Acyldiglucosyldiacylglycerol-Containing Lipoteichoic Acid with a Poly(3-*O*-galabiosyl-2-*O*-galactosyl-*sn*-glycero-1-phosphate) Chain from *Streptococcus lactis* Kiel 42172†

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ABSTRACT: The lipoteichoic acid of *Streptococcus lactis* Kiel 42172 was isolated. The lipid portions were released by HF and were established to be 3-*O*-[*O*-α-D-galactopyranosyl-(1 → 6)-α-D-galactopyranosyl]-2-*O*-α-D-galactopyranosyl-*sn*-glycero-1-phosphate; they are joined by phosphodiester bonds nosyl)]glycerol. The repeating units of the hydrophilic chain were established to be 3-*O*-[*O*-α-D-galactopyranosyl-(1 → 6)-α-D-galactopyranosyl]-2-*O*-α-D-galactopyranosyl-*sn*-glycero-1-phosphate; they are joined by phosphodiester bonds at carbon atom 6 of the galabiosyl residues. The innermost unit is linked to the glycolipid by a phosphodiester presumably at C-6 of the outer glucosyl moiety. The hydrophilic chain is 7.4–11.8 units in length, measuring 12–19 nm in extended conformation. The content of 2.7–2.96 acyl groups per 2 glucosyl residues indicates that 70–96% of the glycolipid consists of acyldiglucosyldiacylglycerol. The novel poly(glycosylglycerophosphate) structure provided for the first time the opportunity to prove chemically that the glycerophosphates of lipoteichoic acids are the *sn*-1 isomer which has previously been suggested from biosynthetic studies (Glaser, L., & Lindsay, B. (1974) *Biochem. Biophys. Res. Commun. 59*, 1131–1136).

Group N *Streptococci* can be divided into two subgroups on the basis of their differing glycerophosphoglycolipid pattern (Fischer et al., 1978a) which attracted our attention since glycerophosphoglycolipids are related to lipoteichoic acids, being their enzymic breakdown products or biosynthetic intermediates (Fischer, 1976; Fischer et al., 1978a,b). The major polar glycerophosphoglycolipid that is characteristic of one subgroup was isolated from *S. lactis* NCDO 712 and proved to be *sn*-glycero-1-phospho-3-*sn*-glycero-1-phospho → 6Glcα1 → 2Glc(6 ← acyl)-α1 → 3-acyl$_2$Gro (Laine & Fischer, 1978). Its structure reflects, therefore, the elongation principle of the poly(glycerophosphate) chain of lipoteichoic acids (Kelemen & Baddiley, 1961; Toon et al., 1972; Button & Hemmings, 1976). The major compound typical for the second subgroup has meanwhile been isolated from *S. lactis* Kiel 42172 and was established to be Galα1 → 6Galα1 → 3-*sn*-glycero(2 ← 1αGal)-1-phospho → 6Glcα1 → 2Glc(6 ← acyl)-α1 → 3-acyl$_2$Gro (Fischer, W., et al., unpublished data). If this compound were related to the lipoteichoic acid of this organism, its structure would not be compatible with a poly(glycerophosphate) chain which has so far been considered as characteristic of lipoteichoic acids (Knox & Wicken, 1973; Lambert et al., 1977). In the work described here, studies on the lipoteichoic acid of *S. lactis* 42172 revealed that it contains indeed galabiosyl residues as integral parts of the hydrophilic chain and is, therefore, not of the classical poly(glycerophosphate) type.

Materials and Methods

Materials. Enzymes and cosubstrates were from Boehringer Mannheim GmbH. Reference glycolipids were from *S. lactis* Kiel 42172 (Fischer et al., 1978c). Dipolyol phosphates (Fischer & Landgraf, 1975) and partially methylated alditol acetates (Nakano & Fischer, 1977) were prepared as in the references given. Galα1 → 2Gro was released from the lipoteichoic acid of *S. lactis* NCDO 712 by hydrolysis with HF. GlcNAcα1 → 3Gro was a deacylated glycolipid from *Streptococcus hemolyticus* (Ishizuka & Yamakawa, 1969), Galβ1 → 3Gro and Galα1 → 6Galβ1 → 3Gro are deacylated plant lipids, and Galα1 → 6Galα1 → 6Galβ1 → 3Gro is the deacylation product of a synthetic glycolipid (Gent & Gigg, 1975).

Growth of Bacteria. S. lactis Kiel 42172 was grown and harvested as described previously (Fischer et al., 1978c). Three batches (30 L each) were harvested at the late growth phase (7–9 h) when the pH of the medium had dropped from 7 to 4.8 ± 0.1 and the absorbance (578 nm) of the culture was 2.4 ± 0.1.

Extraction and Purification of Lipoteichoic Acid. The lipoteichoic acid of one batch (preparation 1) was extracted according to the method of Coley et al. (1975), that of the other two batches (preparations 2 and 3) by a recently described procedure (Nakano & Fischer, 1978). After phenol extraction and digestion with nucleases, which were performed as previously described (Nakano & Fischer, 1978), in preparation 1, 50%, and, in preparations 2 and 3, 10% of the total phosphorus consisted of nondialyzable nucleic acid material. Final purification of lipoteichoic acid by column chromatography on Sepharose 6B is shown in Figure 1. Rechromatography in 25% aqueous methanol containing 0.02 M ammonium acetate (Coley et al., 1975) was necessary to remove larger amounts of residual nucleic acid fragments from preparation 1.

Analytical Procedures. Unless otherwise specified, analytical methods were as described earlier (Nakano & Fischer, 1977, 1978). Glycolipids and glycosides were measured by an anthrone procedure (Ough, 1964). Glycerophosphate released on acid hydrolysis was calculated from the measured content of *sn*-glycero-3-phosphate (Fischer, 1977). Acyl groups of glycolipids were measured by a hydroxamate method (Snyder & Stephens, 1959), those of lipoteichoic acids as described

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under deacylation. Optical rotations were determined with a Präzisionspolarimeter Zeiss 0.005° using a cuvette of 10-cm optical path.

Thin-Layer Chromatography. The following solvents were used: (A) chloroform–acetone–methanol–acetic acid–water (80:20:10:10:4, v/v); (B) chloroform–methanol–water (65:35:4, v/v); (C) petroleum ether–diethyl ether–acetic acid (60:40:4, v/v); (D and E) propan-1-ol–pyridine–water (7:4:3 and 7:4:2, v/v, respectively); (F) propan-1-ol–ethyl acetate–water (7:2:2, v/v); (G) phenol–water–acetic acid–ethanol (75:33:4.5:4.5, w/v/v/v). Solvents A–F were used on precoated silica gel plates (Merck, Kieselgel 60); solvent G was used on precoated cellulose plates (Merck). The reagents used for the detection of lipids, glycolipids, glycosides, and phosphates were the same as described (Fischer et al., 1973). Preparative thin-layer chromatography and elution from silica gel of glycolipids were carried out as in previous work (Fischer, 1977). Glycosides were purified by chromatography on silica gel sheets (Merck); they were eluted by sonicating the silica gel in methanol–water (1:1, v/v) followed by centrifugation at 12 000*g* for 30 min. Residual silica gel was removed by passing the supernatant through small columns of charcoal–Celite (1:1, w/w) and eluting the glycosides with 50% ethanol.

Gas–Liquid Chromatography. Fatty acid methyl esters, partially methylated alditol acetates, and trimethylsilylated mono- and dipolyolphosphates were analyzed as in previous work (Nakano & Fischer, 1977; Fischer et al., 1978b). Glycosides were trifluoroacetylated (Shapira, 1969) or trimethylsilylated (Fischer, 1977) and analyzed on 2% SE 30 silicone on Chromosorb G, AW, DMCS (80–100 mesh) in a glass column (210 cm × 2 mm). Trimethylsilyl derivatives of monohexosylglycerols were separated at 210 °C, and isothermally, trifluoroacetates of mono-, di-, and trihexosylglycerols at a programmed temperature rise of 4 °C per min from 135 to 260 °C. For quantitating glycosides, the trifluoroacetyl derivatives were used.

Deacylation. Lipoteichoic acid (12 μmol of phosphorus) was dissolved in 0.2 M NaOH in 50% aqueous methanol (1 mL) and kept at 37 °C for 16 h. Cation exchange resin (Merck S 1080), H^+ form, was added and fatty acids were extracted with petroleum ether. The water–methanol layer was withdrawn, neutralized with ammonia, and concentrated by rotary evaporation. For quantitation of fatty acids, prior to deacylation, methyl heptadecanoate (100 nmol/μmol of phosphorus) was added as an internal standard.

Hydrolysis with HF. Lipoteichoic acid (5–15 μmol of phosphorus) was hydrolyzed in 48% (w/w) HF (1 mL) at 2 °C for the times given under Results. After freezing the samples and drying them at 0 °C over NaOH in vacuo overnight, lipid and water-soluble compounds were separated by Folch partition using 0.05 M NH_4OH as the water phase. The phosphorus was completely recovered in the aqueous layer in which the extent of hydrolysis was determined as the ratio of inorganic and total phosphorus. After incomplete hydrolysis, the aqueous layer was fractionated by chromatography on columns (25 × 1.5 cm) of Sephadex G-50 which were eluted with water. Native and poorly degraded material eluted near the void volume, glycosides and phosphorus at the inner volume of the column.

Smith Degradation. Deacylated lipoteichoic acid (5 μmol of phosphorus) was dissolved in 0.1 M $NaIO_4$ (2 mL) and kept at 37 °C in the dark for 48 h. Ethylene glycol (1.8 M, 0.15 mL) was added and after 1 h the oxidation product was reduced with $NaBH_4$ (40 mg) at room temperature overnight. Excess $NaBH_4$ was oxidized by addition of acetone (0.5 mL) in ice. Then the mixture was concentrated to a small volume in vacuo and applied for desalting to a column (25 × 1.5 cm) of Sephadex G-25. On elution with water the phosphorus emerged completely at the void volume. The pooled phosphorus-containing fractions were freeze-dried and studied further as described under Results.

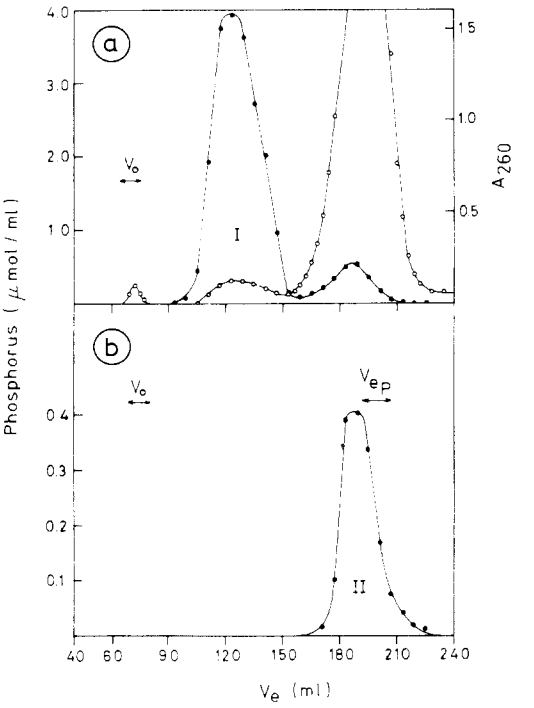

FIGURE 1: Chromatographic behavior of lipoteichoic acid (I) and its deacylation product (II). Column chromatography on Sepharose 6B was performed as described (Nakano & Fischer, 1978), but using water for elution. (a) Phenol extract; (b) lipoteichoic acid after mild alkaline treatment (see Materials and Methods); (●—●) phosphorus; (○—○) nucleic acid material (A_{260} of nondiluted samples). The exclusion (V_0) and total fluid volume (V_{ep}) of the column were determined in a separate run, using blue dextran and K_2HPO_4, respectively.

Enzymic Hydrolyses. Dephosphorylation with alkaline phosphomonoesterase (EC 3.1.3.1) and determination of the products were essentially as described (Nakano & Fischer, 1978). Glcα1 → 2Glcα1 → 3Gro was hydrolyzed with α-glucosidase (10 U/mL reaction mixture, EC 3.2.1.20) in 0.2 M sodium acetate buffer, pH 6, at room temperature for 12 h. Native or deacylated lipoteichoic acid (12–14 μmol of phosphorus) was dissolved in the same buffer (4 mL) containing 0.02% NaN_3 and was treated with α-galactosidase (0.8 U/mL, EC 3.2.1.22) at 37 °C. By following the release of galactose with galactose dehydrogenase (EC 1.1.1.48) the hydrolysis was found to be finished after 72 h. The enzyme was removed by heating the mixture (100 °C, 5 min) and subsequent centrifugation. The lipoteichoic acid derivatives were separated from galactose and buffer by column chromatography on Sephadex G-25. Trigalactosylglycerol was hydrolyzed with α-galactosidase (0.4 U/mL) within 48 h, under which conditions cleavage of Galβ1 → 3Gro was less than 5%.

Results and Interpretation

Properties and Composition of Lipoteichoic Acid. Lipoteichoic acid was isolated from three batches of late growth phase cells of *S. lactis* 42172 by two different procedures (see Materials and Methods). Chromatography of the phenol extract of defatted cells is shown in Figure 1a. The material corresponding to peak I had the composition of a carbohydrate-rich lipoteichoic acid (Table I). It contained negligible amounts of nucleic acid, less than 1% protein and was free of

TABLE I: Analyses of Lipoteichoic Acid.

prep no.	molar ratios to phosphorus: D-glucose[a]	D-galactose[b]	glycerophosphates[a]	labile glycerol[a]	total glycerol[c]	acyl groups	hexosamine	repeating units per chain[d]	acyl groups per molecule[d]
1	0.17	nd[e]	0.90	0.20	1.12	nd	0.02	11.8	*f*
2	0.23	2.82	0.94	0.10	0.95	0.34	0.08	8.7	2.96
3	0.27	2.93	0.91	0.26	1.17	0.36	nd	7.4	2.70

[a] Determined after short acid hydrolysis (2 M HCl, 100 °C, 2.5 h). [b] Determined after short acid hydrolysis and subsequent enzymic dephosphorylation. [c] Measured after drastic acid hydrolysis (2 M HCl, 125 °C, 48 h). [d] Calculated from the values of glucose, on the basis of glucose being restricted to the glycolipid portion in the form of acylated diglucosylglycerols (see text). [e] After hydrolysis with HF (see text), the main water-soluble product was Gal_3Gro. nd, not determined. [f] Content similar to that of preparation 3 as judged from Table III.

TABLE II: Fatty Acid Composition of Lipoteichoic Acid.[a]

prep no.	14:0	14:1	16:0	16:1	18:0	18:1	19cy
1	14.5	1.4	30.2	6.8	1.4	44.2	1.4
2	12.4	1.5	27.5	6.5	2.1	43.7	7.0
3	19.8	2.1	25.9	5.5	2.9	41.8	1.9

[a] Values are given as mol %; cy, cyclopropanecarboxylic acid.

low-molecular-weight lipids as was evidenced by extracting aqueous solutions with water-saturated butanol or chloroform–methanol (1:1, v/v). On mild alkaline treatment (see Materials and Methods) fatty acids were released which were converted to methyl esters (Fischer et al., 1973) and analyzed by gas–liquid chromatography. Their composition (Table II) was similar to that of glycolipids of *S. lactis* strains (Fischer et al., 1978c). The elution profile on Sepharose 6B of the deacylated product is shown in Figure 1b. As compared with lipoteichoic acid, the K_d value[1] was shifted from 0.43 to 0.98, which indicates a micellar solution of the native material.

The lipoteichoic acid preparations were similar in their ratios of glycerol and phosphorus (approximately 1.1:1) and the high proportion of D-galactose but differed in the content of D-glucose and acyl groups (Table I). No other constituents, besides some hexosamine, were observed. The optical rotation $[M]_D^{20}$ of preparation 3 was + 662° (*c* 1.03 mM H_2O).

On short acid hydrolysis, approximately 92% of the phosphorus was released as glyceromonophosphates (Table I). The rest was apparently liberated as galactose phosphate since after enzymic dephosphorylation an increase in galactose of approximately 5% was observed. These hydrolysis products contrasted with those of a poly(glycerophosphate)-containing lipoteichoic acid (Nakano & Fischer, 1978) among which glyceromonophosphates and glycerobisphosphate accounted for 60 and 40% of the phosphorus (not shown).

The polymer was largely resistant against alkaline treatment (0.2 M NaOH, 100 °C, 24 h) since only 9% of the phosphorus became susceptible to phosphomonoesterase. When the neutralized (Fischer & Landgraf, 1975) hydrolysate was chromatographed on a column of Sephadex G-50, 93% of the initial phosphorus eluted at the void volume, the rest at the inner volume of the column, whereas fragments of intermediate chain length were not detected.

HF Degradation and Basic Structure. On treatment with HF for 48 and 96 h, 56 and 91% of the lipoteichoic acid was hydrolyzed. It was therefore more resistant than poly(glycerophosphate) lipoteichoic acids, 97% of which was hydrolyzed within 36 h under the same conditions (Nakano & Fischer, 1978).

The lipid products released by HF and their proportions are listed in Table III. Besides lyso-glycolipids two major products were observed which chromatographed with Glcα1 → 2Glc(6 ← acyl)-α1 → 3-acyl$_2$Gro and Glcα1 → 2Glcα1 → 3-acyl$_2$-Gro. The complete absence of diacylglycerol (solvent C) rules out phosphatidyl glycolipids as lipid anchors (Toon et al., 1972; Ganfield & Pieringer, 1975). The two major glycolipids were isolated by preparative thin-layer chromatography in solvent A. They consisted of D-glucose, glycerol, and fatty acid ester in the molar ratio of 1.97:1.00:3.12 and 1.92:1.00:1.99, respectively. Deacylation of both lipids gave the same glycoside which chromatographed with authentic Glcα1 → 2Glcα1 → 3Gro (solvents D and F) and was completely hydrolyzed by α-glucosidase. The third fatty acid of acyldiglucosyldiacylglycerol was located by methylating the originally esterified and free hydroxyl groups with C^2H_3I and CH_3I, respectively (Nakano & Fischer, 1977; Fischer et al., 1978c). The partially methylated alditol acetates derived therefrom were identified by gas chromatography–mass spectral analysis (Table IV) as A, 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetylglucitol, and C, 3,4,6-tri-*O*-methyl-1,2,5-tri-*O*-acetylglucitol. This substitution confirms the (1–2) linkage in the disaccharide portion, whereas the restriction of trideuteriomethoxy groups to peak C (Table IV) with shifted signals at *m/e* 48, 104, 148, 164, and 208 and nonshifted signals at *m/e* 189 and 129 locate the third fatty acid exclusively at C-6 of the inner glycerol-linked glucosyl moiety (Nakano & Fischer, 1977).

Independent of the time of hydrolysis with HF one main water-soluble glycoside was released with the chromatographic properties given in Table V. After purification by preparative thin-layer chromatography (solvent E), it showed: on analysis carbohydrate (anthrone), galactose and glycerol in the molar ratio of 2.88:3.04:1.00; on periodate oxidation, no formaldehyde was released. Permethylation analysis (Hakomori, 1964; Sandford & Conrad, 1966) gave two partially methylated alditol acetates (Table IV) which were identified as B, 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetylgalactitol, and E, 2,3,4-tri-*O*-methyl-1,5,6-tri-*O*-acetylgalactitol. These substitutions and the ratio of the peak areas *B/E* of 2:1 indicate a galactopyranosyl and a galactopyranosyl(1–6)galactopyranosyl residue at the glycerol portion. The optical rotation $[M]_D^{20}$ of the native trigalactosylglycerol was +112° (*c* 1.77 mM, H_2O). After oxidation of the acetylated derivative with CrO_3 (Angyal & James, 1970; Laine & Renkonen, 1975), no loss of galactose was observed, which indicates in accord with the high dextrorotatory $[M]_D$ value that the three galactopyranosyl residues are in the α-anomeric form (Oshima & Ariga, 1976). Furthermore, trigalactosylglycerol was hydrolyzed by α-galactosidase yielding galactose and glycerol in the ratio 3:1. In order to locate the galactosyl and the galabiosyl residue, trigalactosylglycerol was subjected to partial

[1] $K_d = (V_e - V_0)/V_i$ where $V_i = V_{ep} - V_0$ (Figure 1).

TABLE III: Lipid Products of HF Hydrolysis of Lipoteichoic Acid.[a]

identification (chromatography with ref lipids)	chromatographic mobility[b] R_1	R_2	mol % in prep 1	2	3
Glcα1 → 2Glc(6 ← acyl)-α1 → 3-acyl$_2$Gro	2.81	1.45	56	85	53
Glcα1 → 2Glcα1 → 3-acyl$_2$Gro	1.00	1.00	30	15	38
lyso-glycolipids[c]	1.19	1.10	14	nd[d]	9
	0.33	0.52			

[a] Preparations 1 and 2 were hydrolyzed for 24 h; preparation 3 was hydrolyzed for 48 h. After longer hydrolysis, the lipid composition was the same but the proportion of the deacylation product increased. [b] R_1, R_2, chromatographic mobility relative to Glcα1 → 2Glcα1 → 3-acyl$_2$Gro in solvents A and B, respectively. [c] Glycolipids with R_1 values of 1.19, 1.00, and 0.33 were formed when *authentic* Glcα1 → 2Glc(6 ← acyl)-α1 → 3-acyl$_2$Gro was partially deacylated (8 mM NaOH in chloroform/methanol (1:1 v/v), 20 °C, 5 min) or treated with HF. [d] nd, not determined.

TABLE IV: Gas Chromatography–Mass Spectral Analysis of Partially Methylated Alditol Acetates Derived from Permethylated Acyldiglucosyldiacylglycerol and Trigalactosylglycerol.

parent compd	peak[a]	t_R[b]	peak area ratios	mass no. of primary and some secondary fragments[c] 45	101	117	129	145	161	173	189	205	233
acyldiglucosyldiacylglycerol	A	1.01	1.00	x	x	x	x	x	x			x	
	C	1.92	0.97	↑	↑		x	▲	▲		x	▲	
trigalactosylglycerol	B	1.22	2.05	x	x	x	x	x	x			x	
	E	3.15	1.00		x	x	x		x	x	x		x

[a] For designation and retention times of authentic partially methylated alditol acetates, cf. Nakano & Fischer (1977). [b] t_R, retention time relative to 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetylglucitol. [c] x, signal at the mass number given; ↑, signal shifted upward by three mass units.

TABLE V: Chromatographic Behavior of Galactosylated Glycerols Obtained on Degradation of Lipoteichoic Acid.

	R_1[a]	R_2[a]	t_R[b]	t_R[c]
ref compds				
Galα1 → 2Gro	1.43	2.40	0.40	0.77
Galβ1 → 3Gro	1.30	2.09	0.49	1.00
GlcNAcα1 → 3Gro	1.55	2.53		1.87
Galα1 → 6Galβ1 → 3Gro	1.00	1.00	1.00	
Galα1 → 6Galα1 → 6Galβ1 → 3Gro	0.61	0.39	1.30	
glycosides from lipoteichoic acid				
Galα1 → 6Galα1 → 3Gro(2 ← 1αGal)[d]	0.71	0.44	1.25	
Galα1 → 6Galα1 → 3Gro[e]	1.11	1.09	0.89	
Galα1 → 3Gro[e]	1.32	2.17	0.41	0.91

[a] R_1 and R_2, mobility relative to Galα1 → 6Galβ1 → 3Gro on thin-layer chromatography in solvents D and F, respectively. [b] Retention time on gas–liquid chromatography of trifluoroacetylated glycosides relative to nonakis(trifluoroacetyl)Galα1 → 6Galβ1 → 3Gro. [c] Retention time of trimethylsilyl derivatives relative to hexakis(trimethylsilyl)Galβ1 → 3Gro. [d] Major glycoside obtained on HF degradation of lipoteichoic acid. [e] Released from Galα1 → 6Galα1 → 3Gro(2 ← 1αGal) by partial acid hydrolysis (cf. text).

acid hydrolysis (0.015 M HCl, 100 °C). After 6 h native material and galabiosylglycerol were found in the proportion 1:4; after 10 h trigalactosylglycerol was no longer detectable and galabiosylglycerol and monogalactosylglycerol were observed in the proportions 2:1. Both novel glycosides were tentatively identified by a rapid reaction with the periodate–Schiff reagent and by their chromatographic behavior (Table V). Very small amounts of two other glycosides were detectable which were on the basis of their chromatographic and staining properties possibly Galα1 → 6Gal and Galα1 → 3Gro(2 ← 1αGal). The predominant galabiosylglycerol was isolated by preparative thin-layer chromatography in solvent F. It showed on analysis D-galactose, glycerol, and 1,2-glycol groups in the molar ratio of 1.99:1.00:0.94, which locates the galabiosyl residue at a primary hydroxyl group of the glycerol moiety. Since, as will be shown below, the glycerol is substituted at position 1 by the phosphate ester, the galabiosyl-, galactosylglycerol has the structure depicted in Figure 2.

In summary, the products obtained on hydrolysis with HF and the data in Table I lead to the following conclusions. (i) The lipid anchors of the lipoteichoic acid are Glcα1 → 2Glc(6 ← acyl)-α1-3-acyl$_2$Gro and Glcα1 → 2Glcα1 → 3-acyl$_2$Gro, the former accounting for 70–96% (Table I). Somewhat lower values are calculated from the lipid products of hydrolysis with HF (Table III) owing to partial deacylation. (ii) The basic units of the hydrophilic chain are galabiosyl-, galactosylglycerophosphate residues which must be joined to each other and to the glycolipid portion by phosphodiester bonds. This interunit linkage is consistent with the release of almost all the phosphorus as glyceromonophosphate on hydrolysis with HCl (Table I), whereas the double glycosylation of the glycerophosphates accords with the observed resistance to alkaline treatment (see above). (iii) Since glucose is restricted to the glycolipid portion, the hydrophilic chains of preparations 1–3 can be calculated to have an average size of 11.8, 8.8 and 7.4 units, respectively (Table I).

The Point of Attachment of Adjacent Units. Native or deacylated lipoteichoic acid was subjected to exhaustive hydrolysis with α-galactosidase (see Materials and Methods) which released 34 and 35% of the galactose from preparations 2 and 3, respectively. The resultant lipoteichoic acid derivatives, freed from enzyme, galactose, and buffer (see Materials and Methods), were hydrolyzed with HF for 96 h. The released water-soluble glycoside was on chromatography (Table V) and analysis identical with the Galα1 → 6Galα1 → 3Gro char-

acterized above, whereas Galα1 → 3Gro could not be observed. These findings prove that the galabiosyl residues are the linkage units throughout the hydrophilic chain (Figure 2).

Another sample of deacylated lipoteichoic acid (preparation 3) was oxidized with $NaIO_4$, reduced with $NaBH_4$, and desalted as described under Materials and Methods. The reaction product was hydrolyzed under mild acidic conditions (Figure 2) which break acetal bonds leaving phosphodiester linkages essentially intact (Fischer et al., 1973; Fischer & Landgraf, 1975). The hydrolysate was applied to a small column of anion exchange resin (Merck MP 5080), acetate form. After elution of nonionic products with water, 95% of the initial phosphorus was eluted with 2 M formic acid. The phosphorus-containing fraction was rotary evaporated, and the residue dissolved in water, neutralized with ammonia and applied to a small column of DE 32-cellulose, HCO_3^- form (Laine & Fischer, 1978); 88.5 and 11.5% of the phosphorus eluted with 0.03 and 0.06 M triethylamine carbonate buffer, pH 8.5, respectively. As evidenced by thin-layer chromatography in solvent G, and by gas–liquid chromatography of the trimethylsilyl derivatives (Fischer et al., 1973), the major fraction consisted of diglycerol phosphate, whereas the minor fraction was a mixture of α- and β-glycerophosphate (4:1) which had been formed by partial phosphodiester cleavage.[2] On analysis the major product showed phosphorus, glycerol, and 1,2-glycol groups in the molar ratio of 1.00:2.02:2.05 and is therefore identified as di-α-glycerol phosphate. Its formation confirms the phosphodiester linkage between adjacent units, one glycerol being that of the original glycerophosphate, whereas the other can only be derived from C-4, C-5, and C-6 of a hexopyranosyl residue with the phosphodiester at C-6 (Figure 2). Since the linkage unit between two glycerophosphates is the (1–6)-joined galabiosyl residue, it follows that the phosphodiester is located at C-6 of the outer galactosyl moiety.

Based on the chain length of 7.4 units (preparation 3), the phosphodiester linkage between the hydrophilic chain and the glycolipid portion contributes 13.5% to the phosphorus-containing products. The recovery of 95% of the initial phosphorus in the form of diglycerol phosphate (84%) and glyceromonophosphate (11%) suggests therefore that the chain is joined to C-6 of the glycolipid moiety and, if so, to the outer glucosyl residue since C-6 of the inner one is largely occupied by the third fatty acid.

In another experiment the periodate oxidation product of the polymer was desalted by dialysis, an equal volume of 0.5 M sodium glycinate buffer, pH 10.5, was added, and the mixture was allowed to stand at room temperature for 5 h (Brown et al., 1955). Thereupon no inorganic phosphate was present but 86% of the initial phosphorus had become susceptible to phosphomonoesterase. Since mild alkaline treatment is well known to cause ester elimination from β- rather than from α-hydroxy-carbonyl derivatives (Linstead et al., 1953; Brown et al., 1955), the above finding confirms independently that the glycerophosphates are joined to C-6 of neighboring hexopyranosyl residues.

The Stereochemical Configuration of the Glycerophosphate Residues. Since the forementioned elimination did not lead to α-glycerophosphate,[3] as suggested by Archibald & Coapes (1971), the stereochemical analysis was performed by subjecting the di-α-glycerol phosphate obtained on Smith degradation to alkaline hydrolysis (0.1 M NaOH, 100 °C, 6 h). On analysis of the hydrolysate (Fischer et al., 1973; Fischer & Landgraf, 1975), glycerol, glycerophosphates, α-glycerophosphate,[4] and *sn*-glycero-3-phosphate were found in the molar ratio 0.99:1.03:0.45:0.01. Based on the fact that the alkali-released α-glycerophosphate retains the original stereochemical configuration (Baer & Kates, 1950; Fischer et al., 1973; Brotherus et al., 1974; Fischer & Landgraf, 1975), the absence of *sn*-glycero-3-phosphate establishes the diglycerol phosphate to be *sn*-1-glycerophospho-1′-*sn*-glycerol. This configuration proves definitely that the glycerophosphates of the hydrophilic chain are the *sn*-1 isomer and is consistent with the other glycerol of the diglycerol phosphate being derived from C-4 through C-6 of a D-hexopyranosyl moiety.

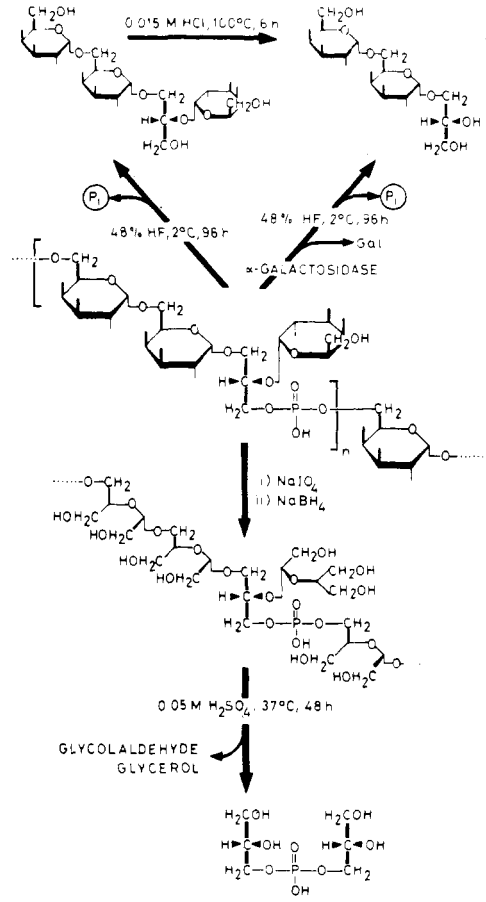

FIGURE 2: Degradative sequence for structural analysis of the hydrophilic chain. Glycolaldehyde and glycerol released on Smith degradation were not analyzed; the stereochemical configuration of di-α-glycerol phosphate was established by analysis of the alkali hydrolysis products (see text).

The complete structure of the repeating unit is therefore established to be Galα1 → 6Galα1 → 3-*sn*-glycero(2 ← 1αGal)-1-phosphate (Figure 2).

The Structure of the Hydrophilic Terminus. On periodate oxidation (0.1 M $NaIO_4$, 37 °C, 24 h) native, deacylated, and enzymically degalactosylated lipoteichoic acid (preparation 3) released per 2 mol of glucose 0.06, 0.97, and 0.83 mol of formaldehyde, respectively. This proves an unbranched hydrophilic chain, confirms that a diester-linked α-glycerophosphate is the terminus (Figure 3), and shows in accord with the high content of galactose (Table I) that the terminal glycerophosphate is galactosylated.

[2] In a control experiment, 13.6% of deacylated phosphatidylglycerol was hydrolyzed under the same conditions.

[3] After enzymic dephosphorylation, only 0.04 mol of glycerol per mol of phosphorus was detected.

[4] The difference between glycerophosphates and α-glycerophosphate is accounted for by β-glycerophosphate which is formed by phosphodiester cleavage via a five-membered cyclic glycerophosphate (Baer & Kates, 1950).

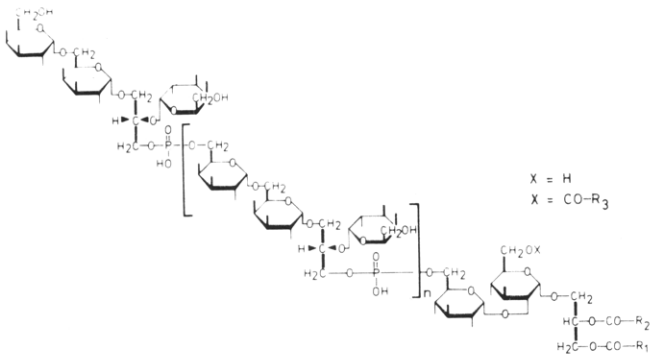

FIGURE 3: Structure of the lipoteichoic acid from *S. lactis* 42172. Part of the glycerophosphate terminus contains only the galabiosyl residue. The triacylglycolipid accounts for 70–96%. n = 6.4–10.8.

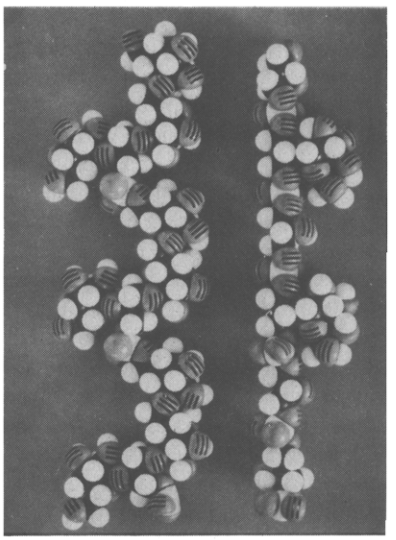

FIGURE 4: Space-filling model of a tri(Galα1 → 6Galα1 → 3-*sn*-glycero(2 ← 1αGal)-1-phosphate) in comparison with a (1–3) linked hexa-(glycerophosphate) carrying two α-D-galactopyranosyl residues. Both chain fragments are shown in extended conformation. The lengths per unit are 1.63 and 0.75 nm, respectively.

To detect minor glycosides, possibly located at the terminus, lipoteichoic acid (preparation 3) was subjected to exhaustive hydrolysis with HF for 96 h. Analysis of the water-soluble products revealed that 0.94 mol of galactose had been liberated per mol of lipoteichoic acid (less than 5%). After trimethylsilylation and trifluoroacetylation, the released glycosides were identified (cf. Table V) and quantitated by gas–liquid chromatography. Galα1 → 3Gro, Galα1 → 2Gro, and compounds with retention times identical with or similar to that of GlcNAcα1 → 3Gro were not observed, whereas Galα1 → 6Galα1 → 3Gro and Galα1 → 6Galα1 → 3Gro(2 ← 1αGal) were found in a molar ratio of 1.0:4.29. Thus, based on the chain length of 7.4 units (Table I) and corrected for the released galactose, the chain can be calculated to contain on the average 0.46 galabiosylglycerol and 6.94 galabiosyl-, galactosylglycerol moieties.

When the low-molecular-weight fraction, which was obtained by chromatography on Sephadex G-50 of the alkali-treated polymer (see above), was enzymically dephosphorylated, chromatographic analyses confirmed the absence of monogalactosylglycerol and revealed the liberation of Galα1 → 6Galα1 → 3Gro. This let us suggest that part of the terminal glycerophosphate bears only a galabiosyl residue, whereas the remainder seems to be fully galactosylated.

Discussion

The structure of the lipoteichoic acid of *S. lactis* Kiel 42172 is summarized in Figure 3. Having a poly(galabiosyl-, galactosylglycerophosphate) chain it is thus far unique since lipoteichoic acids, in contrast to wall teichoic acids, have been thought to have uniformly a poly(glycerophosphate) backbone (Knox & Wicken, 1973; Wicken & Knox, 1975; Lambert et al., 1977). Furthermore, most of the examined lipoteichoic acid is anchored in the membrane with three fatty acids and belongs therefore to the novel subclass which has recently been discovered in *Lactobacillus casei* and discussed already in detail (Nakano & Fischer, 1978). Judged from the glycerophosphoglycolipid pattern (see introductory section) the poly(galabiosyl-, galactosyl glycerophosphate)–lipoteichoic acid is more widespread among *S. lactis* strains. It should be noted, however, that *S. diacetilactis* and *S. lactis* NCDO 712 have lipoteichoic acids with a partially galactosylated poly(glycerophosphate) chain (Koch, H. U., & Fischer, W., unpublished data).

Poly(glycosyl glycerophosphates) have been described as wall teichoic acids of *Bacillus licheniformis* (Burger & Glaser, 1966), *Bacillus stearothermophilus* (Anderson & Archibald, 1975), and *Lactobacillus plantarum* (Archibald & Coapes, 1971). These polymers were found to bear mono- or dihexosyl residues also at the primary hydroxyl group of the glycerophosphate but none of them was glycosylated at position 2. The repeating units of two distinct polymers from *L. plantarum* have been demonstrated to be Glcα1 → 1-*sn*-glycero-3-phosphate and Glcα1 → 2Glcα1 → 1-*sn*-glycero-3-phosphate (Archibald & Coapes, 1971). The *sn*-glycero-3-phosphate configuration accords with the fact that the glycerophosphate of wall teichoic acids is biosynthetically derived from CDP glycerol (Burger & Glaser, 1964). The enantiomeric glycerophosphate as building block of lipoteichoic acids has recently been suggested by in vitro and in vivo studies in which phosphatidylglycerol was shown to turn over into water-soluble polymers having the properties of lipoteichoic acids (Glaser & Lindsay, 1974; Emdur & Chiu, 1974, 1975). This pathway is now corroborated by the direct proof that the glycerophosphates of the lipoteichoic acid studied here are indeed the *sn*-1 isomer and their arrangement in the polymer chain (Figure 3) is compatible with a transfer of *sn*-glycero-1-phosphate on extension of the growing chain from the glycolipid portion. Studies on the hydrophilic terminus showed that it is galactosylated but failed to reveal Galα1 → 3Gro and Galα1 → 2Gro. Nevertheless prior assembly of repeating units, which is thought to be operative in the biosynthesis of poly(glycosyl glycerophosphate)–wall teichoic acids (cf. Archibald, 1974) seems to be not likely since Galα1 → 6Galα1 → 3Gro was found at the terminus of the lipoteichoic acid and as possible biosynthetic precursors nongalactosylated, mono-, di-, and trigalactosylglycerophosphoglycolipids have been observed in *S. lactis* 42172 (Fischer et al., 1978a, and unpublished data).

With the model depicted in Figure 4 one galabiosyl-, galactosyl glycerophosphate unit was found to be 1.63 nm in length and the whole chain of the novel lipoteichoic acid, consisting of 7.4–11.8 units, measures therefore 12–19 nm in extended conformation. A similar length of 14 nm can be calculated for the partially galactosylated poly(glycerophosphate)–lipoteichoic acid of *S. lactis* NCDO 712 which contains 19 glycerophosphate units of 0.75 nm each (Figure 4). Both these lipoteichoic acids act as group N antigen, the determinants of which are not as originally suggested galactose phosphates (Elliot, 1963) but α-D-galactopyranosyl residues

(Wicken & Knox, 1975). Accordingly, in both lipoteichoic acids α-galactosyl residues are joined to the terminus and to position 2 of the intrachain units. As shown by electron microscopy the wall of *S. lactis* is 20 nm thick and contains 20-nm holes which are thought to be partially filled by protrusions from the membrane (Hurst & Stubbs, 1969). Thus, lipoteichoic acids can act here as antigen either by extending from the membrane through the wall at the surface of the cell (van Driel et al., 1973; Wicken & Knox, 1975) or within the holes by full contact with the environment.

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