated from the GLF by acid hydrolysis in 1 N HCl at 100 C for 20 min showed a thin-layer chromatogram similar to that of the "Lipid A" fraction of *Escherichia coli* LPS described by Kasai and Yamano (6, 7).

A 20.5 mg portion of the chloroform-soluble fraction was obtained from 30.4 mg of the GLF by mild acidic hydrolysis with 0.1 N acetic acid. The chloroform-soluble fraction was separated into acetone-soluble and acetone-insoluble fractions and applied to TLC. As shown in Fig. 9, the acetone-insoluble fraction still showed a chromatogram similar to that of the original GLF. The acetone-soluble material seemed to be a mixture of fatty acids, as found by TLC. The KDO content of the chloroform-soluble but acetone-insoluble fraction was about 4.6%.

The results of the elementary quantitative chemical analysis as well as the KDO and glucosamine contents are summarized in Table 2. Since nitrogen, corresponding to the glucosamine content of the GLF, was calculated as 1%, the remaining nitrogen is probably due to the presence of amino acids and ethanolamine in the preparation.

The ratio of phosphorus to hexosamine was approximately 1:1, indicating a higher phosphorus content than that of partially purified "Lipid A" fractions (1, 8, 16).

The rate of decomposition of KDO in the GLF and the CFI during acid hydrolysis in 0.02 N H_2SO_4 at 100 C is shown in Fig. 10. There are no significant differences between these glycolipid fractions with regard to the rate of decomposition of KDO, but it should be noted that the KDO content of the CFI showed some decreased value as compared with the original glycolipid fraction. The KDO content, which was estimated by extrapolation of the acidic hydrolysis to zero time, showed approximately 16% in the GLF and 13% in the CFI preparations.

Biological properties. The chick embryo lethal-

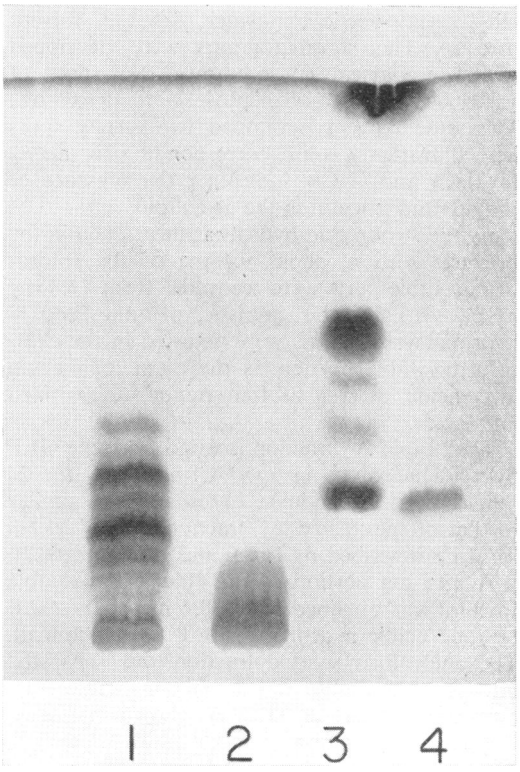

FIG. 9. *TLC of different components; (1) "Lipid A" fraction from Serratia marcescens soluble in chloroform, insoluble in acetone; (2) chloroform-soluble but acetone-insoluble fraction of GLF after acetic acid hydrolysis; (3) acetone-soluble and chloroform-soluble fraction of 2; and (4) a purified "Lipid A-2" component from Escherichia coli, isolated by Kasai and Yamano (8).*

ity test showed that both the GLF and a trichloroacetic acid-extracted endotoxin of *S. marcescens* (batch 403) have essentially similar toxicities (Table 3).

The Shwartzman skin reactivity of the GLF is shown in Table 4. Two series of experiments were carried out to test Shwartzman reactivity. The abdominal skin of all rabbits was prepared with GLF and with toxic endotoxin from *S. marcescens*, using 20, 10, 5, and 2.5 μg doses. Four rabbits were challenged 24 hr later with 20 μg of GLF, applied iv. Five rabbits received 20 μg of toxic endotoxin from *S. marcescens*, iv. All combinations gave positive results, indicating that the GLF is equally potent in the preparatory intradermal injection and in the provocative iv application. At 5- and 2.5-μg doses, the GLF showed even higher reactivity than the toxic endotoxin if used in the preparative injection, and the animals were challenged with endotoxin (Fig. 11). No difference could be detected between the two preparations if GLF was used as the iv challenge.

The CFI was barely soluble in water; therefore, only a few bioassays were carried out. Figure 12 shows an example of the Schwartzman reaction examined by using a fine suspension which was made by the same procedure as the GLF. A reaction similar to those of the GLF was again observed, but, in general, the reactivity appears to be slightly less, probably due to the relative insolubility of this preparation.

The febrile responses in rabbits were examined by inoculating the GLF at doses of 1, 0.1, and 0.01 μg per rabbit. The results are shown in Fig. 13. A 0.1-μg dose of the GLF showed febrile responses very similar, from both qualitative and quantitative standpoints, to those of any partially purified endotoxin of *S. marcescens* (18).

N. Ueta and T. Yamakawa (Department of Biochemistry, Faculty of Medicine, University of Tokyo) carried out carbohydrate and fatty acid analyses of the GLF fraction using gas-liquid chromatography. The GLF was hydrolyzed in a sealed tube with methanol containing 3% HCl for 3 hr in a boiling-water bath. The hydrolysate was extracted with petroleum ether and separated into petroleum ether-soluble and methanol-soluble parts. The former fraction was applied for fatty acid analysis using a column (2 m × 3 mm) which was packed with EGSS-X (15%)

TABLE 2. *Chemical composition of the glycolipid fractions*

	Glycolipid fraction GLP	Chromatographic fraction CFI
Nitrogen	1.64%	1.8%
Phosphorus	2.7%	2.2%
2-Keto-3-deoxy octonic acid	~16%	~13%
Glucosamine	12–13%	13–14%
Fatty acids[a]	55%	not determined
Unidentified carbohydrates and hydroxy acids[b]	3–4 components	not determined
Aspartic acid, glutamic acid[c]	trace	not detectable
Neutral and basic amino acids[c]	trace	trace
Ethanolamine[c]	(+)	(+)
Cation (Na, Ca)	(++)	(++)

[a] Principal acids; lauric, myristic, palmitic, β-hydroxymiristic
[b] By gas chromatography
[c] By high-voltage paper electrophoresis

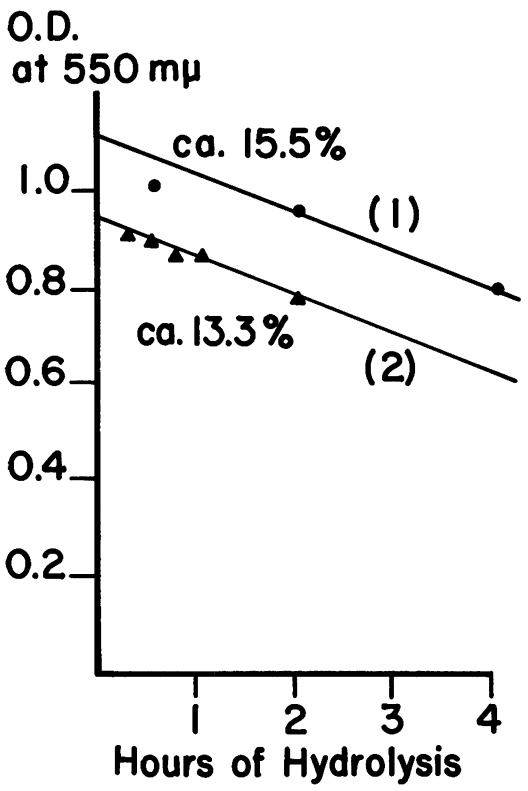

FIG. 10. *Rate of decomposition of KDO during weak acid hydrolysis. Quantitative KDO estimation done by extrapolation of the hydrolysis curve to 0 time.*

TABLE 3. *Chick embryo lethality of the glycolipid fraction*

Dose injected	Purified glycolipid fraction	Trichloroacetic acid-endotoxin from *Serratia marcescens* (No. 403)	Control (saline)
μg			
2.5	4/10[a]	2/10	
5	4/10	5/10	
10	9/10	5/10	1/10
25	9/10	8/10	
50	10/10	8/10	
100	—	9/10	

[a] Number of deaths/total number of chick embryos.

absorbed on Gaschrom RP. The methanol-soluble part was treated with trimethylsilyl (TMS) reagents. The resultant TMS derivative was applied to gas-liquid chromatography by using a metal column (2 m × 3 mm) which was packed with Ucon LB550 (5%) absorbed on Gaschrom CLH (800-1,000 mesh) at 192 (C 32).

TABLE 4. *Shwartzman skin reactivity of the GLF*

Intradermal reaction with	Intravenous challenge with 20 μg			
	Glycolipid fraction (GLF)		Toxic endotoxin	
	Dose	Reactivity	Dose	Reactivity[b]
	μg		μg	
GLF	20	4/4	20	5/5
	10	4/4	10	5/5
	5	4/4	5	5/5
	2.5	2/4	2.5	5/5
Toxic endotoxin	20	4/4	20	5/5
	10	4/4	10	5/5
	5	4/4	5	2/5
	2.5	3/4	2.5	1/5

[a] Toxic endotoxin was a trichloroacetic acid extracted and partially purified endotoxin from *Serratia marcescens* 08.

[b] Reactivity means positive skin reactions/total animals tested.

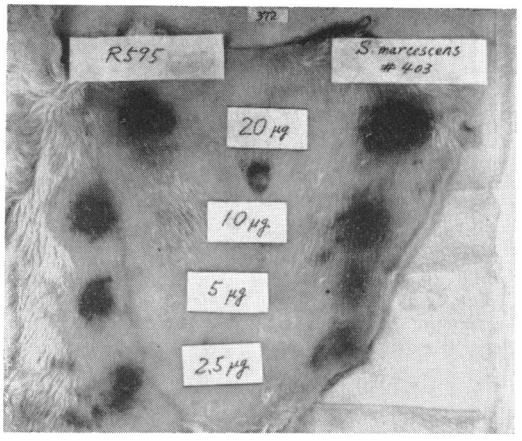

FIG. 11. *The Shwartzman reaction of the GLF and Serratia marcescens endotoxin. (R595 = GLF; No. 403 = S. marcescens endotoxin.)*

A Hitachi-Perkin Elmer gas chromatograph model F-6 was used for these analyses.

Four peaks of KDO and the peak of β-hydroxymyristate were detected as major components. In addition, several minor peaks which showed a retention time shorter than that of β-hydroxymyristic acid were also observed, suggesting the presence of three or more minor components (Fig. 14). For the identification of the KDO components, Ueta and Yamakawa used a column chromatographically purified KDO preparation isolated from *E. coli* O111 according to the method of Heath and Ghalambor (4).

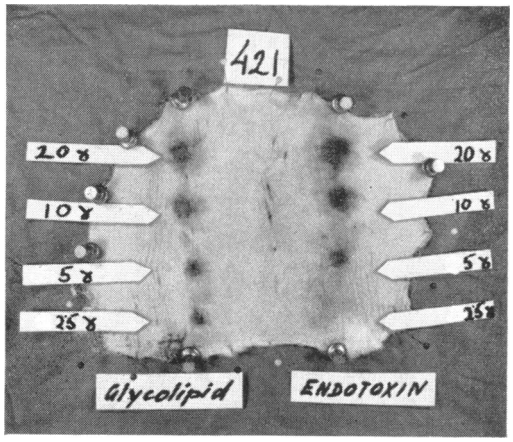

FIG. 12. *Shwartzman reaction of the CFI compared to Serratia marcescens endotoxin No. 403.*

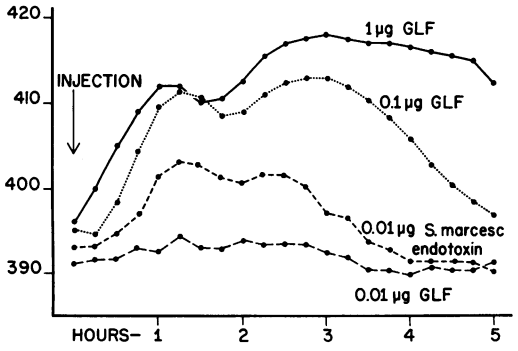

FIG. 13. *Pyrogenicity of GLF and a trichloroacetic acid extracted and partially purified endotoxin from Serratia marcescens.*

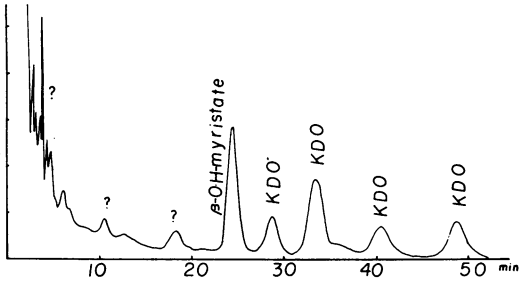

FIG. 14. *Gas-liquid chromatography of trimethylsilyl derivatives from methanolysate of the glycolipid fraction (GLF). Column: 10% Ucon-LB-550 X on Gaschrom CLH, length 2 m. Temperature: 195 C.*

The results of fatty acid analysis are shown in Fig. 15. The relative proportions, taking palmitic acid as 1.00, are: lauric acid, 1.40; myristic acid, 1.29; and β-hydroxymyristic acid, 1.56.

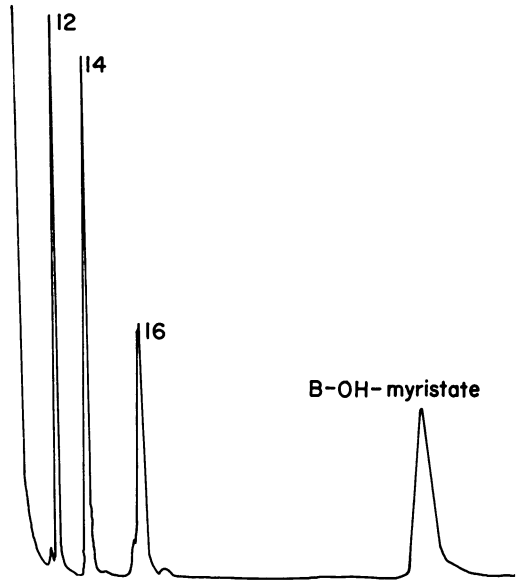

FIG. 15. *Gas-liquid chromatography of fatty acid methyl esters from the glycolipid fraction (GLF).*

DISCUSSION

The endotoxic glycolipid of the *S. minnesota* R595 mutant strain was distributed mainly in the insoluble fractions. In possible connection with this observation, it is of interest that a phenol-soluble LPS, isolated from *Xanthomonas campestris* by Hickman and Ashwell (5), has a unique chemical composition which is probably different from that of the water-soluble LPS (27). More recently, Wheat et al. (Bacteriol. Proc., p. 123, 1967) reported a similar result in extracting several gram-negative species including *Chromobacterium violaceum*, *Citrobacter* species, and *Shigella flexneri* 2b. These observations indicate that LPS may be found in fractions other than the water phase, depending primarily upon the chemical composition. The origin of the bacterial cells, and the materials and methods used for the extraction and purification, may also influence the distribution.

The yield of LPS of most smooth gram-negative strains is 2 to 6% which contains 10 to 20% (30) of the firmly bound lipid. The yield of the glycolipid was approximately 1% of the dry *S. minnesota* R595 cells. The low yield of the endotoxin seems to be a common feature of rough mutant strains (11, 14). The analytical results described here also suggest that the major part of the R595 glycolipid may be similar to the "Lipid A" part of the smooth LPS. Accordingly, it might be said that the endotoxic glycolipid yield of the R595 cells is close to usual values.

The TLC of the purified glycolipid gave us some information about the homogeneity and physico-chemical properties, but since the chromatographic pattern of the glycolipid varied markedly depending upon the quality of the silica gel preparation and the solvent system, more suitable conditions for TLC should be further studied. Although the data presented here did not reveal a great deal of inhomogeneity, further experiments are necessary to rule out this possibility.

The glycolipid fraction showed a single precipitation line in gel diffusion with some rabbit antisera, but showed double lines with others. This underlines the uselessness of this assay for proving homogeneity unless it is carried out with individual hyperimmune sera of several rabbits.

Although it is of interest that the GLF showed a very slow rate of diffusion in Ouchterlony's double diffusion test, one of the authors (7) has emphasized the close relationship between endotoxic activity and the presence of a somatic antigen which showed similarly slow diffusion. Other results have shown a similar relationship (3, 8, 12, 13, 15, 19, 23).

Regarding the solubility of the preparations, it is of interest that the partially purified glycolipid GLF was soluble in both chloroform-methanol mixtures and in water. The chromatographically purified glycolipid CFI was barely soluble in water. It seems likely that the removal of the polar substances, such as amino compounds and KDO, or both, from the GLF during purification resulted in reduced solubility in water.

The results, which showed that the GLF consists mainly of phosphoric acid, KDO, glucosamine, and fatty acids, are in accord with the findings of Luderitz and co-workers (10).

The acidic amino acids such as aspartic and glutamic acids which were found in the acid hydrolysate of the GLF were almost absent in the CFI. These amino acids have been found in most lipopolysaccharides of gram-negative bacteria (25, 30).

Since ethanolamine was released from the intact CFI on high-voltage paper electrophoresis, it is, at least in part, not linked covalently in the glycolipid structure. The hydrolysates of several purified *S. marcescens* and *E. coli* endotoxins were also found to be free of ethanolamine, as analyzed by high-voltage paper electrophoresis in authors' laboratories.

A KDO-containing component, soluble in sodium lauryl sulfate, could be separated from the glycolipids which were insoluble in the detergent. In the water-solubility of the GLF, the role of this component, as well as of those which were removed by silicic acid column chromatography (Fraction II), is apparent.

The minor components found in the gas-liquid chromatography of the TMS derivatives could not be identified. Their presence may indicate small amounts of carbohydrates in the glycolipid preparation, but they may also be other volatile products of the trimethylsilylation procedure.

It is also of interest that weak acid hydrolysis isolated a relatively undegraded lipid fraction from the GLF, which fraction was soluble in chloroform. This observation indicates the possibility of the isolation of a lipid consisting of glucosamine, fatty acids, and phosphoric acid in relatively intact condition. The glycolipids seem, therefore, to be similar to the lipid moiety of the parent smooth LPS.

The results of the biological assays correspond to the previous observations of Tripodi and Nowotny (26), who reported that different R-mutants of *S. minnesota* strain showed endotoxic activity somewhat lower than, but comparable to, the smooth lipopolysaccharides. Similar observations have been reported by Y. B. Kim and D. W. Watson (Bacteriol. Proc., p. 50, 1966). Biological assays carried out with the glycolipids as reported here showed that the GLF preparation was as active in the chick embryo lethality, Shwartzman reactivity, and pyrogenicity tests as a partially purified *S. marcescens* endotoxin. The mouse lethality test did not show the same degree of toxicity as an average endotoxin preparation. This is in accord with the observations of previous experiments (10, 26). In contrast, Kessel, Freedman, and Braun (9) found the heptoseless mutant lipopolysaccharide of *Salmonella typhimurium* to be neither toxic nor protective. This may be due to the different strain used, but it may well be attributed to methodological differences in the isolation, fractionation, and solubilization. Incompatibility among various toxicity assays has been found and studied recently (K. R. Cundy and A. Nowotny, Bacteriol. Proc., p. 79, 1967). These results indicate differences in the mode of endotoxic action in the numerous endotoxicity assays.

On the basis of the results reported here, it is supposed that some of the active sites of *S. minnesota* lipopolysaccharide preparations may reside in their lipid moieties.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the valuable help of Anne M. Nowotny for the quantitative chemical analysis, of Francine Borden for the pyrogenicity measurements, and of K. R. Cundy for chick embryo lethality determinations.

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