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Lipoteichoic Acid from *Bacillus licheniformis* 6346 MH-1. Comparative Studies on the Lipid Portion of the Lipoteichoic Acid and the Membrane Glycolipid†

David Button* and Norman L. Hemmings

ABSTRACT: A lipoteichoic acid and a membrane glycolipid were isolated from *Bacillus licheniformis* 6346 MH-1. The fatty acid composition of the two preparations were similar. Most of the fatty acids were of the branched chain type. The glycolipid was shown to be a diacyl derivative of *O*-β-D-glucopyranosyl-(1→6)-*O*-β-D-glucopyranosyl-(1→3)-glycerol. The lipoteichoic acid contained lipid, polyglycerol phosphate, and glucosamine. The lipid was released by treatment with hydrofluoric acid and by hydrolysis in dilute acid and was shown to have a structure identical with that of the membrane glycolipid.

1,2-diglycerides, substituted with either monosaccharide or oligosaccharide residues, are components of the membranes of gram-positive bacteria (Shaw, 1970). Diglycosyl diglycerides comprise a major class of glycolipids and have taxonomic significance, in that the disaccharide residues, which are contained in the glycolipids of member organisms of a given genus, are identical (Shaw and Baddiley, 1968). The sugar components of glycolipids frequently occur as structural units of polymers, that are located in the cell envelope. For example, in *Micrococcus lysodeikticus,* the same dimannosyl residue occurs in both glycolipid and membrane polysaccharide (Lennarz and Talamo, 1966) and the glycolipid and capsular polysaccharide of *Pneumococcus* type XIV contain D-galactopyranosyl-(1→2)-*O*-α-D-glucopyranosyl residues (Kaufman et al., 1965; Barker et al., 1961).

Lipoteichoic acids from *Lactobacillus fermenti* and *Streptococcus faecalis* NCIB 8191 consist of membrane teichoic acid, covalently linked to lipid. In *L. fermenti,* the lipid is identical with the membrane glycolipid (Wicken and Knox, 1970). In *S. faecalis,* the lipid moiety in the lipoteichoic acid is phosphatidylkojiobiosyl diglyceride (Ganfield and Pieringer, 1975). This lipid is a minor component of the membrane lipids and is a derivative of the principal glycolipid of *S. faecalis,* kojiobiosyl diglyceride (Fischer et al., 1973; Brundish et al., 1966).

Structural information on lipoteichoic acids from other organisms is required before the extent of the relationship between membrane lipid and lipoteichoic acid lipid can be assessed. However, chromatographic evidence has been presented, which indicates that, in both *Bacillus subtilis Marburg* strain 168 and *Micrococcus sp.* 24, the lipid portion of the lipoteichoic acid differs from either of the respective membrane glycolipid or a phosphatidyl derivative of the glycolipid (Coley et al., 1972).

In the following communication, a partial structure for the lipoteichoic acid from *B. licheniformis* 6346 MH-1 is reported, and comparative studies of the membrane glycolipid and the lipid portion of lipoteichoic acid are described.

Materials and Methods

Materials. Alkaline phosphatase (EC 3.1.3.1), hexokinase (EC 2.7.1.1), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), glycerokinase (EC 2.7.1.30), and glycerolphosphate dehydrogenase (EC 1.1.1.8) were obtained from the Boehringer Corporation (London) Ltd. α-Glucosidase (EC 3.2.1.20) and β-glucosidase (EC 3.2.1.21) were obtained

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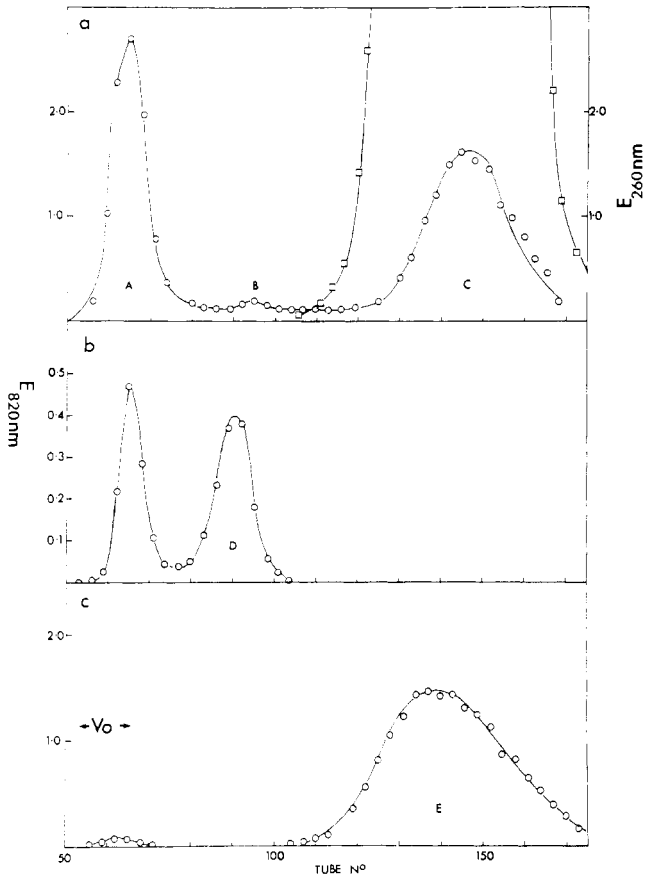

FIGURE 1: Chromatography of lipoteichoic acid and its deacylation products. Samples were applied to Sepharose 6B (140 cm × 8 cm^2). The column was eluted with 0.2 M ammonium acetate, pH 7, containing 0.02% sodium azide at a rate of 16.6 ml/hr. Fractions (5.5 ml) were collected and assayed for phosphorus (—○—) and, in some cases, their optical density at 260 nm was measured (—□—): (a) phenol extract; (b) lipoteichoic acid treated with 0.5 M hydroxylamine, pH 7, at 45 °C; (c) lipoteichoic acid treated with 0.2 M sodium methoxide.

from Sigma, London, Ltd. Sophorose was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks. Chromosorb W, OV-1, OV-101, ECNSS-M, ethylene glycol succinate, and poly(ethylene glycol succinate) were obtained from Phase Separations Ltd., Flintshire. Methyl esters of fatty acids were obtained from Supelco, Inc., Bellefonte, Pa. SilicAR-CC7 (Mallinckrodt) was obtained from Camlab, Cambridge, England.

Methods. Growth of Bacteria. B. licheniformis 6346 MH-1 was grown on Spizizen minimal medium supplemented with glucose (0.5%) and L-histidine hydrochloride (0.01%), as described by Forsberg et al. (1973). Growth was arrested by cooling to 4 °C over a period of 30 min. Cells were harvested in a refrigerated Sharples centrifuge, suspended in water at 2 °C, and immediately freeze dried. The resulting material was stirred with chloroform–methanol (2:1 v/v) (30–40 mg/ml) for 18 h at 4 °C. Defatted cells were removed by filtration and were dried in a vacuum desiccator. The filtrate was evaporated to dryness and stored at −20 °C.

Isolation of Lipoteichoic Acid. Defatted cells were extracted with 40% aqueous phenol (30 mg/ml) at 68 °C, using the procedure of Osborn (1966). The combined aqueous phase was stirred with an equal volume of chloroform for 1 h at room temperature. The organic phase was discarded and the remaining material was dialyzed against water. The nondiffusible material was freeze dried, and the resulting solid was taken up in a solution (40 ml) containing 0.01 M Tris-HCl, pH 7.5, 0.1 mM magnesium chloride, ribonuclease (0.05 mg/ml), and deoxyribonuclease (0.05 mg/ml) and was dialyzed against 0.01 M Tris-HCl, pH 7.5, containing 0.1 mM magnesium chloride (5 l.), at 35 °C for 2 hr. The nondiffusible material was dialyzed further, against distilled water (5 l.), and was freeze dried. Ammonium acetate (0.2 M) containing 0.02% sodium azide, pH 7 (10 ml), was added to the residue and the suspension was mixed by sonication. Insoluble material was removed by centrifugation (12 000g for 5 min), and a sample of the supernatant (3 ml) was applied to a column (140 cm × 8 cm^2) of Sepharose 6B, that had been equilibrated with 0.2 M ammonium acetate, pH 7, containing 0.02% sodium azide. The column was eluted with the same buffer, by upward flow at a rate of 16.6 ml/h. Fractions (5.5 ml) were collected and assayed for phosphorus and their optical density at 260 nm was measured. Peak fractions were pooled, dialyzed against water (5 l.), and freeze dried.

Deacylation of Lipoteichoic Acid. Lipoteichoic acid (30 mg) was treated with 0.5 M hydroxylamine, pH 7, at 45 °C for 16 h. The solution was dialyzed against water, and the nondiffusible material was freeze dried. The residue (27 mg) was dissolved in 0.2 M ammonium acetate containing 0.02% sodium azide, pH 7 (2 ml), and was chromatographed on Sepharose 6B as described above (Figure 1a).

A sample of lipoteichoic acid (107 mg) was stirred with 0.2 M sodium methoxide in methanol (5 ml) for 40 min, at 25 °C. Water (5 ml) was added, the solution was passed over a column (10 cm × 1 cm^2) of Amberlite IR 120 (H^+ form) resin, and methanol was removed from the eluate in vacuo. The resulting solution was extracted with chloroform and the aqueous phase was freeze dried. The residue was dissolved in 0.2 M ammonium acetate containing 0.02% sodium azide (2 ml) and was chromatographed on Sepharose, as described above (Figure 1b). Peak fractions were pooled, freeze dried, dissolved in water, and applied to a column (90 cm × 2 cm^2) of Sephadex G-25. The column was eluted with an upward flow of water, at a rate of 19.4 ml/h and fractions (8 ml) were collected. A sample (100 μmol of P) of deacylated lipoteichoic acid, from the Sephadex G-25 column, was applied to a column (28 cm × 4 cm^2) of Dowex 1 × 8 (OH^- form) resin. The column was washed with water (200 ml) and was eluted with a linear gradient of sodium chloride at a rate of 40 ml/h. The mixing vessel contained water (300 ml) and the reservoir contained 2M sodium chloride (300 ml). Fractions (5 ml) were collected and assayed for phosphorus (Figure 2).

Deacylation of Glycolipids. Samples of glycolipids (1–5 μmol) were dissolved in chloroform–methanol (1:1 v/v) (1 ml) and an equal volume of 0.2 M sodium methoxide in methanol was added. After 30 min at room temperature, water (5 ml) was added and the mixture was passed through a small column of Amberlite IR 120 (H^+ form) resin. Glycosides were eluted with water and were freeze dried.

Treatment of Glycosides with Glucosidases. Samples of glycosides in water (0.5 μmol/ml) were mixed separately with equal volumes of α-glucosidase in 0.1 M Tris-HCl, pH 6.8, (0.2 mg/ml), and β-glucosidase in 0.02 M sodium acetate, pH 5.2 (0.2 mg/ml). The solutions were kept at 35 °C and aliquots were withdrawn, at intervals, and assayed for glucose and glycerol.

Analytical Methods. Unless otherwise stated, acid hydrolysis was carried out using 3 M HCl at 100 °C for 3 h. The following analytical procedures were employed: D-glu-

cose using D-glucose-6-phosphate dehydrogenase (Wieland, 1963a); glycerol, using glycerol phosphate dehydrogenase (Wieland, 1963b)—when necessary, samples were dephosphorylated as described by Forsberg et al. (1973); phosphorus was determined by the method of Ames (1966); formaldehyde was determined using chromotropic acid (Hanahan and Olley, 1958); formic acid was measured by the method of Barker and Somers (1966); esters were determined by the method of Snyder and Stephens (1959); amino acids and amino sugars were estimated using a Beckman-Spinco automatic amino acid analyzer.

Chromatographic Procedures. Whatman No. 1 paper was used for chromatography and was eluted with butan-1-ol–pyridine–water (6:4:3 by volume; solvent A). Glycosides were detected with alkaline silver nitrate using the procedure described by Brundish et al. (1965). Periodate–Schiff reagent was used to detect α-glycol groupings (Baddiley et al., 1956).

Glass plates coated with Kieselgel H were used for TLC and were developed in the following solvents: B, chloroform–methanol (3:1 v/v); C, chloroform–methanol–water (65:25:4 by volume); and D, chloroform–methanol–acetic acid–water (80:12:18:5 by volume). Compounds were detected with the following reagents: 18 M sulfuric acid; α-naphthol (Siakotos and Rouser, 1965); ninhydrin (Marinetti, 1964).

Gas–liquid chromatography was carried out using a Perkin-Elmer F-11 gas chromatograph, fitted with a flame ionization detector. Nitrogen was used as carrier gas. A flow rate of 35 ml/min was maintained in packed columns and an operating pressure of 2.7 kg/cm^2 was used with the wall-coated column. Stainless steel columns were used, and acid washed, silanized, Chromosorb W (100–120 mesh) was employed as support material. In all analyses the injection port was kept at a temperature 50 °C above that of the column.

Preparation of Derivatives for GLC and Analysis Conditions. Monosaccharides. Sugars were converted to the corresponding alditol acetates, as described by Albersheim et al. (1967), and these were chromatographed on a column (1.8 m × 2 mm) of 3% ECNSS-M at 175 °C.

Glycosides. Trimethylsilyl ethers of glycosides were prepared using a mixture of pyridine–hexamethyldisilazane–trimethylchlorosilane (10:2:1 by volume; Sweeley et al., 1963) and were chromatographed at 210 °C using an open tubular column (25 m × 0.25 mm), the wall of which was coated with OV-101.

Fatty Acids. Methyl esters of fatty acids were prepared by heating, in sealed tubes, samples, containing either acids or esters, with 2 M HCl in methanol, at 100 °C for 3 h and were chromatographed on 15% poly(ethylene glycol succinate) (1.8 m × 2 mm) at 120 °C. For quantitative determinations, methyl heptadecanoate (10 μg/μmol of P) was added to samples, prior to methanolysis.

Methylation Analysis. Glycosides were methylated as described by Archibald and Coapes (1971). The products were treated with 2 M HCl in anhydrous methanol at 60 °C and were examined by GLC on 15% ethylene glycol succinate (1.8 m × 2 mm), at 160 °C. As standards, methyl glycosides of 2,3,4-tri-*O*-methylglucose, 2,3,6-tri-*O*-methylglucose, 2,4,6-tri-*O*-methylglucose, and 3,4,6-tri-*O*-methylglucose were prepared from gentiobiose, maltose, nigerose, and sophorose, respectively.

Results

The chloroform–methanol extract of whole cells was examined by two-dimensional TLC in solvents C and D. One

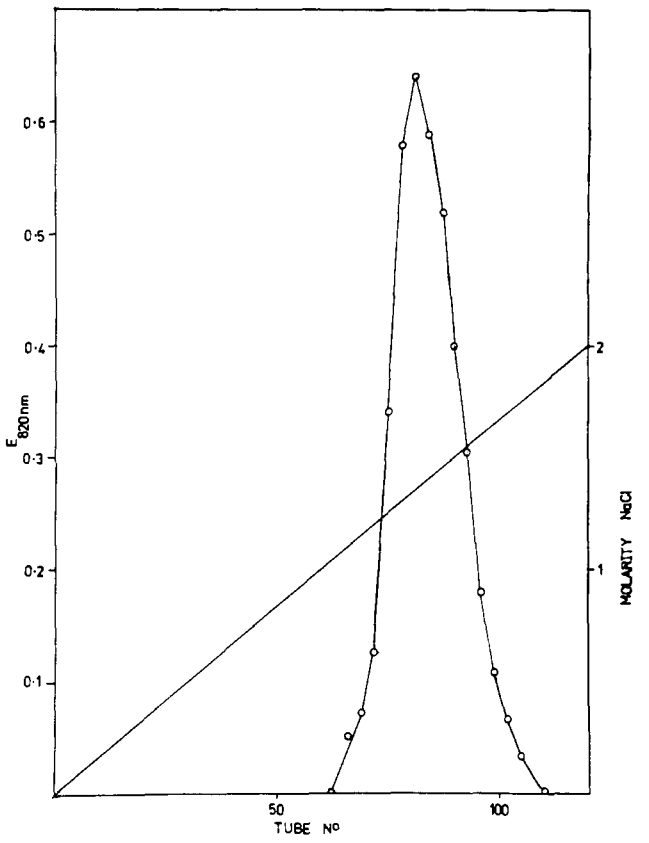

FIGURE 2: Chromatography of deacylated lipoteichoic acid on Dowex 1 (OH^-). The product of the reaction between lipoteichoic acid and sodium methoxide was desalted on Sephadex G-25 and chromatographed on Dowex 1 × 8 (OH^- form) (25 cm × 4 cm^2). The column was washed with water (200 ml) and was eluted with a linear gradient of sodium chloride at a rate of 40 ml/h. Fractions (5 ml) were collected and assayed for phosphorus (—o—).

lipid reacted with α-naphthol and had a mobility corresponding to a diglycosyl diglyceride. The following phospholipids were detected: phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin. A portion of the chloroform–methanol extract was evaporated to dryness, and the residue (200 mg) was stirred with silicAR CC-7 (2 gm) in chloroform–methanol (2:1 v/v; 100 ml). Solvent was removed in vacuo and the impregnated silica was suspended in chloroform and added to a column of silicAR CC-7 (25 × 2.2 cm) that had been prepared in chloroform. The column was eluted with chloroform (500 ml), acetone (500 ml), and chloroform–methanol (1:1 v/v; 1 l.). The acetone eluate contained a single component that accounted for over 95% of the applied glucose. The glycolipid (GL1) was subjected to acid hydrolysis and methanolysis, and the products were examined by GLC. Glucose was the only sugar detected; glycerol (ratio to Glc 0.5) and fatty acids were also present (Tables I and III).

A glycoside (G1) was formed from GL1 by deacylation. G1 (R_{Glc} 0.48 in solvent A) responded quickly to periodate–Schiff reagent and gave a positive reaction with alkaline silver nitrate, after steaming. The trimethylsilyl ether of G1 was chromatographed on OV-101 with a retention time of 6.2 min. Glucose and glycerol (ratio 2:1) were released, on acid hydrolysis of G1. α-Glucosidase did not hydrolyze the glycoside, but treatment with β-glucosidase for 1.5 h resulted in complete degradation of G1 to glucose and glycerol (ratio 2:1) Oxidation of G1 (0.92 μmol of glycerol) with 0.002 M sodium periodate (4 ml) was followed spectrophotometrically. Samples (50 μl) of the reaction mixture were

Table I: Properties of Glycolipids GL1, GL2, GL3, and GL3b.

Glycolipid	GL1	GL2	GL3a	GL3b
R_f in solvent B	0.73	0.73	0.73	0.34
Ratio, glucose–glycerol	2.2:1	ND[a]	1.8:1	2.0:1
Ratio, ester–glycerol	1.9:1	1.9:1	2.1:1	1.4:1

[a] Not determined.

Table II: Properties of Glycosides G1, G2, G3a, and G3b.

Glycoside	G1	G2	G3a	G3b
R_{Glc} in solvent A	0.48	0.48	0.48	0.48
Retention time (min) of Me_3Si–glycoside on OV-101 wall coated column (25 m × 0.25 mm) at 210 °C	6.2	6.2	ND[a]	6.2
Ratio, glucose–glycerol	1.95:1	1.9:1	1.7:1	ND[a]
Action of β-glucosidase	Hydrolyzed to glucose and glycerol			
Action of α-glucosidase	Remained unchanged			

[a] Not determined.

withdrawn and diluted with water (0.95 ml), and the cal density of the solution at 223 nm was measured. Allowance was made for the contribution to absorbance due to iodate (Aspinall and Ferrier, 1957). The reaction reached completion after 90 h. Periodate (5 molecular proportions) were reduced and formaldehyde (1.1 molecular proportions) were released. In a separate experiment, a sample of G1 (4.1 μmol of glycerol) was oxidized with 0.03 M sodium periodate (1 ml) for 48 h; 2.1 M proportions of formic acid were released. The methanolysis products of fully methylated G1 were examined by GLC. Methyl glycosides of 2,3,4-tri-*O*-methylglucose and 2,3,4,6-tetra-*O*-methylglucose were detected (Table IV).

Chromatography of the phenol extract of defatted cells, on Sepharose 6B, is shown in Figure 1a. Fraction C contained material of low molecular weight, which showed absorbance at 260 nm and was probably degradation products of nucleic acids. Material corresponding to fraction B was absent from some extracts. When present, fraction B was small and, in terms of total phosphorus, accounted for no more than 4% of fraction A. Phosphorus, glycerol, glucose, glucosamine, and fatty acids were detected in fraction B. Fraction A contained lipoteichoic acid and its composition is given in Table V. Amino acids were absent from fraction A but trace amounts of galactose and galactosamine were detected. Fatty acids, which were identified by GLC (Table III), accounted for 10% of the dry weight. The differences in detector response to the individual acids were neglected in the estimation of total fatty acid.

Chloroform–methanol (2:1 v/v) (50 ml) was added to a sample of lipoteichoic acid (20 mg), and the suspension was stirred at 0 °C for 6 h and centrifuged (12 000*g* for 20 min). The supernatant was examined by TLC in solvent B and was found to be devoid of components that react with either 18 M sulfuric acid or α-naphthol reagent. Hydrolysates (10% trifluoroacetic acid at 100 °C for 3 h) of dried supernatant and pellet were assayed. Neither phosphorus nor glucose was present in the supernatant; the pellet contained both phosphorus and glucose in the ratio 1:0.1.

The elution profile of hydroxylamine treated lipoteichoic acid is shown in Figure 1b. The peak, at the void volume of

Table III: Fatty Acid Compositions of Lipoteichoic Acid and Glycolipids.[a]

Fatty Acid	Relative Retention Time	Area under Peak[d] Lipoteichoic Acid	GL1	GL2	GL3a	GL3b
iso-C_{14}	0.36	6	2	8	5	TR
C_{14}	0.44	TR[b]	TR	TR	TR	TR
iso-C_{15}	0.55	45[c]	41	33	31	22
anteiso-C_{15}	0.58					
C_{15}	0.66	TR	TR	TR	TR	TR
iso-C_{16}	0.83	28	31	31	32	39
C_{16}	1.0	6	12	13	18	20
iso-C_{17}	1.26	13[c]	14	13	13	18
anteiso-C_{17}	1.33					

[a] Fatty acids were converted to their methyl esters and were chromatographed on 15% polyethylene glycol succinate (1.8 m × 2 mm) at 120 °C. Methyl hexadeconoate had a retention time of 27.1 min. [b] TR signifies less than 1% of total area. [c] Separation of peaks was incomplete. Area under combined peak was measured. [d] As percentage of total area.

the column, was identical, in composition, with the starting material. Peak D differed from the starting material in that it contained a lower level of fatty acids (Table V).

The products of the reaction between sodium methoxide and lipoteichoic acid were chromatographed on Sepharose 6B, as shown in Figure 1c. Some unchanged lipoteichoic acid was present together with peak E, which accounted for over 90% of the applied material. Fractions corresponding to peak E were dried and chromatographed on Sephadex G-25. The applied phosphorus was eluted in the void volume of the column. The resulting material was chromatographed on Dowex 1 as shown in Figure 2. Peak fractions were pooled, dried, and rechromatographed on Sephadex G-25 to remove salt. Deacylated lipoteichoic acid, with the composition shown in Table V, was eluted in the void region of the column. HF (60%, w/v; 1 ml) was added to a sample (60 μmol of P) of deacylated lipoteichoic acid, and the mixture was kept at −2 °C in a polyethylene tube. After 18 h, the pH was adjusted to 7 by addition of lithium hydroxide, and the precipitate which formed was removed by centrifugation (12 000*g* for 10 min). The supernatant was applied to Whatman 3mm paper and chromatographed in solvent A. The glycoside, G4 (R_{Glc} 0.47), was detected with silver nitrate reagent, after exposure to steam. G4 was eluted and on acid hydrolysis was converted to glycerol and glucose. Methanolysis of fully methylated G4 gave methyl glycosides of 2,3,4-tri-*O*-methylglucose and 2,3,4,6-tetra-*O*-methylglucose (Table IV).

A sample of lipoteichoic acid (109 mg) was treated with 60% (w/v) HF (2 ml) as described above. After neutralization, the precipitate was removed and the supernatant was extracted with an equal volume of chloroform. The aqueous portion was freeze dried, and the residue and the original precipitate were extracted with chloroform–methanol (1:1 v/v) (20 ml). Of the three extracts, only that of the original precipitate contained esters (18.6 μequiv). When examined by TLC, in solvent C, this fraction contained one component (GL2) that had a mobility identical with that of GL1. Glycerol and glucose were identified in acid hydrolysates of GL2 by paper chromatography and GLC. The ratio of glycerol to ester in GL2 was found to be 1:1.9. On deacylation, GL2 gave a glycoside (G2) which had chromatographic properties similar to those of G1 (Table II) and contained

Table IV: Products of Methanolysis of Methylated Glycosides.[a]

Methyl Ethers	Relative Retention Time of Derivative of				
	Standard	G1	G2	G3b	G4
Methyl 2,3,4,6-tetra-*O*-methyl-D-glucoside	1, 1.57	1, 1.57	1, 1.57	1, 1.57	1, 1.59
Methyl 2,4,6-tri-*O*-methyl-D-glucoside	4.07, 6.33				
Methyl 2,3,4-tri-*O*-methyl-D-glucoside	3.08, 4.7	3.14, 4.8	3.15, 4.7	3.15, 4.8	3.2, 4.9
Methyl 2,3,6-tri-*O*-methyl-D-glucoside	4.2, 6.1				
Methyl 3,4,6-tri-*O*-methyl-D-glucoside	3.8, 4.7				

[a] Products chromatographed on 15% ethylene glycol succinate (1.8 m × 2 mm) at 160 °C. Methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucoside had a retention time of 5.8 min.

Table V: Composition of Lipoteichoic Acid and Deacylated Lipoteichoic Acid Preparations

Preparation	Ratio to Phosphorus			
	Glycerol	Glucose	Glucosamine	Fatty Acid (as C_{17})
Lipoteichoic acid (peak A)	0.91	0.11	0.1	0.12
Partially deacylated lipoteichoic acid (peak D)	1.0	0.11	0.11	0.08
Deacylated lipoteichoic acid eluted from Sephadex G-25	n.d.	0.1	n.d.	0
Deacylated lipoteichoic acid eluted from Dowex 1 (OH^-)	0.9	0.12	0.09	0

glucose and glycerol (ratio 1.95:1). The glycoside was not a substrate for α-glucosidase but was converted to glucose and glycerol when incubated with β-glucosidase. Methyl glycosides of 2,3,4-tri-*O*-methylglucose and 2,3,4,6-tetra-*O*-methylglucose were products of methanolysis of fully methylated G2 (Table IV).

A sample of lipoteichoic acid (17.6 mg) was treated with 0.1 M HCl (10 mg/ml) at 100 °C and the optical density of the mixture, at 550 nm, was measured at intervals (Figure 3). The turbidity reached a maximum after 50 min, and this reaction time was chosen for a large-scale hydrolysis (478 mg of lipoteichoic acid in 0.1 M HCl (47.8 ml)). Less than 1% of the total phosphorus was converted to inorganic phosphate during the reaction. Insoluble material was removed by centrifugation (12 000*g* for 10 min), washed with water, and dried in vacuo, and the residue was dissolved in chloroform–methanol (1:1 v/v) and examined by TLC in solvent C. Two components GL3a (R_f 0.73) and GL3b (R_f 0.34) were detected with α-naphthol reagent. The extract was absorbed to silicAR CC-7, as previously described, and was added to a column (27 × 2.2 cm) of silicAR CC-7 in chloroform. The column was eluted with chloroform (350 ml), acetone (440 ml) and the following mixtures of chloroform and methanol: (98:2 v/v, 300 ml); (10:1 v/v, 330 ml); and (10:2 v/v, 400 ml). GL3a (6.5 μmol of Glc) and GL3b (7.5 μmol of Glc) were eluted with acetone and chloroform–methanol (10:2 v/v), respectively. Analytical data for GL3a and GL3b are given in Table I, and the fatty acid compositions are listed in Table III.

The presence of esters was indicated by an absorption band at 1730 cm^{-1} in the infrared spectra of both lipids but, whereas the ratio of ester to glycerol in GL3a was about 2:1, in GL3b it was closer to unity (Table I). On deacylation, GL3a and GL3b were converted into glycosides G3a and G3b, respectively. These were indistinguishable from one another and from G1 and G2 by paper chromatography; both reacted with silver nitrate, after steaming, and gave a fast, pink coloration with periodate–Schiff reagent. Neither G3a nor G3b were hydrolyzed by α-glucosidase but, on treatment with β-glucosidase, both were hydrolyzed to glucose and glycerol. Methanolysis of fully methylated G3b gave methyl glycosides of 2,3,4-tri-*O*-methylglucose and 2,3,4,6-tetra-*O*-methylglucose (Table IV).

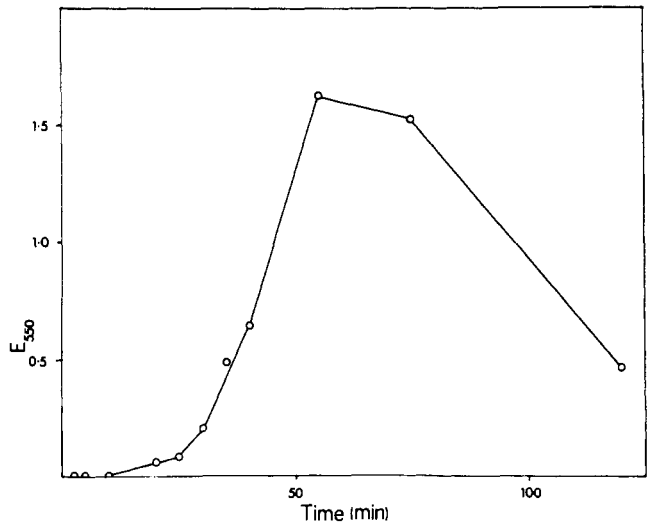

FIGURE 3: Release of lipid from lipoteichoic acid on hydrolysis in 0.1 M HCl. Lipoteichoic acid was treated with 0.1 M HCl (10 mg/ml) at 100 °C. (—o—) Optical density at 550 nm of the suspension was measured at times shown.

A sample (5 μmol of Glc) of glycolipid GL1 was hydrolyzed in 0.1 M HCl (1 ml) at 100 °C for 50 min. The hydrolysate was centrifuged (12 000*g* for 10 min) and the pellet was washed with water, dissolved in chloroform–methanol (1:1 v/v), and examined by TLC, in solvents C and B.

Two components with mobilities identical with those of GL3a and GL3b were detected.

Discussion

These studies show that a portion of the lipoteichoic acid of *B. licheniformis* 6346 MH-1 has a structure similar to that of the glycolipid, which is present in the membrane of this organism. Membrane glycolipid, GL1, was extracted from freeze dried cells with chloroform–methanol and glycolipids, GL2 and GL3a, were released from lipoteichoic acid by treatment with HF and hydrolysis in dilute acid, respectively. The lipids have identical mobilities on TLC and their deacylation products, glycosides G1, G2, and G3a, have identical mobilities on paper chromatography. Trimethylsilyl ethers of G1 and G2 have similar retention times on GLC. Analytical data for the glycosides and glycolipids indicate that GL1, GL2, and GL3a are diglucosyl diglycerides. A second lipid, GL3b, was isolated from the dilute acid hydrolysate of the lipoteichoic acid. GL3b has a lower ester content and a lower mobility on TLC than GL3a. However, the corresponding glycoside, G3b, is identical with G3a. It seems probable that GL3b is a monoglyceride and is formed from GL3a during hydrolysis. This conclusion is supported by the observation that GL1, when treated with 0.1 M HCl at 100 °C for 50 min, is converted into a lipid with chromatographic properties similar to those of GL3b. Further proof of identity of the glycosides was obtained by methylation analysis. α- and β-methyl 2,3,4,6-tetra-*O*-methylglucosides and α- and β-methyl 2,3,4-tri-*O*-methylglucosides were formed from G1, G2, and G3b. The tetramethyl esters are indicative of a nonreducing terminal glucopyranosyl residue and the trimethyl ethers show the presence of a glucopyranosyl residue that is substituted at the primary hydroxyl group. The β configuration is assigned to all linkages as G1, G2, and G3b are hydrolyzed to glucose and glycerol by β-glucosidase, whereas α-glucosidase is without effect. The response of the glycosides to periodate–Schiff reagent indicates that formaldehyde is produced (Roberts et al., 1963) and, thus, the secondary hydroxyl group of glycerol is unsubstituted. Confirmation of the structure of the membrane glycolipid was obtained from studies of the oxidation of G1 with sodium periodate; 5 mol of periodate was reduced per mole of G1, with the concomitant release of 1.1 mol of formaldehyde and 2.1 mol of formic acid. The only structures that are consistent with the above data are the two isomeric forms of *O*-β-D-glucopyranosyl-(1→6)-*O*-β-D-glucopyranosyl-(1→3)-glycerol. Thus, a common diglucosyl glycerol residue is present in the glycolipids of *B. licheniformis, B. subtilis* (Brundish et al., 1966), and *Bacillus cereus* (Lang and Lundgren, 1970).

The intact lipoteichoic acid and the glycolipid fractions have similar fatty acid compositions. 12-Methyltetradecanoic acid, 13-methyltetradecanoic acid, and 14-methylpentadecanoic acid are the major fatty acids present (Table III). A preponderance of fatty acids, of the branched chain type, is a common feature of bacilli (Kaneda, 1967). The location of the acids, on the glycolipids, has not yet been established but comparison with other glycolipids, from gram-positive bacteria, suggests that they are esterified to a glycerol residue.

Lipoteichoic acid eluted from Sepharose 6B in the region of the void volume. The observations that phosphorus and glycerol are the principal components of the molecule and that they are present in similar amounts suggests that the lipoteichoic acid of *B. licheniformis* MH-1 contains 1,3-polyglycerol phosphate. In this respect, it is similar to all other lipoteichoic acids that have, thus far, been examined. Diglycerol triphosphate has been identified among the products of alkaline hydrolysis of the lipoteichoic acid (D. Button, unpublished). Its formation is diagnostic for 1,3-polyglycerol phosphate (Kelemen and Baddiley, 1961). The purified material did not contain amino acids. The location of glucosamine, galactosamine, and galactose within the polymer has not been determined. However, the latter two sugars are found in trace amounts and may be impurities. The observation that lipoteichoic acid is eluted from Sepharose 6B near the exclusion volume of the column indicated a high degree of aggregation. Thus, it is possible that the lipoteichoic acid preparation contains a number of molecular species, which, in solution, form a mixed micelle. However, free gentiobiosyl diglyceride was not present in the lipoteichoic acid preparation, as glucose was not removed by treatment with chloroform–methanol.

It appeared that ion exchange chromatography would establish whether or not glycolipid and polyglycerol phosphate are contained in the same molecule. Lipoteichoic acid has been shown to bind irreversibly to ion exchange resins (Wicken et al., 1973). Thus, it was decided to examine deacylated material and to relate its glucose and phosphorus content to glycolipid and polyglycerol phosphate of the lipoteichoic acid. Treatment of lipoteichoic acid with neutral hydroxylamine resulted in the formation of a partially deacylated fraction (peak D) that was included in Sepharose 6B and was probably aggregated but to a lesser extent than the starting material. It is of interest that a small portion of the phenol extract of *B licheniformis* (peak B) had a mobility, on Sepharose 6B, similar to that of partially deacylated lipoteichoic acid and that corresponding extracts from other organisms contained material that chromatographed in this region of the column (Coley et al., 1972). It is possible that, in the course of isolation, some deacylation of lipoteichoic acid occurs, as a result of either the action of esterases or hydrolysis during the phenol treatment (Tsang et al., 1974).

Complete deacylation of lipoteichoic acid was achieved by treatment with sodium methoxide. The resulting product was of relatively low molecular weight, as judged by its chromatographic behavior on Sepharose 6B, but was excluded from Sephadex G-25. Deacylated lipoteichoic acid was eluted from Dowex 1 (OH^- form) resin, as one peak, with sodium chloride and the resulting material contained phosphorus, glycerol, glucose, and glucosamine in ratios similar to those found in the initial lipoteichoic acid preparation. The glycoside, G4, was liberated from the deacylated lipoteichoic acid by treatment with HF and was shown to contain a gentiobiosyl residue by methylation analysis. These findings confirm that gentiobiosyl diglyceride is linked to polyglycerol phosphate and indicate that the lipoteichoic acid preparation is essentially free of other polymers.

About 60% of the glucose, of the lipoteichoic acid, was recovered as diglucosyl diglyceride, after the polymer was treated with HF. A minimum value of 18 can be calculated for the degree of polymerization of the polyglycerol phosphate if it is assumed that all of the glucose is present as diglucosyl diglyceride and that each chain of polyglycerol phosphate is coupled to one lipid residue.

Fractions, designated as lipoteichoic acid carriers, that serve as acceptors in the synthesis of polyribitol phosphate, have been isolated from membranes of a variety of organisms, by extraction with Triton X-100 (Fiedler and Glaser, 1974a). In composition, the lipoteichoic acid carriers from

Staphylococcus aureus H and *B. subtilis* are similar to one another and to the lipoteichoic acid of *B. licheniformis* (Mauck and Glaser, 1974; Fiedler and Glaser, 1974b). It remains to be shown that lipoteichoic acid and lipoteichoic acid carriers are different structural entities.

While these studies were in progress, it was reported that polar lipids were released from the lipoteichoic acid of *S. faecalis* ATCC 9790, by treatment with dilute acid (Pieringer and Ganfield, 1974). The hydrolysis products were found to include a lipid that consisted of phosphatidylkojiobiosyl diglyceride covalently linked to small segments of polyglycerol phosphate, together with phosphatidylkojiobiosyl diglyceride and degradation products of the latter lipid (Ganfield and Pieringer, 1975). On the basis of these findings, it was concluded that the only lipid moiety present in the lipoteichoic acid of *S. faecalis* was phosphatidylkojiobiosyl diglyceride. This lipid occurs, in a free form, in the membrane of this organism (Fischer et al., 1973; Toon et al., 1972).

Phosphatidyldiglucosyl diglyceride does not occur in *B. licheniformis*. It was not detected in a chromatographic examination of the chloroform–methanol extract of whole cells, and studies of deacylated lipids have shown that none of the phospholipids contain glucose (D. Button, unpublished). Furthermore, gentiobiosyl diglyceride and gentiobiosyl monoglyceride were the only lipids present in mild acid hydrolysates of the lipoteichoic acid. The specific reactions that occur during the hydrolysis remain to be determined. Turbidity measurements indicate that a lag occurs prior to the liberation of lipid, which is subsequently degraded, probably by cleavage of ester bonds. Neither inorganic phosphate nor a phosphomonoester of gentiobiosyl diglyceride are released during the reaction and, thus, it seems unlikely that the hydrophobic and hydrophilic portions of the lipoteichoic acid are joined through a pyrophosphate bond. It has been suggested that, in the lipoteichoic acids of *Strep. faecalis* and *Staph. aureus* H. glycolipid and polyglycerol phosphate are linked by a phosphodiester bond (Toon et al., 1972; Ganfield and Pieringer, 1975: Duckworth et al., 1975). The finding that gentiobiosyl diglyceride is cleaved from the lipoteichoic acid of *B. licheniformis* MH-1, by treatment with HF, is consistent with the existence of a similar type of linkage in this organism.

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