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Characterization of Group N Streptococcus Lipoteichoic Acid

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Lipoteichoic acid was extracted from the group N organism *Streptococcus lactis* ATCC 9936 with hot aqueous phenol and purified by gel chromatography followed by affinity chromatography using *Ricinus communis* lectin as the specific absorbent. The teichoic acid moiety of the lipoteichoic acid was calculated to contain 16 to 17 glycerol phosphate units, approximately half of which were substituted with α-D-galactosyl residues; the glycolipid moiety contained *O*-α-D-glucosyl-1 → 2-*O*-α-D-glucosyl-1 → 1-glycerol. The finding of 2-*O*-α-D-galactosyl glycerol in the lipid fraction of hydrofluoric acid hydrolysates suggests that fatty acids also occur as substituents on the main chain of the lipoteichoic acid. The reactivity of the lipoteichoic acid with *R. communis* lectin was studied by the quantitative precipitin method and compared with the reactivity of *Lactobacillus fermenti* lipoteichoic acid, which has a lower degree of α-D-galactosyl substitution. Group N antiserum reacted strongly with the *S. lactis* lipoteichoic acid and cross-reacted with *L. fermenti* lipoteichoic acid. From inhibition studies it is concluded that the antibodies are specific for α-D-galactosyl substituents. In addition to lipoteichoic acid, a fraction was obtained by gel chromatography which contained galactose and reacted with group N antiserum but could be distinguished from the lipoteichoic acid by immunoelectrophoresis.

Structural studies on a number of membrane lipoteichoic acids have shown the lipid moiety to be a glycolipid and the teichoic acid moiety generally to consist of 25 to 30 glycerol phosphate units joined by 1 → 3 phosphodiester linkages and variously substituted with glycosyl and D-alanyl ester groups (8, 29). Where antibodies that are specific for the glycosyl substituents are formed, the lipoteichoic acid may function as a grouping antigen, examples being the group D streptococci (8, 20, 29) and the group F lactobacilli (5). The probable inclusion of group N streptococci in this category was indicated by Elliott's finding that "intracellular" teichoic acid isolated from disintegrated cells reacted with grouping antisera (2). Intracellular teichoic acid is now recognized to be membrane-associated lipoteichoic acid (8, 20, 22, 29), but the product isolated by Elliott differed from all subsequently characterized lipoteichoic acids in that "galactose phosphate" was reported to be among the products of acid hydrolysis of the polymer, with both galactose-1-phosphate and galactose-6-phosphate acting as inhibitors in the precipitin reaction with group antisera.

This study reports more definitive investigations into the chemical structure and serological properties of the group N streptococcal lipoteichoic acid isolated from *Streptococcus lactis* and presents evidence for a product containing α-D-galactosyl residues as the sole carbohydrate substituent on the teichoic acid moiety. The lipoteichoic acid does, however, differ from those previously described (8, 20, 29) in that the carbohydrate substituent of the glycolipid moiety differs from that of the teichoic acid.

MATERIALS AND METHODS

Organism. *S. lactis* ATCC 9936 was obtained from the American Type Culture Collection. This strain is the same as the original Lancefield group N strain C559 and Shattock strain O.J. (17). Organisms were routinely grown in Todd-Hewitt broth (Oxoid) supplemented with 2% glucose and 0.5% yeast extract for 16 h in a New Brunswick 14-liter Microferm batch fermentor without aeration at 30 C; 500 to 600 mg (dry weight) of saline-washed cells per liter was routinely obtained.

Preparation of cell walls. The cell wall fraction from organisms disintegrated in the Braun cell homogenizer was purified, as described previously (7), by repeated washing with 0.85% NaCl and distilled water followed by treatment with 4% sodium dodecyl sulfate at 100 C; this procedure proved the most effective for removing contaminating lipoteichoic acid.

Extraction of cells. Freeze-dried cells were ex-

tracted with 45% aqueous phenol at 65 to 68 C as previously described (26). Acid extracts of whole cells were prepared by the method of Lancefield (10).

Column chromatography. Gel chromatography was routinely carried out on columns (45 by 2.6 cm) of Sephadex G-75, Sepharose 4B (Pharmacia), and 6% agarose (Litex, Denmark) at ambient temperature with 0.2 M ammonium acetate, pH 6.9, as elutant. Fractions (about 4 ml) were collected automatically by drop-counting using standard LKB equipment, and samples were analyzed for phosphorus and extinction at 280 nm. Column void volumes were determined with blue dextran (Pharmacia).

Preparation of Ricinus communis lectin. The galactose-specific castor bean lectin was extracted from locally harvested *R. communis* seeds (18) and purified by affinity chromatography (19) on columns of Sepharose 2B (Pharmacia); adsorbed lectin was eluted with 0.1 M galactose in phosphate-buffered saline (pH 7.0), dialyzed, and freeze-dried; 891 mg of lectin was obtained from 750 g of seeds.

Lectin affinity chromatography. CNBr-Sepharose 4B (Pharmacia) (30 g) was allowed to swell for 15 min in 10^{-3} M HCl and washed thoroughly on a glass filter with the same solvent. Lectin (500 mg) was dissolved in 250 ml of a solution containing 10^{-2} mol of $NaHCO_3$, 5×10^{-1} mol of NaCl, and 10^{-3} g ions of Mn^{2+}, Mg^{2+}, and Ca^{2+} per liter, mixed with the drained gel, and agitated by gentle rotation at 20 C for 2 h. Unbound lectin (17 mg) was removed by washing with the coupling solution, and the remaining reactive groups were destroyed by treatment with 1 M ethanolamine in 0.5 M NaCl containing 10^{-3} M divalent cations. Noncovalently bonded protein was removed from the gel as suggested by Pharmacia by six cycles of successive washes with 0.1 M acetate, pH 4.0, and 0.1 M borate, pH 8.0, buffers in 1 M NaCl containing 10^{-3} g ions of Mn^{2+}, Ca^{2+}, and Mg^{2+} per liter. The final product was stored in 0.01 M imidazole buffer, 0.5 M NaCl containing 10^{-3} g ions of Mg^{2+}, Mn^{2+}, and Ca^{2+}, 0.02% NaN_3, pH 6.85, in the cold. Before use in affinity chromatography, the gel was washed with the same buffer less the addition of divalent cations.

Analytical methods. Conditions for the acidic and alkaline hydrolysis of column fractions, enzymatic dephosphorylation, characterization of glycosides, and identification of products by paper chromatography, unless stated in the text, were essentially as described previously (6, 7, 26, 27). Procedures for quantitative analyses have also been previously detailed (6, 7, 26, 27). Hydrolysis with 60% hydrofluoric acid (HF) was by the procedure of Burger and Glaser (1).

The following solvent systems were used for paper and thin-layer chromatography: (i) propan-1-ol/aqueous ammonia (specific gravity 0.88)/water (6:3:1, by volume), Whatman paper no. 4, ascending; (ii) butan-1-ol/pyridine/water (6:4:3, by volume), Whatman paper no. 1, descending; (iii) ethyl acetate/pyridine/water (5:2:5, by volume, upper phase), Whatman paper no. 1, descending; (iv) chloroform/methanol/water (65:25:4, by volume), silica gel thin-layer chromatography plates. Chromatographic spray reagents, including periodate-Schiff reagent, were as detailed previously (27).

Gas chromatographic analysis of fatty acids. Gas chromatographic analyses of fatty acids were kindly performed by D. G. Bishop, C.S.I.R.O. Division of Food Preservation, Sydney, on columns of 25% diethylene glycol succinate/2% phosphoric acid on Gas-Chrom P (100 to 200 mesh) at 165 C.

Preparation of α-kojibiosyl-1-glycerol. Lipoteichoic acid isolated from hot aqueous phenol extracts (26) of *Streptococcus faecalis* NCIB 8191 was hydrolyzed in N NaOH for 3 h at 100 C. The hydrolysate was passed through a column of Dowex 50 (NH_4^+) to remove Na^+ ions and chromatographed in solvent system A as a band. The glycoside region of the chromatogram was eluted with water and further purified by chromatography in solvent system B into two glycosides, which gave fast reactions with the periodate- Schiff reagent, of $R_{glucose}$ values of 0.58 and 0.40, corresponding to reported values of 0.56 and 0.39 (4), respectively, for α-kojibiosylglycerol and α-kojitriosylglycerol. Characterization of the major glycoside of the glycolipid moiety of *S. faecalis* lipoteichoic acid as α-kojibiosylglycerol (*O*-α-D-glucosyl-1 → 2-*O*-α-D-glucosyl-1 → 1-glycerol) has been described previously (20).

Serological methods. The procedures used for isolating lipoteichoic acids and for obtaining antisera to organisms and lipoteichoic acid preparations have been described previously (5 to 7, 28); the method of Slade and Slamp (16) was followed for obtaining antisera to disintegrated *S. lactis* cells. Commercial group N antiserum was obtained from Burroughs Wellcome. Serological reactivity was examined by the quantitative precipitin method (3) and gel immunoelectrophoresis (26). Experiments using *R. communis* lectin were carried out in 0.85% NaCl containing 10^{-3} M $CaCl_2$, 10^{-3} M $MgCl_2$, and 10^{-3} M $MnCl_2$. For inhibition studies, methyl-α- and β-D-galactopyranosides were obtained from Pfanstiehl Laboratories Inc., Waukegan, Ill., and Schwarz-Mann, Orangeburg, N.Y.; glycerol-phosphoryl-glycerol-phosphoryl-glycerol (G_2P_2) was prepared from cardiolipin (28, 30).

RESULTS

Fractionation of aqueous phenol extracts of whole cells of S. lactis. Chromatography of a hot aqueous phenol extract of whole cells of *S. lactis* on 6% agarose gave three phosphate-containing fractions (Fig. 1). Fraction I, on acid hydrolysis, gave degradation products typical of a glycerol teichoic acid and was designated as crude lipoteichoic acid. Fraction II was further fractionated on a column of Sephadex G75 (Fig. 2) into IIA, IIB, and IIC. Fraction III proved to be a mixture of low-molecular-weight nucleotides and peptides as shown by paper chromatographic analysis of acid hydrolysates. Fractions IIB and IIC were similar mixtures of nucleotide and peptide material, but IIC showed in addition traces of glycerol phosphate and glycerol in

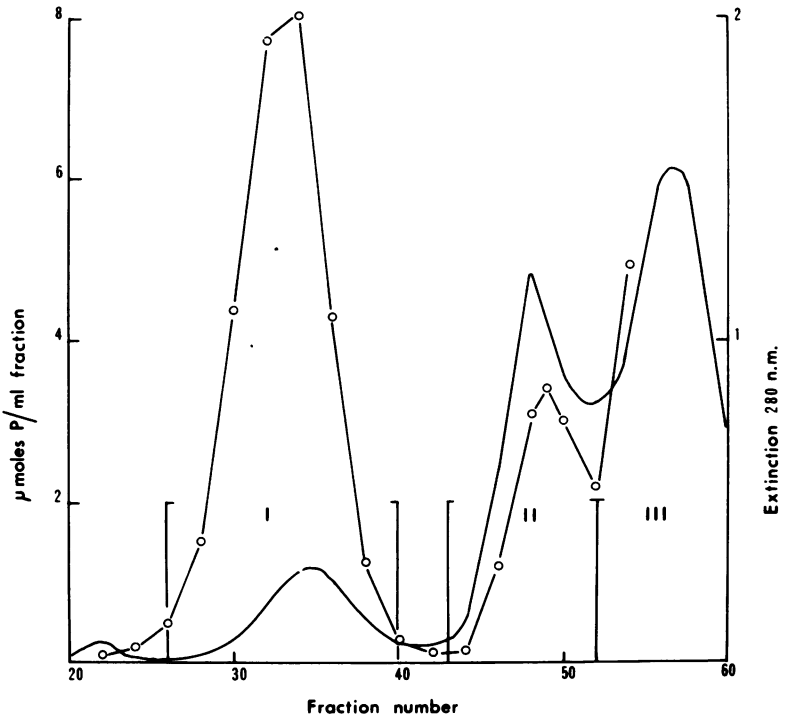

FIG. 1. *Agarose gel (6%) chromatography of aqueous phenol extracts of S. lactis. Symbols: O, micromoles of phosphorus/milliliter of fraction; ——, extinction at 280 nm.*

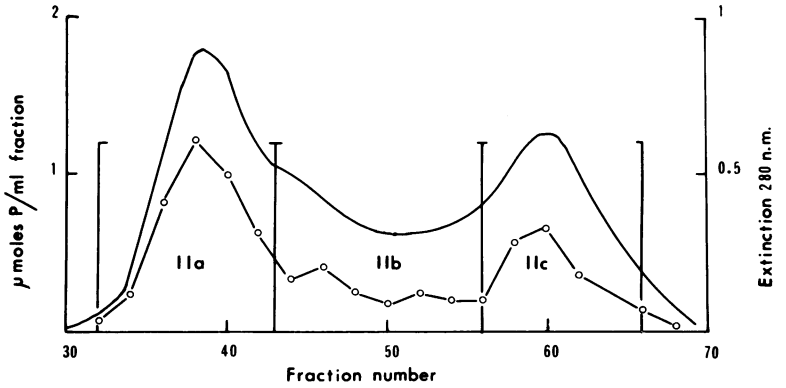

FIG. 2. *Sephadex G-75 chromatography of fraction II from agarose gel columns (Fig. 1). Symbols are as in Fig. 1.*

acid hydrolysates; these fractions were not investigated further. Acid hydrolysates of fraction IIA contained a range of amino acids, nucleotide degradation products, inorganic phosphate, and the sugars glucose, galactose, rhamnose, and ribose; sugar phosphates were not detectable. Teichoic acid degradation products were absent from this fraction and rhamnose was absent from the other fractions.

Purification of lipoteichoic acid. Gel chromatography of crude lipoteichoic acid on columns of Sepharose 4B removed much of the contaminating ultraviolet light-absorbing material. Further purification of lipoteichoic acid was achieved by affinity chromatography on a column of Sepharose 4B linked to *R. communis* lectin. Lipoteichoic acid (240 μmol of organic P) was applied to the column in starting buffer,

and a small fraction (8 μmol of organic P) eluted with the same buffer. Chromatographic examination of acid hydrolysates of this fraction indicated that it was a mixture of nucleotide, peptide, and oligosaccharide material similar in composition to fraction IIA. Lipoteichoic acid (156 μmol) was eluted from the affinity column with 0.1 M galactose in starting buffer. This represents a total recovery of 68% of the applied organic P. Increasing the concentration of galactose in the eluting buffer to 1 M did not increase the yield of lipoteichoic acid.

Analysis of lipoteichoic acid. Quantitative analysis of purified lipoteichoic acid showed a mole ratio of P/glucose/galactose/D-alanine of 1.00:0.12:0.53:0.14. Preparations from affinity chromatography columns still contained 4.85% protein but less than 0.5% nucleotide material. The fatty acid ester content was 5.7% (wt/wt) made up of 14:0 (14.3%); 16:0 (40.1%); 16:1 (1.9%); 18:0, trace: 18:1 (27.5%); 19:0 Δ (16.0%). Preparations from Sepharose 4B columns showed the same molar proportions of phosphorus to sugar but were higher in protein content.

Paper and thin-layer chromatographic analysis of acid hydrolysates of lipoteichoic acid showed α-glycerol phosphate, glycerol diphosphates, glycerol, alanine, fatty acids, glucose, and galactose. Alkaline hydrolysis followed by phosphomonoesterase treatment gave only a partial breakdown of the polymer, as would be expected for a 1 → 3 phosphodiester-linked glycerol phosphate polymer having a high degree of glycosidic substitution.

Hydrolysis of lipoteichoic acid in 60% HF at 0 C was used to characterize the glycosidic substitution of the polymer. Neutralized hydrolysates were extracted three times with chloroform/methanol (2:1) (lipid fraction) and three times with water (aqueous fraction). The lipid fraction was further purified by chromatography on Sephadex G-25 in chloroform/methanol/water.

Paper chromatographic analysis of the aqueous fraction showed the presence of glycerol and a single glycoside that reacted slowly with the periodate-Schiff reagent. This glycoside had $R_{glucose}$ values of 1.00 and 0.88 in solvents B and C, respectively, identical to authentic galactosyl-2-glycerol, and gave galactose and glycerol on acid hydrolysis or treatment with α-galactosidase but not β-galactosidase. Small quantities of diglycerol phosphate were found in the aqueous fraction; this component had an $R_{glycerol}$ of 0.70 in solvent A, gave a fast reaction with the periodate-Schiff reagent, and was hydrolyzed in alkali to a mixture of glycerol phosphate and glycerol. A further phosphorus-containing component present in trace amounts was partially characterized as galactosyl-2-glyceryl-phosphoryl-glycerol, being hydrolyzed in alkali to galactosyl-2-glycerol and glycerol phosphate. No glucose was detected in the aqueous fraction.

The purified lipid fraction of HF hydrolysates was deacylated (30) and shown to contain all of the glucose of the original polymer as a single glycoside having $R_{glucose}$ values of 0.58 and 0.32 in solvents B and C, respectively. This component gave a fast reaction with the periodate-Schiff reagent characteristic of a 1-substituted glycerol. Partial acid hydrolysis (0.025 N HCl for 5 h at 100 C) of the glycoside gave a mixture of glucose, glycerol, glucosyl-1-glycerol ($R_{glucose}$ of 0.89 in solvent C), and unchanged original glycoside. Treatment with α-glucosidase gave complete breakdown of the glycoside to glucose and glycerol. The glycoside cochromatographed as a single component with α-kojibiosylglycerol, prepared from *S. faecalis* lipoteichoic acid, in solvents B and C.

Comparable amounts of 2-*O*-α-D-galactosyl-glycerol, characterized as above, were also found in the deacylated lipid fraction of HF hydrolysates; this product, being 2-substituted, would derive from the teichoic acid "backbone" rather than from a glycolipid.

Serological reactivity of S. lactis lipoteichoic acid. Antiserum 218 to *Lactobacillus casei* lipoteichoic acid, which is specific for the glycerol phosphate "backbone" (28), reacted with *S. lactis* lipoteichoic acid purified by lectin affinity chromatography, the maximum amount of antibody precipitated being 50% of that precipitated in the homologous reaction.

Antisera to *L. fermenti* lipoteichoic acid exhibit variable specificity (5). Two of the rabbits (132 and 147) had antibodies that were primarily galactose specific, and their antisera were examined for their cross-reactivity with *S. lactis* lipoteichoic acid. With both antisera, the amount of antibody precipitated was 39% of that precipitated in the homologous reaction. The reaction between antiserum 147 (0.2 ml) and *S. lactis* lipoteichoic acid (10 μg) was inhibited 59% by 100 μmol of methyl-α-D-galactoside and 43% by 100 μmol of methyl-β-D-galactoside. These results may be compared with those obtained in the homologous reaction where the reaction between antiserum 147 (0.2 ml) and *L. fermenti* lipoteichoic acid (20 μg) was inhibited 36% by 100 μmol of the α-galactoside and 19% by 100 μmol of the β-galactoside.

Reaction of lipoteichoic acids with R. communis lectin. The reactions between 200 μg of *R. communis* lectin and lipoteichoic acids from *S. lactis* and *L. fermenti* are compared in Fig. 3. *L. fermenti* lipoteichoic acid has a lower degree of galactose substitution (27), which probably accounts for its lesser reactivity. This difference between the two preparations was also manifested (Fig. 4) in the amounts of methyl-α- and β-D-galactosides required to inhibit the precipitin reaction between the lectin and the lipoteichoic acids, 0.2 μmol of methyl-β-D-galactoside being as effective in inhibiting the reaction with 30 μg of *L. fermenti* lipoteichoic acid as was 5 μmol in the reaction with 40 μg of the *S. lactis* preparation.

Preparation of group N antisera. Antibodies that were group specific were obtained in two of three rabbits injected with whole organisms. However, compared with sera obtained by injecting disrupted organisms (see below), these sera were relatively weak (0.6 and 1.3 mg of antibody/ml) and were not examined in detail. The third rabbit injected with whole organisms yielded antiserum that reacted strongly with *S. lactis* lipoteichoic acid (4.5 mg of antibody/ml); however, the serum was not group specific, cross-reacting with lipoteichoic acids from group D streptococci and lactobacilli of groups A, B, C, D, and F.

Antisera obtained by injecting disintegrated organisms into four rabbits contained, respectively, 1.7, 2.2, 4.0, and 4.3 mg of antibody per ml; results are provided for the most potent antiserum (rabbit 309), but the other antisera displayed a similar specificity.

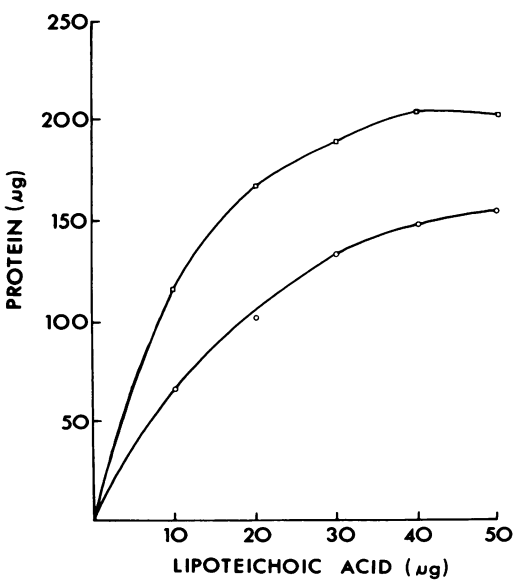

FIG. 3. *Precipitation of lipoteichoic acids from S. lactis* (□) *and L. fermenti* (○) *by 200 μg of R. communis lectin.*

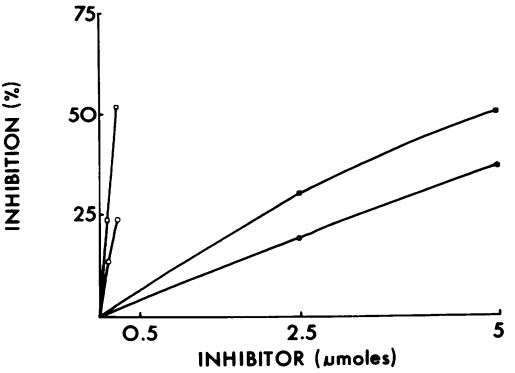

FIG. 4. *Comparison of the inhibition of the reaction between R. communis lectin (200 μg) and S. lactis lipoteichoic acid (40 μg) by methyl-α-D-galactoside* (●) *and methyl-β-D-galactoside* (■) *with the inhibition by the methyl galactosides* (α, ○; β, □) *of the reaction with L. fermenti lipoteichoic acid (30 μg).*

Injection of *S. lactis* lipoteichoic acid with Freund complete adjuvant (5) into three rabbits did not give a detectable antibody response.

Reactivity of group N antiserum with whole organisms and cell wall. To 1 ml of suspension containing 10 mg of *S. lactis* cells was added 0.25 ml of antiserum 309; the cells agglutinated and, after 2 h at room temperature, were removed by centrifugation. As determined by the quantitative precipitin method, absorption had removed 46% of the antibody from the serum. In contrast, 1 mg of cell wall preparation did not lower the antibody content of the serum.

Specificity of group N antiserum. Antisera to disintegrated organisms cross-reacted with *L. fermenti* lipoteichoic acid but not with *L. casei* lipoteichoic acid, which lacks glycosyl substituents on the teichoic acid backbone. The results with antiserum 309 are shown in Fig. 5; *L. fermenti* lipoteichoic acid precipitated 46% of the amount of antibody precipitated in the homologous reaction.

The inhibition of the precipitin reaction between antiserum 309 (40 μl) and *S. lactis* lipoteichoic acid by galactose and methyl-α- and β-D-galactosides is compared in Fig. 6 and indicates that the antibodies were reacting with α-D-galactosyl substituents on the lipoteichoic acid. G_3P_2 is an effective inhibitor of the precipitin reaction when antibodies are specific for the glycerol phosphate backbone (28), but in this

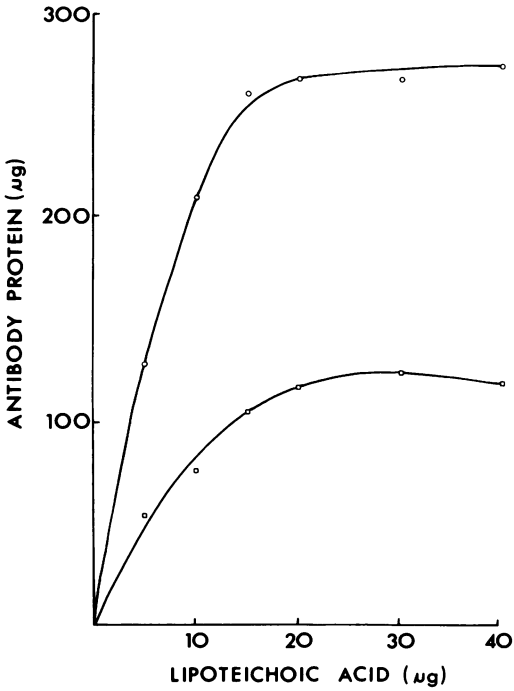

FIG. 5. *Precipitation of lipoteichoic acids from S. lactis* (O) *and L. fermenti* (□) *by antiserum 309 to S. lactis (75 μl).*

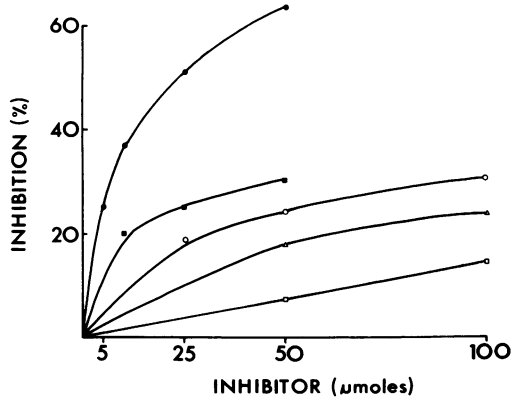

FIG. 6. *Inhibition of the reaction between 40 μl of antiserum 309 and 10 μg of S. lactis lipoteichoic acid by galactose* (Δ), *methyl-α-D-galactoside* (O), *and methyl-β-D-galactoside* (□); *inhibition of the cross-reaction between 100 μl of antiserum 309 and 20 μg of L. fermenti lipoteichoic acid by the methyl galactosides* (α, ●; β, ■).

system 2 μmol of G_3P_2 gave only 7% inhibition.

The cross-reaction of antiserum 309 (100 μl) with *L. fermenti* lipoteichoic acid (20 μg) was also examined for its specificity. The results obtained in the homologous reaction were supported; methyl-α-D-galactoside was a considerably more effective inhibitor than the β-anomer (Fig. 6), and 2 μmol G_3P_2 failed to give a detectable inhibition.

Immunoelectrophoresis. *S. lactis* lipoteichoic acid, fraction IIA, and a Lancefield acid extract of whole organisms were compared by immunoelectrophoresis against commercial group N antiserum, antiserum 309, and antiserum to *L. casei* lipoteichoic acid. The pattern of precipitin arcs obtained is shown schematically in Fig. 7. It is evident that the slower-migrating component in Lancefield extracts corresponded to lipoteichoic acid, whereas the faster component was equivalent to fraction IIA isolated from aqueous phenol extracts of *S. lactis*. Fusion of the two precipitin arcs obtained against commercial group N antiserum and serum 309 indicated a common antigenic specificity (galactose) of the two components.

DISCUSSION

By using the procedures previously developed with lactobacilli, lipoteichoic acid has been isolated from the group N streptococcus *S. lactis* ATCC 9936 and characterized chemically and serologically. The presence of a group-specific lipoteichoic acid had been implied by earlier studies; Elliott isolated a glycerol teichoic acid from disintegrated cells (2), and Smith and Shattock showed that the grouping antigen was membrane rather than wall associated (17); a report implicating a cell wall component (16) can probably be accounted for by the presence of contaminating lipoteichoic acid (7).

The purified lipoteichoic acid from *S. lactis* has properties similar to those of other organisms (6, 7, 20, 27). Elution close to the void volume of 6% agarose columns indicates an apparent high molecular weight common to lipoteichoic acid micelles, and the components found in acid hydrolysates, including fatty acids, are those expected for a lipoteichoic acid. The isolation and characterization of diglycerol phosphate and 2-*O*-α-D-galactosyl glycerol as HF degradation products of the polymer shows that the polyglycerol phosphate backbone of the polymer is 1 → 3 phosphodiester linked and that approximately half of the glycerol residues are substituted on C-2 with α-D-galactopyranose groups. There are sufficient glycerol phosphate residues unsubstituted with galactose to accommodate the D-alanyl ester substituents.

The lipid moiety of lipoteichoic acids is, characteristically, a glycolipid (8, 20, 29) that is joined by a phosphodiester linkage involving a

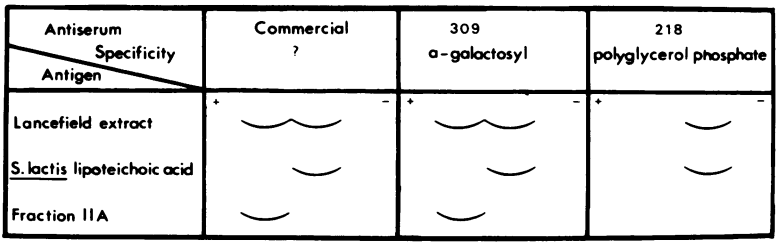

FIG. 7. *Schematic representation of precipitin arcs formed on immunoelectrophoresis of Lancefield acid extracts and aqueous phenol extracts of S. lactis.*

sugar hydroxyl group of the glycolipid and the terminal phosphate group of the teichoic acid. In *S. faecalis* (20) and *L. fermenti* (27), the disaccharide substituent of the glycolipid also occurs as a substituent on the teichoic acid. In *S. lactis* lipoteichoic acid, the glycolipid glycoside has been characterized as *O*-α-D-glucosyl-1 → 2-*O*-α-D-glucosyl-1 → 1-glycerol in contrast to exclusively α-D-galactosyl substitution of the teichoic acid. The finding of 2-*O*-α-D-galactosylglycerol in the lipid fraction from HF hydrolysates of the teichoic acid suggests that fatty acid residues can occur as substituents on the main chain of the lipoteichoic acid. We have postulated (29) that the galactose residue on the terminal glycerol phosphate bears a fatty acid ester substituent.The phosphodiester linkage between teichoic acid and glycolipid in lipoteichoic acid is known to be more acid labile (20, 27) than the other phosphodiester linkages in the glycerol phosphate backbone of these polymers. We suggest that the hydrophobic environment engendered by fatty acid residues in substituents on either side of the phosphodiester bond may contribute to its acid lability.

Assuming that diglucosyl-1-glycerol represents all of the glucose in *S. lactis* lipoteichoic acid, the length of the glycerol phosphate backbone of the teichoic acid portion can be calculated as 16 to 17 glycerol phosphate residues. This chain length is considerably shorter than that reported for other lipoteichoic acids (6, 7, 20, 27).

The chemical evidence on the structure of the teichoic acid moiety of *S. lactis* lipoteichoic acid has been supported by serological studies using the quantitative precipitin method. The presence of a backbone of 1 → 3-linked glycerol phosphate residues is indicated by the cross-reaction with antisera to *L. casei* lipoteichoic acid, which is specific for this structure (28). The presence of D-galactose substituents, which had been suggested by the cross-reaction with type XVI pneumococcal antiserum (3), is supported by the strong cross-reaction with antisera to *L. fermenti* lipoteichoic acid and the reaction with *R. communis* lectin. Antibodies to the *L. fermenti* lipoteichoic acid are specific for the α-D-galactopyranosyl substituents and in both the homologous and heterologous reaction methyl-α-D-galactopyranoside is a better inhibitor than the β-anomer. The specificity of *R. communis* lectin is directed against D-galactose and sterically related structures (11); both α- and β-D-galactopyranosyl residues of polymers will react with the lectin (11, 24), though the results with the lipoteichoic acids from *L. fermenti* and *S. lactis* confirm previous observations (23) that the β-anomer is the more effective inhibitor of the reaction. It has recently been shown that the lectin preparation used in this and most other studies can be fractionated into two components, one with the above specificity and one for which the α- and β-anomers are equally inhibitory (14).

More definitive serological evidence on the linkage of the D-galactose residues to the glycerol phosphate backbone of *S. lactis* lipoteichoic acid has been provided by using homologous antiserum to disintegrated organisms. With such sera it was shown that methyl-α-D-galactopyranoside is a more effective inhibitor of the precipitin reaction than the β-anomer. This result together with those on the cross-reaction of the antisera with *L. fermenti* lipoteichoic acid would indicate that the antibodies are specific for α-D-galactosyl substituents and are reacting with such substituents on the glycerol phosphate backbone of *S. lactis* and *L. fermenti* lipoteichoic acids.

The cross-reaction of *L. fermenti* lipoteichoic acid with *S. lactis* antiserum is particularly susceptible to inhibition by methyl-α-D-galactoside, 5 μmol being as effective in the cross-reaction as 50 μmol in the homologous reaction, where half as much lipoteichoic acid was used; i.e., there is a 20-fold difference in inhibitor concentration relative to antigen. A similar effect was observed with the inhibition of the reaction of the two lipoteichoic acids with *R.*

communis lectin. These results indicate the competition between the glycoside and the antigen for combining sites, reflecting the fact that *S. lactis* lipoteichoic acid has a fourfold higher degree of substitution with galactosyl residues than does *L. fermenti* lipoteichoic acid.

Elliott (2) reported that galactose was not inhibitory in the system he used for studying the specificity of the group N antigen, though both galactosyl-1-phosphate and galactose-6-phosphate did inhibit. Rather than this result indicating, as Elliott concluded, that "galactose phosphate constitutes part at least of the immunologically determinant group" (2), it is more likely that it is an example of nonspecific inhibition by an ionized compound (9). We found no evidence of galactose phosphate as a component of *S. lactis* lipoteichoic acid or fraction IIA.

The cellular origin of fraction IIA, which was isolated from aqueous phenol extracts of *S. lactis* ATCC 9936, remains unknown. Its reaction with commercial group N antiserum and serum 309 is presumably due to galactose residues, but the failure of a cell wall preparation to absorb antibodies from serum 309 indicates that there is not an accessible cell wall component with the same antigenic determinant. Since routine classification of an organism in group N depends on a positive reaction by an acid extract in the qualitative ring precipitin method, clarification of the extent to which this carbohydrate fraction contributes to the reaction by extracts of other strains requires that they be examined by immunoelectrophoresis; there is no a priori reason for both components occurring in all strains reacting with the grouping sera. The possibility that different organisms in a given serological group may actually contain different antigens reacting with the grouping sera has been indicated by studies on *S. mutans* strains, where there are reports of different serotype *a* strains containing either a specific polysaccharide (12) or membrane lipoteichoic acid (21), and of serotype *b* strains containing either a specific polysaccharide (13) or wall teichoic acid (25).

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