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Glycerolphosphate-Containing Cell Wall Polysaccharides from *Streptococcus sanguis*

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Six glycerolphosphate-containing tetraheteroglycans, a, b-1, b-2, b-3, b-4, and b-5, have been purified from the formamide extracts of *Streptococcus sanguis* by alcohol and acetone precipitations, Sephadex G-75, and diethylaminoethyl-cellulose column chromatography. The polysaccharides were judged as at least 95% pure by analytical disc gel electrophoresis and immune double diffusion against rabbit antiserum. They were shown to be cell wall polysaccharides, since they formed a single band of identity in immune double diffusion with partially purified polysaccharide extracted from a purified cell wall preparation of *S. sanguis*. The polysaccharides were composed of L-rhamnose, D-glucose, and *N*-acetyl D-glucosamine in a similar molar ratio, but varied in their glycerol and phosphate contents. They exhibited four different mobilities in polyacrylamide disc gel electrophoresis at pH 8.9. When they were treated with formamide at 170 C for 20 min, the faster moving polysaccharide(s) yielded polysaccharides with mobilities corresponding to the other slower moving polysaccharides. These results indicate that the polysaccharides originated from the same cell wall polysaccharide and were produced as a result of breakage in the phosphodiester bonds during the formamide extraction procedure. A preliminary structural study shows that the terminal reducing sugar is L-rhamnose and that the glycerol moiety is probably linked to the polysaccharide through a phosphodiester bond.

Glycerolphosphate-containing polymers have been found in many streptococcal cell envelopes in the form of teichoic acids (9, 22, 23, 28). Heymann et al. (14) have also reported the presence of glycerolphosphate in a cell wall polysaccharide-peptidoglycan complex isolated from group .A streptococcal cell walls. The glycerolphosphate was shown to be involved in the linkage between the reducing terminal of C-polysaccharide and the *N*-acetylmuramic acid moiety of peptidoglycan.

Most recently we have isolated a glycerolphosphate-containing cell wall polysaccharide from *Streptococcus sanguis;* however, our results showed that glycerolphosphate is not linked to the reducing terminal L-rhamnose by a glycosidic bond as was reported by Heymann et al. (14) for *Streptococcus pyogenes*.

MATERIALS AND METHODS

Bacterial strain. *S. sanguis* was obtained from the American Type Culture Collection, Rockville, Md. (strain 10556) and was maintained on blood agar slants in our laboratory.

Materials. Todd-Hewitt broth and complete Fruend adjuvant were obtained from Difco (Detroit, Mich.); Sephadex G-75 from Pharmacia (Piscataway, N.J.); and diethylaminoethyl(DEAE)-cellulose and the phases and supports for gas liquid chromatography from Applied Science (State College, Pa.). D-[U-^{14}C]glucose (180 mCi/mmol) and sodium [^{3}H]borohydride (143 mCi/mmol) were obtained from New England Nuclear (Boston, Mass.), and [2-^{3}H]glycerol (2 Ci/mmol) came from International Chemical and Nuclear (Irvine, Calif.). Deoxyribonuclease (deoxyribonucleate oligonucleotide-hydrolase, EC 3.1.4.5.), ribonuclease (polyribonucleotide 2-oligonucleotide-transferase [cyclizing], EC 2.7.7.16), and Pronase were purchased from Calbiochem (Los Angeles, Calif.); glycerol dehydrogenase (EC 1.1.1.6) and Glucostat were purchased from Worthington (Freehold, N.J.). New Zealand White rabbits were purchased from Hilltop Animal Laboratories (Scotdale, Pa.). All other chemicals were obtained from commercial sources.

Growth of culture. *S. sanguis* was grown routinely in 24 2-liter Erlenmeyer flasks, each containing 1 liter of Tood-Hewitt broth. The cultures were incubated at 37 C overnight with shaking in a model G-25 Incubator Shaker (New Brunswick Scientific, New Brunswick, N.J.). In most cases, 1 mCi of [^{3}H]glycerol or D-[^{14}C]glucose was added to four of the flasks to label the polysaccharide(s). Cells were harvested at 6,000 × *g* for 10 min and washed two times with saline.

Preparation of cell walls. All the following procedures were carried out at 0 to 4 C unless otherwise specified. The washed cells (40 g) were mixed with one-third their volume of glass beads (5 μm in diameter), suspended in 100 ml of saline, and sonically treated with a Sonifier Cell Disruptor model W185 equipped with a 0.5-in (ca. 1.3-cm) disruptor horn (Plainview, N.Y.) for three 10-min periods. Cell walls were separated from cytoplasmic material by centrifugation at 6,000 × *g* for 10 min. The precipitate (4 g dry weight) was washed two times with 0.02 M of tris(hydroxymethyl)aminoethane-hydrochloride buffer, pH 7.5 (Buffer A), suspended in 250 ml of the same buffer, and left standing for 3 to 4 h to allow the glass beads to sediment. The supernatant was then centrifuged at 6,000 × *g* for 10 min. The precipitate (crude cell wall) was used for the extraction of cell wall polysaccharide. In one experiment the crude cell wall preparation was washed three additional times and suspended in 60 ml of buffer A for further purification as follows: Samples (1 ml) were layered on 30-ml sucrose gradients (20 to 70%) and centrifuged at 915 × *g* for 60 min in a Beckman model L centrifuge using an SW25.1 rotor (Palo Alto, Calif.). The band corresponding to the cell wall material was mixed with an equal volume of buffer A and centrifuged again at 6,000 × *g* for 10 min. The precipitate was suspended in 20 ml of buffer A, treated with 5 mg each of deoxyribonuclease and ribonuclease for 60 min at 37 C after the addition of 20 μmol of $MgCl_2$ and 2 μmol of disodium ethylenediaminetetraacetate. It was further treated with 20 mg of Pronase for 120 min at 37 C and dialyzed overnight against 2 liters of buffer A. The sucrose gradient centrifugation was repeated on 35 to 70% sucrose gradients at 1,630 × *g* for 90 min. The band corresponding to cell wall material was mixed with an equal volume of buffer A and centrifuged at 6,000 × *g* for 10 min. The precipitate was suspended in 2 ml of buffer A and dialyzed overnight against 2 liters of buffer A.

Chromatography and electrophoresis procedures. Paper chromatography was performed on Whatman 3-MM filter paper in the following solvent systems: (A) pyridine-water-ethyl acetate-acetic acid (5:3:5:1, vol/vol/vol/vol); (B) butanone-acetic acid-water (75:25:10, vol/vol/vol); and (C) ethyl acetate-pyridine-water (6:3:1, vol/vol/vol). Paper electrophoresis was carried out on Whatman no. 1 filter paper at 26 V/cm for 3 h in 0.04 M sodium borate, pH 9.2. Sugars were detected with silver nitrate-sodium hydroxide (25), sugar phosphates with molybdate-perchloric acid (1), and sugars containing vicinyl hydroxyl groups with a benzidine-periodate stain (12). Polyacrylamide disc gel electrophoresis was performed in 7% acrylamide gel (2 mA/gel) at pH 8.9 (6) using the periodate-Schiff reagent (26) to stain gels. The gels were scanned at 574 nm in a Gilford 2400 spectrophotometer fitted with a 2410-s linear transport mechanism and a 6040 recorder (Oberlin, Ohio).

Alditol acetates were prepared according to the method of Griggs et al. (13) and injected into a Perkin-Elmer 900 gas chromatograph (Norwalk, Conn.) equipped with a hydrogen flame detector. A glass-coiled column (183 by 0.318 cm) was filled with packings of 3% OV-17 on Gas Chrom Q (100 to 120 mesh). Chromatography was conducted at a temperature program between 210 to 240 C, with an initial time of 2 min and a program rate of 1 C/min. The injector port temperature and detector temperatures were 220 and 250 C, respectively. The flow rate of the nitrogen carrier gas was 32 ml/min. Peak areas were determined by triangulation.

Chemical and radiometric procedures. Glucose was determined with Glucostat (32) and by the method of Dische (7); rhamnose by the procedure of Dische and Shettles (8); glucosamine according to a modified procedure of Elson and Morgan (2); *N*-acetyl group determination as described by Ludowieg and Dorfman (20); phosphate according to the procedure of Chen, Toribara, and Warner (4); and glycerol with glycerol dehydrogenase as described by Burger and Glaser (3).

Radioactive compounds separated by paper chromatography were located with a Tracer lab radiochromatogram scanner (Waltham, Mass.). The radioactivity of compounds was determined by counting them in a Tri-Carb liquid scintillation spectrophotometer (Packard Instruments Co., Inc., Downers Grove, Ill.) using a Triton scintillation fluid. The scintillation fluid was prepared by dissolving 5.5 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene in 333 ml of Triton X-100 and 667 ml of toluene.

Immunological methods. A 1-liter overnight culture of *S. sanguis* was centrifuged at 6,000 × *g* for 20 min and washed with saline. The bacteria were then treated with 0.067 M of formalin for 3 to 4 h, washed two times with saline, centrifuged (6,000 × *g*, 20 min), and suspended in saline to the concentration of 4 mg (dry weight)/ml. A sample of this bacterial suspension was mixed with the same volume of complete Freund adjuvant, and 0.5 ml was injected intramuscularly into 2.3-kg New Zealand White rabbits. After 5 days, 0.25 ml of the bacterial suspension was injected intravenously, followed by 0.25 ml subcutaneously every 3 days. The subcutaneous injections were repeated five to eight times, and animals were bled approximately 10 days after the last injection. The immune double diffusion analyses were performed as described by Tan et al. (30).

RESULTS

Extraction and purification of cell wall polysaccharide. The polysaccharide was extracted from both cell wall preparations and intact cells with formamide at 170 C for 20 min, followed by acid alcohol and acetone precipitation (11). A typical result obtained from the purification of [2-^{3}H]glycerol-labeled polysaccharide extracted from 8 g of crude cell walls is as follows: The acetone precipitate was dissolved in a 4 ml of water and dialyzed overnight against 2 liters of buffer A. The retentate was treated with deoxyribonuclease, ribonuclease, and Pronase, as described in the preparation of cell walls, and placed in a 100-C water bath for 3

min. Denatured protein was removed by centrifugation (6,000 × *g* for 10 min). The supernatant fluid was then dialyzed for 12 h against 2 liters of buffer A. The retente (8.7×10^6 counts/min) was chromatographed on a Sephadex G-75 column (2.5 by 57 cm) as described in Fig. 1. One major and one minor peak were obtained. Fractions 36 to 50 were combined, concentrated under reduced pressure to 3.5 ml, and dialyzed overnight against 2 liters of distilled water. A total of 7.8×10^5 counts/min were recovered. The dialyzed preparation was loaded onto a DEAE-cellulose column (2 by 37 cm) equilibrated with distilled water. The column was eluted with 150 ml of water, followed by elution with a linear salt gradient produced by mixing 200 ml of water in the mixing chamber and 200 ml of 1 M NaCl solution in the reservoir. The eluates were collected in fractions of 6.8 ml. The polysaccharide was separated into three peaks (Fig. 2). The first one was eluted with water, whereas the other two were eluted with NaCl solution. Fractions 16 to 18 (polysaccharide[PLS] a, 8.5×10^4 counts/min) and 39 to 41 (PLS b, 6×10^5 counts/min) were combined separately, evaporated under reduced pressure to 2 ml, and dialyzed overnight against 2 liters of distilled water. PLS b was rechromatographed with the same DEAE-cellulose column, except using 0 to 0.1 M of linear NaCl gradient produced in the same manner. PLS b was further fractionated into five peaks, b-1, b-2, b-3, b-4 and b-5, respectively (Fig. 3). Peak fractions corresponding to each peak (b-1, 16 to 18; b-2, 40 to 41; b-3, 43 to 46; b-4, 49 to 52; and b-5, 56 to 60) were combined, concentrated under reduced pressure to appropriate volumes (1 to 2 ml), and dialyzed against 2 liters of distilled water. The total amount of polysaccharides obtained were (in milligrams and counts per minute, respectively): PLS a, 36.3, 8.5×10^4; PLS b-1, 8.1, 3.1×10^4; PLS b-2, 4.8, 1.8×10^4; PLS b-3, 35.5, 2.2×10^5; PLS b-4, 14.4, 1.3×10^5; PLS b-5, 2.8, 4.3×10^4. Non-radioisotope-labeled and ^{14}C-labeled polysaccharides were isolated in a similar manner from cells grown in media without and with D-[U-^{14}C]glucose, respectively. The polysaccharide has also been extracted from the purified cell wall preparation and partially purified with a Sephadex G-75 column.

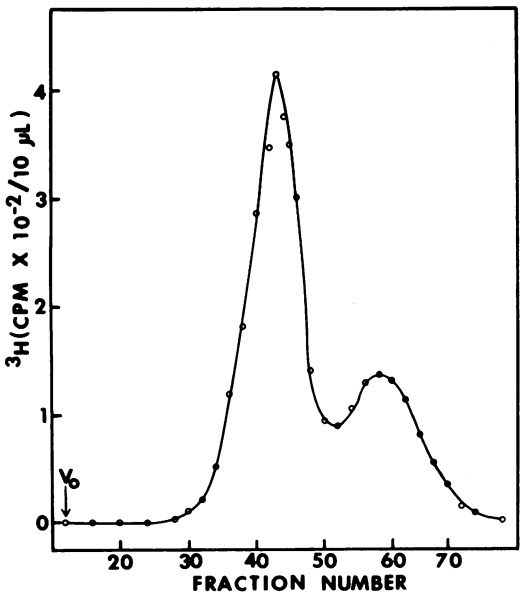

FIG. 1. *Sephadex G-75 column chromatographic purification of cell wall polysaccharide extracted from S. sanguis. Crude [^3H]glycerol-labeled polysaccharide (8.7×10^6 counts/min) was loaded onto a Sephadex G-75 column (2.5 by 57 cm) equilibrated with buffer A. The column was eluted with buffer A, and fractions of 2 ml were collected. Portions of each fraction were removed and counted in a liquid scintillation counter. V_0, void volume.*

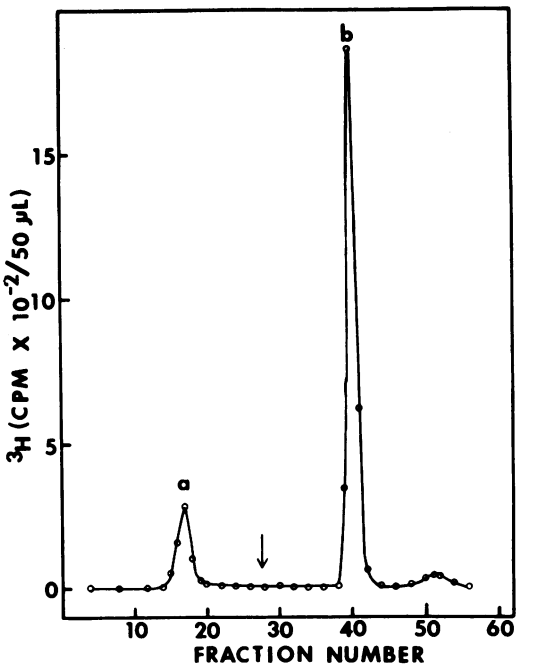

FIG. 2. *DEAE-cellulose column chromatographic purification of cell wall polysaccharide. Polysaccharide partially purified by Sephadex G-75 column chromatography was fractionated with a DEAE-cellulose column (2 by 37 cm) as described in the text. Portions of each fraction were removed and counted. At the arrow, the linear NaCl gradient (0 to 1 M) was started.*

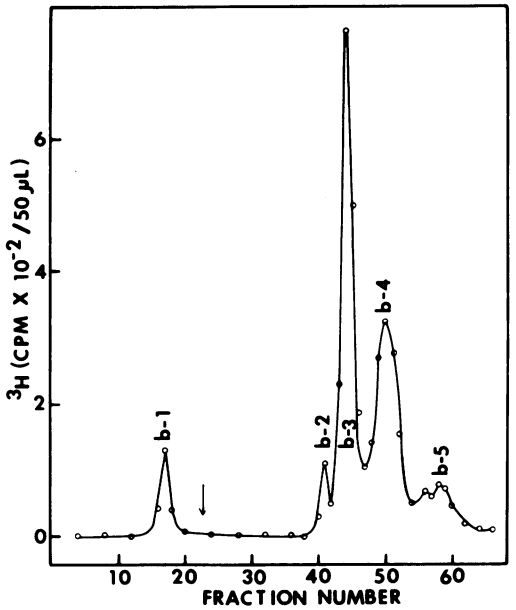

FIG. 3. *Second DEAE-cellulose column chromatographic purification. Dialyzed PLS b (6 × 10⁵ counts/min) was fractionated with a DEAE-cellulose column as described in Fig. 2, except a 0 to 0.1 M NaCl gradient was used. At the arrow, the linear NaCl (0 to 0.1 M) gradient was started.*

Characterization of polysaccharide. The purified polysaccharides extracted from intact cells are considered to be cell wall polysaccharides, since they formed a single band of identity in immune double diffusion in agarose gel with polysaccharide extracted from purified cell walls (Fig. 4). Moreover, no significant amount of such polysaccharides can be extracted from the cytoplasm and cell membrane preparations. Therefore, in some preparations intact cells were used directly for the extraction of the cell wall polysaccharide. After analytical disc gel electrophoresis of the cell wall polysaccharides, only a single band for each preparation was detected with the periodate-Schiff reagent (Fig. 5). The spectrophotometric scans of the gels are shown in Fig. 6. The polysaccharide preparations exhibited four different mobilities in which PLS a, b-1, and b-2 have a similar mobility. When PLS b-2, b-3, b-4, and b-5 were heated at 170 C in formamide for 20 min, dialyzed, and subjected to polyacrylamide disc gel electrophoresis, all except b-2, which has the slowest mobility, yielded bands corresponding to the mobilities of the slower moving polysaccharide preparations (Fig. 7, 8).

Approximately 624 µg each of nonlabeled and ^{14}C-labeled polysaccharides (a, b-1 to b-5) were hydrolyzed with 2 N HCl for 120 min at 100 C, dried in vacuo over NaOH and $CaCl_2$ pellets, and chromatographed overnight in solvent A. Each hydrolysate yielded three compounds whose mobilities corresponded to those of L-rhamnose, D-glucose, and D-glucosamine (R_{Glc} 1.40, 1.00, and 0.73, respectively) (Fig. 9B). Purified ^{14}C-labeled polysaccharides were also hydrolyzed (5 × 10⁵ counts/min) and chromatographed in solvent B. Three radioactive peaks with mobilities similar to those of L-rhamnose, D-glucose, and D-glucosamine were again detected (R_{Glc} 3.05, 1.00, and 0.61, respectively). A typical radiochromatographic profile is shown in Fig. 9A. When 687 µg of purified unlabeled PLS b-4 was hydrolyzed, converted to alditol acetates, and subjected to gas-liquid chromatography as described in Materials and Methods, three peaks corresponding in retention time to the alditol acetates of the three sugars mentioned above (R_{Glc} 0.40, 1.00, and 1.63 for L-rhamnose, D-glucose, and D-glucosamine, respectively) along with a small but distinct unidentified peak (R_{Glc} 0.23) were detected.

To demonstrate that the polysaccharides contain glycerol, purified ^{3}H-labeled polysaccharides were hydrolyzed with either 2 N HCl for 120 min at 100 C or 1 N KOH for 60 min at 100 C and neutralized. Upon chromatography

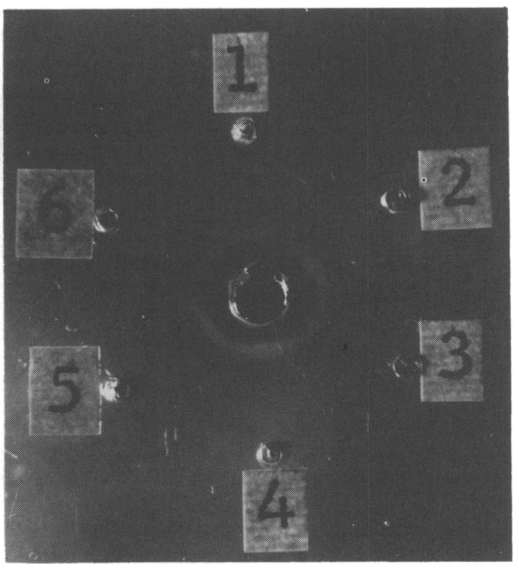

FIG. 4. *Immune double diffusion of cell wall polysaccharide in agarose gel. Center well, antiserum prepared against formalin-treated S. sanguis. Approximately 10 µg of polysaccharide preparations was added to the side wells: 1, partially purified polysaccharide extracted from purified cell wall; 2, PLS a; 3, PLS b-2; 4, PLS b-3; 5, PLS b-4; 6, PLS b-5.*

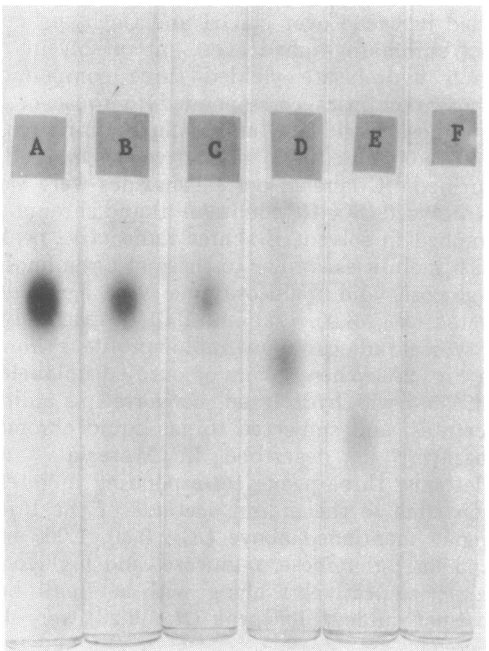

FIG. 5. *Polyacrylamide disc gel electrophoresis of purified polysaccharides. The electrophoresis was performed in 7% gels at pH 8.9. The direction of electrophoresis was from the cathode (top) toward the anode (bottom). A current of 2 mA/gel was applied for 80 min. Approximately 10 to 60 μg of polysaccharide was used in each electrophoresis. Gels were stained with the periodate-Schiff reagent (26). A, PLS a; B, PLS b-1; C, PLS b-2; D, PLS b-3; E, PLS b-4; F, PLS b-5.*

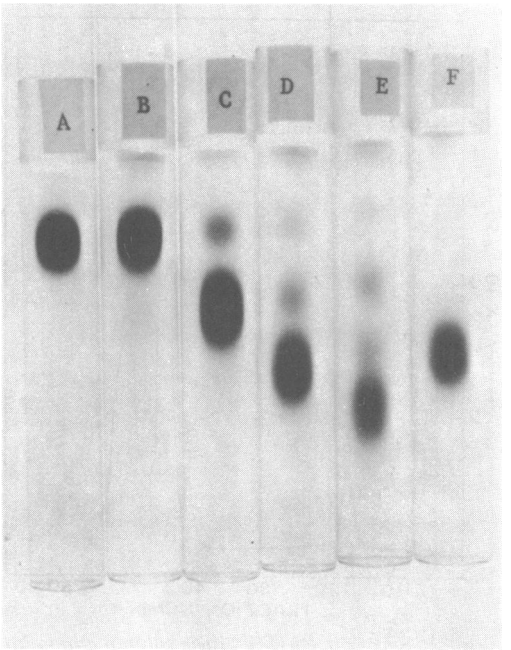

FIG. 7. *Polyacrylamide disc gel electrophoresis of formamide-treated polysaccharides. The electrophoresis was carried out as was described in Fig. 5. A, untreated PLS a; B, formamide-treated PLS b-2; C, formamide-treated PLS b-3; D, formamide-treated PLS b-4; E, formamide-treated PLS b-5; F, untreated PLS b-4.*

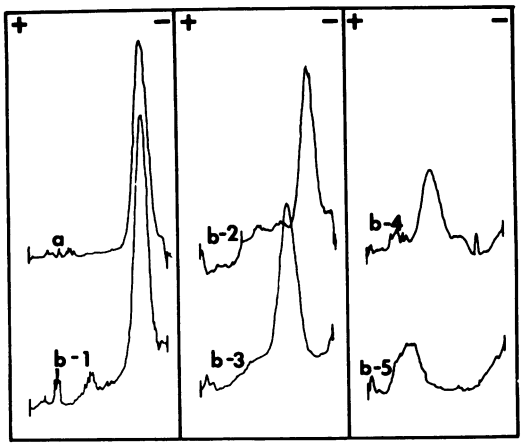

FIG. 6. *Spectrophotometric scan of cell wall polysaccharides in polyacrylamide gels. The gels were scanned as described in Materials and Methods. The direction of the electrophoresis was from the top of the gel shown on the right toward the anode on the left. a, PLS a; b-1, PLS b-1; b-2, PLS b-2; b-3, PLS b-3; b-4, PLS b-4; b-5, PLS b-5. The full-length absorption was 0.5 optical density unit, except for PLS a in which 1 optical density unit was used.*

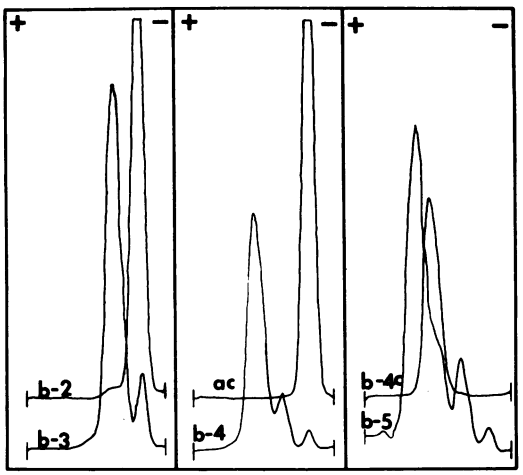

FIG. 8. *Spectrophotometric scan of formamide-treated polysaccharides in polyacrylamide gels. The gels were scanned as was described in Fig. 6. The full-length absorption was 2 optical density units, except 1 optical density unit for PLS b-5. The direction of electrophoresis is the same as Fig. 6. ac, untreated PLS a; b-2, formamide-treated PLS b-2; b-3, formamide-treated PLS b-3; b-4, formamide-treated PLS b-4; b-5, formamide-treated PLS b-5; b-4 c, untreated PLS b-4.*

in solvent A, both acid and alkali hydrolysates yielded a major ^{3}H-labeled compound which had the same mobility as that of authentic α-glycerolphosphate (R_{Glc} 0.42) (Fig. 9C). When the ^{3}H-labeled compound was eluted, treated with alkaline phosphatase, and rechromatographed in solvent A, a ^{3}H-labeled compound with the same mobility as that of authentic glycerol (R_{Glc} 1.41) was released (Fig. 9D). This indicates that the original ^{3}H-labeled compound released by acid and alkaline hydrolysis was α-glycerolphosphate.

To determine whether glucosamine existed in the polysaccharide as *N*-acetylglucosamine, ^{14}C-labeled PLS b-4 (10^5 counts/min) was hydrolyzed with 0.1 N HCl for 60 min at 100 C, dried in vacuo (over NaOH pellets), chromatographed in solvent B, and scanned. A ^{14}C-labeled compound with the same mobility as that of authentic *N*-acetylglucosamine (R_{Glc} 2.2) was detected (Fig. 9E). The compound was eluted from the paper and chromatographed in solvent A. The labeled compound again exhibited the same mobility as that of authentic *N*-acetylglucosamine (R_{Glc} 1.27) (Fig. 9F).

The compositions of the purified polysaccharides are shown in Table 1. It should be pointed out that the glycerol-to-phosphate molar ratio is 1. Also, the molar ratio between the glucosamine and the *N*-acetyl group determination was found to be 1.0:0.91. This result indicates that most glucosamine moieties, if not all, are *N*-acetylated.

Preliminary structural analysis. To determine whether glycerolphosphate was attached to the reducing terminal sugars as was reported by Heymann et al. (14), 0.38 mg each of PLS b-4 and PLS b-5 was hydrolyzed with 1 N KOH as described above. The KOH-treated polysaccharides and 0.38 mg of the corresponding intact polysaccharides were reduced separately at 25 C with 60 μmol of [^{3}H]sodium borohydride (143 mCi/mmol) in a total volume of 200 μliters. Excess sodium [^{3}H]borohydride was destroyed by the addition of acetone, which in turn was removed under a stream of nitrogen in a 37-C water bath. The reaction mixtures were dialyzed against distilled water at 4 C, hydrolyzed with 2 N HCl for 2 h at 100 C, and dried in vacuo (over NaOH and $CaCl_2$ pellets). The hydrolysates were electrophoresed in 0.04 M sodium borate, pH 9.2. A ^{3}H-labeled compound with the same mobility as L-rhamnitol was detected in all samples. This compound was eluted and rechromatographed in solvent A and solvent D. In all these three experiments, the ^{3}H-labeled compound had the same mobility as that of authentic L-rhamnitol (R_{Glc} 1.37, 1.88, and 0.87 in solvents A, D, and 0.04 M sodium borate, respectively). Typical profiles are shown in Fig. 10. These results strongly suggest that rhamnose is the terminal reducing sugar of PLS b-4 and b-5 and that a phosphate moiety does not block the reducing terminal.

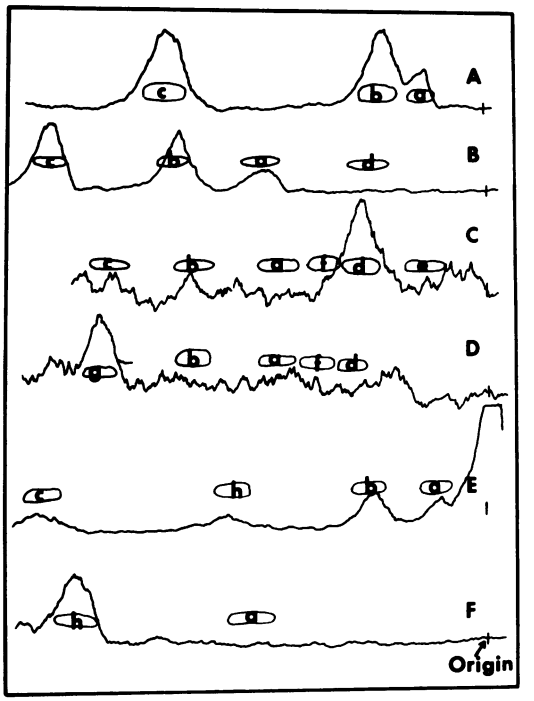

FIG. 9. *Paper chromatography of acid and alkali hydrolysates of purified cell wall polysaccharides. A and B, acid hydrolysate of [^{14}C]polysaccharide developed in solvents B and A, respectively; C, acid hydrolysate of [^{3}H]polysaccharide developed in solvent A; D, eluate of C treated with alkaline phosphatase and developed in solvent A; E, partial acid hydrolysates of [^{14}C]polysaccharide developed in solvent B; F, eluate of E developed in solvent A. a, D-Glucosamine; b, D-glucose; c, L-rhamnose; d, α-glycerolphosphate; e, glycerol 1,2-diphosphate; f, β-glycerolphosphate; g, glycerol; h, N-acetyl D-glucosamine. ^{14}C- and ^{3}H-labeled compounds in the paper chromatograms were located with a Tracer lab radiochromatogram scanner. Lines, radiochromatogram tracings; spot-outlines, locations of reference compounds.*

DISCUSSION

The cell wall polysaccharides extracted from streptococcal walls with 0.5 N HCl (pH 2) at 100 C (19), formamide at 170 C (11) and *Streptomyces albus* enzyme (21) have been found to be composed of rhamnose, glucose, galactose, *N*-acetylglucosamine, and *N*-acetylgalactosamine in various combinations (29). We have fractionated the cell wall polysaccharide(s) of *S. sanguis* into at least four fractions to the purity of 95% or more as judged by polyacrylamide gel

TABLE 1. *Chemical composition of cell wall polysaccharides*

Polysaccharides	Components					
	D-Glucose	L-Rhamnose	D-Glucosamine	Glycerol	Phosphate	*N*-acetyl group
a	1[a]	1.25	0.43	0.023	0.023	0.39
b-1	1	1.26	0.36	0.043	0.045	ND[b]
b-2	1	1.26	0.36	0.047	0.052	ND
b-3	1	1.25	0.31	0.073	0.077	ND
b-4	1	1.22	0.35	0.120	0.123	0.32
b-5	1	1.24	0.30	0.184	0.171	ND

[a] Expressed in molar ratio with glucose as 1.
[b] ND, Not done.

electrophoresis (Fig. 5, 6). Heymann et al. (14) first reported the presence of glycerolphosphate in the streptococcal cell wall polysaccharide. We have subsequently reported the presence of glycerolphosphate in the cell wall polysaccharide of *S. sanguis* (L. I. Emdur, J. G. McHugh, and T. H. Chiu, Abstr. Annu. Meet. Amer. Soc. Microbiol. 1972, G84, p. 44). Karakawa et al. (15, 16, 17) reported that the cell wall polysaccharides of three filamentous streptococci isolated from a human respiratory tract contained phosphate in addition to glucosamine, galactosamine, and glucose, but the presence of glycerol was not reported. Recently Mukasa and Slade (24) and Vaught and Bleiweis (31) reported that both glycerol and phosphate are present in the cell wall polysaccharide of *Streptococcus mutans* but not in equal amount. Our results show that glycerol and phosphate are present in the cell wall polysaccharides isolated from *S. sanguis* in the molar ratio of 1 (Table 1). α-Glycerolphosphate was released upon acid and alkali hydrolysis, suggesting that the glycerol moiety is attached to the polysaccharide via phosphodiester bonds. It is unlikely that the α-glycerolphosphate is due to contamination by teichoic acid in our polysaccharide preparations, since the teichoic acid isolated from *S. sanguis* could not be eluted from DEAE-cellulose columns under the conditions employed in the purification of our polysaccharides (unpublished data). The mobilities of the polysaccharides in polyacrylamide disc gel electrophoresis at pH 8.9 (Fig. 5, 6) are parallel with their phosphate contents (Table 1), suggesting that the phosphate is the sole or major negatively charged moiety in the polysaccharides. When the polysaccharides were hydrolyzed with 2 N HCl, only L-rhamnose, D-glucose, and D-glucosamine were detected chromatographically. Employing milder conditions, using 0.1 N HCl at 100 C for 60 min, *N*-acetylglucosamine was detected chromatographically. The glucosamine-acetyl group ratio was 1.00:0.91, sug-

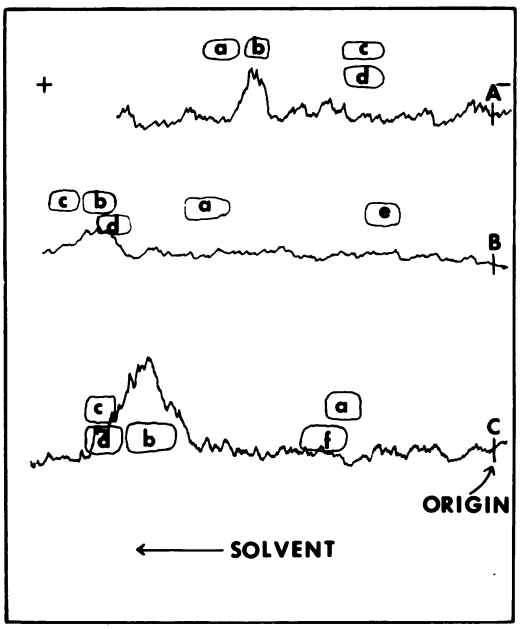

FIG. 10. *Characterization of the reducing terminal sugar. PLS b-5 was reduced with sodium [³H]borohydride, hydrolyzed with HCl, and electrophoresed or chromatographed as described in the text. A, Electrophoresis of the hydrolysate; B, paper chromatography in solvent A; C, paper chromatography in solvent C. a, D-Glucose; b, L-rhamnitol; c, L-rhamnose; d, glycerol; e, D-glucosamine; f, N-acetylglucosamine. Lines and spot outlines were the same as indicated in Fig. 9.*

gesting that most, if not all, of the glucosamine moieties are *N*-acetylated (Table 1).

When KOH-treated and untreated PLS b-4 and b-5 were reduced with sodium [^{3}H]borohydride and hydrolyzed with 2 N HCl, [^{3}H]rhamnitol was detected in all hydrolysates (Fig. 10). This result indicates that in PLS b-4 and b-5 the reducing terminal sugar is L-rhamnose and that glycerol-phosphate does not bind to the reducing group of the rhamnose moiety. This structure is different from that of the C-polysaccharide isolated from *S. pyogenes*, where the reducing terminal is blocked by glycerol-phosphate (14).

It should be pointed out that, although the polysaccharides were fractionated into six different polysaccharides by DEAE-cellulose column chromatography, it is unlikely that they exist as separate polysaccharides in the cell wall. Several lines of evidence suggest that they originated from the same polysaccharide and were produced by breaking phosphodiester bonds during formamide extraction: (i) All purified polysaccharides formed a single band of identity in immune double diffusion against rabbit antiserum (Fig. 4). (ii) All polysaccharides consisted of D-glucose, D-glucosamine, and L-rhamnose in similar proportion (Table 1). (iii) The polysaccharide(s) with lower mobility in the polyacrylamide disc gel electrophoresis can be obtained from the polysaccharide(s) with higher mobility by formamide treatment under the same conditions employed for the polysaccharide extraction (Fig. 7, 8). If it is assumed that a single glycerol-phosphate moiety is present in a chain of PLS a, the number of glycerol-phosphates present per chain in PLS b-1, b-2, b-3, b-4, and b-5 would be approximately 2, 2, 3, 5, and 8, respectively. However, PLS a, b-1, and b-2 are not differentiable by polyacrylamide disc gel electrophoresis. They differ from each other in (i) the glycerolphosphate contents (Table 1) and (ii) the behavior in DEAE-cellulose chromatography (Fig. 2 and 3). Whether they are the same polysaccharide is not clear.

Glycerolphosphate-containing polysaccharides have been previously reported by Coyette and Ghuysen (5) in *Lactobacillus acidophilus* cell walls, by Kennedy et al. (18) in type-specific substance from pneumococcus type II A (43), and by Estrada-Parra et al. (10) in a polysaccharide from type XVIII pneumococcus. Possibly, glycerolphosphate may be present in many other group- and type-specific streptococcal cell wall polysaccharides. The role of the glycerolphosphate in the polysaccharides mentioned above is poorly understood, but four possibilities can be considered: (i) the glycerolphosphate could represent a point of attachment between the polysaccharide and teichoic acid or between two polysaccharide chains; (ii) the glycerolphosphate could represent a point of attachment between the polysaccharide and peptidoglycan as proposed by Heymann et al. (14); (iii) the glycerolphosphate could represent sites of attachment of lipids like fatty acids; or (iv) the glycerolphosphate could just be one of the components of the polysaccharide. Future work will be attempted to differentiate among these possibilities.

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