Carbohydrate Research, 207 (1990) 101–120
Elsevier Science Publishers B.V., Amsterdam

Polysaccharides from *Peptostreptococcus anaerobius* and structure of the species-specific antigen

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(Received November 9th, 1988; accepted for publication, in revised form, March 5th, 1990)

ABSTRACT

The cell-envelope antigens of *Peptostreptococcus anaerobius* were extracted from intact cells by autoclave or alkaline treatment. The purified species-specific antigen (**G**) was identified among several polysaccharides obtained from the extracts by successive treatments with ribonuclease and pronase followed by ion-exchange and gel-filtration chromatography. **G** was investigated by ^{13}C- and ^{31}P-n.m.r. spectroscopy, titrimetry, elemental analysis, and gas-liquid chromatography. Oxidation of **G** with $NaIO_4$ followed by reduction with $NaBH_4$ and mild acid hydrolysis yielded the Smith degradation product of **G** (**GS**). Treatment of **G** and **GS** with 48% HF gave the respective dephosphorylated products **GF** and **GSF**. The structures of **GS**, **GF**, and **GSF** were investigated by ^{13}C-n.m.r. spectroscopy, methylation analysis, and gas-liquid chromatography–mass spectrometry. The principal constituents of **G** were 2-acetamido-2-deoxy-D-glucose (D-GlcNAc), D-glyceric acid, and phosphate as a diester, in the ratio 2:1:1, and a minor amount of D-glucose (β-D-Glc*p*). **GS** contained D-GlcNAc, D-glyceric acid, glycerol, and phosphate in a 1:1:1:1 ratio. **GF** and **GSF** contained D-GlcNAc and D-glyceric acid in the ratios 2:1 and 1:1, respectively. A structure for the principal repeating unit of polymeric **G** compatible with the analytical data consists of α-D-Glc*p*NAc-(1→3)-α-D-Glc*p*NAc-(1→2)-D-glyceric acid units linked through C-6′–C-6″ phosphate diester bridges. This structure is novel for two reasons: (*a*) unsubstituted glyceric acid residues occur as aglycons in the repeating structure, and (*b*) phosphate diester bridges link nonanomeric glycose carbons in a non-nucleic acid polymer. The structural role of the minor amount of β-D-Glc*p* in **G** remains unknown.

INTRODUCTION

The *Peptostreptococcus* genus consists of gram-positive anaerobic cocci comprising the indigenous microbial flora of the human host[1]. The role of *P. anaerobius* in infection has not been completely established, in part because the genus is ill-defined and difficult to differentiate. The situation is complicated by the usual isolation of *P. anaerobius* from mixed infections, but the organism is believed to be involved in a range of infections, some of which may be life threatening[2].

The antigens obtained from members of the genus (*P. anaerobius*, *P. micros*, *P. parvulus*, and *P. productus*) have been reported[3] to be serologically distinct from each

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0008-6215/90/$03.50

other and from the antigens of *Streptococcus* and *Peptococcus*. Bahn *et al.* determined[4] the carbohydrate and amino acid compostion of whole cell walls of different peptostreptococcal isolates, but this attempt to speciate the peptostreptococci was not succesful and it was not pursued in other laboratories. The occurrence of a single, species-specific antigen obtained by the autoclave extraction of intact cells of *P. anaerobius* was reported by Graham and Falkler[5]. The antigen was present in all the isolates of *P. anaerobius* studied, but its presence could not be demonstrated in other species of *Peptococcus*, *Peptostreptococcus*, and *Streptococcus* by serological techniques. These results were confirmed by Wong *et al.*[3] However, neither report[3,5] included the purification or chemical characterization of the serologically active materials. This designation of a species-specific antigen was not in concordance with an earlier report[6] that described strain-specific peptostreptococcal antigens, but few reports have dealt with *P. anaerobius* serology or chemistry. Smith *et al.*[2], without reference to the results of Graham and Falkler[5] or of Wong *et al.*[3], reported the isolation and partial purification of two carbohydrate polymers of the cell envelope of *P. anaerobius* but did not define their chemical structures. Neither polymer described in the latter report corresponded to the serologically active component reported by Graham and Falkler[5].

The present paper describes the isolation and purification of the preponderant cell-envelope polysaccharides and antigens of *P. anaerobius* and presents the structure elucidation of the species-specific antigen originally reported by Graham and Falkler[5]. The structure of the antigen was investigated by ^{13}C- and ^{31}P-n.m.r. spectroscopy, titrimetry, Smith degradation, hydrofluoric acid treatment, g.l.c.–mass spectrometry, and elemental analysis.

EXPERIMENTAL

Analytical methods. — Neutral carbohydrate was determined by the phenol–sulfuric acid method[7], using glucose as standard. Uronic acid analysis employed the *m*-hydroxydiphenyl reaction[8]. Formaldehyde was detected by the chromotropic acid method[9]. The hexosamine composition of the intact polymer was determined by the method of Smith and Gilkerson[10]. The timed release of hexosamine was carried out in 2M trifluoroacetic acid (TFA) at 100°; eight aliquots were taken at 2-h intervals. Free hexosamine was determined in each sample by the method of Elson and Morgan[11], and the hexosamine concentration in the original polymer was estimated by extrapolation of the data to zero time. Protein was estimated by the Coomassie Blue dye-binding assay (Bio-Rad Laboratories)[12]. Total phosphate was determined by the Ames and Dubin modification[13] of the method of Chen *et al.*[14] The ninhydrin degradation for the identification of hexosamines was performed by the method of Stoffyn and Jeanloz[15]. Glyceric acid was determined by the chromotropic acid–sulfuric acid method[16], without previous hydrolysis. The total potential reducing sugar content was determined by the method of Park and Johnson[17] after hydrolysis of the antigen (0.8 mg) with concentrated HCl (1 mL) for 1 h at 100°. The HCl was removed under reduced pressure by perevaporation with absolute ethanol. Elemental analysis was performed by Atlantic

Microlabs Inc., Atlanta, GA. The constituent monosaccharides were identified and quantitated as their per-*O*-acetylated aldononitrile (PAAN) derivatives by gas-liquid chromatography (g.l.c.) after hydrolysis in 2M TFA for 5 h at 120°, followed by nitrous acid deamination[18]. G.l.c. was done with a Perkin–Elmer Sigma 1 gas chromatograph equipped with a flame-ionization detector. The analyzer was fitted with an RSL-300 0.25μm capillary column (30 m × 0.25 mm, Applied Science). Helium was used as the carrier gas. The oven temperature was increased over the ranges and at the rates described in the following programs: (*a*) for PAAN derivatives of neutral and 2,5-anhydrosugars, from 200 to 220° at 7.5 deg.min^{-1}; (*b*) for 1,2,3-tri-*O*-acetylglycerol, from 120 to 220° at 15 deg.min^{-1}; (*c*) for the methylated alditol acetates, from 208 to 230° at 4 deg.min^{-1}; (*d*) for the determination of the configuration of glyceric acid as its (*S*)-*sec*-butyl ester, from 100 to 200° at 2 deg.min^{-1}. G.l.c.–m.s. was done with a separate chromatograph (Perkin–Elmer 8420 Capillary Gas Chromatograph) equipped with a flame-ionization detector, an Ion Trap Detector (Perkin–Elmer GC/ITD), and associated computer software (Perkin–Elmer ITDS for IBM-PC/XT). Separation was accomplished on an SE-54 0.25-μm capillary column (30 m × 0.25 mm, Supelco); using the oven-temperature programs just described. The serological activity of isolated polysaccharides was determined by double immunodiffusion (i.d.) in gels[19]. Specific rabbit antiserum against *P. anaerobius* was a generous gift from Dr. W. A. Falkler, Jr., University of Maryland, Baltimore, MD.

Methylation analysis. — Per-*O*-methylation was done by the method of Hakomori[20] as modified by Lindberg and co-workers[21]. Briefly, 1 mL of Me_2SO was added to 5 mg of sample (previously dried over P_2O_5 at 78° in vacuo), and the vial was then placed in a sonic bath for 30 min or until the sample dissolved. Sodium methylsulfinylmethanide (0.5 mL, 2M, in Me_2SO) was added, and the reaction vessel was kept for 3.5 h at 23° with occasional stirring and sonication. The sample was cooled in an ice bath, methyl iodide (0.4 mL) was added, the sample was sonicated for 1 h, and finally flushed with N_2. Water (5 mL) was added and the methylated polysaccharide was purified on a Sep-Pak C_{18} cartridge[22]. The eluates containing the per-*O*-methylated products were evaporated, hydrolyzed, and derivatized as described[23] by Stellner *et al.* The alditol acetate derivatives of the methylated sugars were characterized by g.l.c.–m.s. using program *c*.

N.m.r. spectroscopy. — Spectra were recorded with a JEOL-GX270 n.m.r. spectrometer equipped with a 5-mm ^1H and ^{13}C dual probe or a 10-mm multinuclear probe, with the deuterium resonance of the solvent serving as an internal lock.

(*a*) *^{13}C-N.m.r. spectra.* The typical solution contained 50 mg of sample in 0.5 mL of D_2O (99.8%, Aldrich Chemical Co.), and adjustments in pD were made with 0.5M KOH when required. Chemical shifts were measured with a digital resolution of 0.0036 p.p.m. (0.24 Hz) relative to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) taken as zero. The spectra were recorded at 70° with a 45-degree pulse repeated at 0.7-s intervals, were 16.025 kHz wide, and were collected with 32k data points; other details were as previously described[24].

(*b*) *^{31}P-N.m.r. spectra.* The typical solution contained 35 mg of sample in 2 mL of 99.8% D_2O in a 10-mm tube, with a concentric inner capillary containing 25% H_3PO_4 as

an external reference, measured at 0.45 p.p.m. relative to external 85% H_3PO_4. The pD was adjusted by adding appropriate increments of 0.5M KOH or 0.5M HCl solutions. The spectra were recorded at 35° with a spectrometer frequency of 108.95 MHz. The spectra were 20 kHz wide and were collected with 16k data points, by use of a 52-degree pulse repeated at 2.4-s intervals, with bilevel proton decoupling (JEOL GX-270 EX-MOD, SGBCM). Fast Fourier transformations were done with a 2.5-Hz broadening factor and zero filling to 64k data points, which provided a digital resolution of 0.0056 p.p.m. (0.61 Hz).

Native serologically-active polysaccharides, antigens, and related derivatives. — *P. anaerobius* (ATCC 27737) was grown in Sterility Test Broth (Baltimore Biological Laboratory) in 100-L batches (Fermenter Model IF-130, New Brunswick Scientific). A 2-L seed culture was used to inoculate the fermenter and N_2 was used to maintain anaerobic conditions. The medium was kept at 37° and the initial pH was 7. Late log phase cells were harvested by continuous flow centrifugation at 4° (Model Z41, Carl Padberg GMBH, Germany).

(*a*) *Polysaccharides from an autoclave extract* (**PA**). The cells were suspended in water (100 g per L) and heated in an autoclave for 35 min at 121°. The cell suspension was centrifuged (20 min, 9000*g*), and the supernatant was exhaustively dialyzed against distilled water. Fraction **PA** was recovered by lyophilization (yield 3.3% based on the wet weight of the whole cells).

(*b*) *Polysaccharides from an aqueous base extract* (**PB**). The residue from the autoclave extraction was suspended in 0.5M NaOH–0.1M $NaBH_4$, then stirred for 4 h at 23°. The suspension was kept overnight at 4°, HCl was added to adjust the pH to 7, and finally the suspension was centrifuged (30 min, 9000*g*). The residue was reserved, and the supernatant solution was dialyzed exhaustively against distilled water, then lyophilized to give **PB** (yield 7.0% based on the wet weight of the whole cells).

(*c*) *Ribonuclease digestion.* **PA** was dissolved in 0.01M 2-amino-2-hydroxymethyl-1,3-propanediol (Tris)–0.1M NaCl, buffer, pH 7.5 (1 g per 50 mL). Ribonuclease A (EC 3.1.27.5, Sigma R-4875, 1 mg per 20 mL of the digestion solution) was added and the mixture was incubated for 20 h at 23°, with dialysis against several changes of Tris buffer. After this, more ribonuclease A (0.5 mg per 20 mL) was added and the dialysis was continued until no further increase in the absorbance (260 nm) of the dialyzate occurred.

(*d*) *Deproteinized polysaccharides from the autoclave* (**A**) *and base* (**B**) *extracts; pronase digestion.* The dialyzed ribonuclease-treated **PA** solution was adjusted to 0.15M Tris and 0.15M $CaCl_2$ by adding the solid reagents, and then the pH was adjusted to 7.8. Pronase E from *Streptomyces griseus* (Sigma P-5147, 1 mg per 20 mL of digestion mixture) was added, followed by toluene to form a continuous layer on the surface of the solution. The mixture was incubated for 24 h at 37°, dialyzed, centrifuged (20 min, 9000*g*), and lyophilized to give **A** (yield 21.0% based on **PA**). **PB** was dissolved in 0.15M Tris–0.15M $CaCl_2$ buffer, pH 7.8, and the solution was digested with pronase E under toluene, dialyzed, centrifuged, and lyophilized to give **B** (yield 16% based on **PB**).

(*e*) *Ion-exchange chromatography.* **A** or **B** was dissolved in 0.01M potassium

phosphate buffer, pH 7.8 (1 g per 60 mL of buffer), and then applied to a column (38 × 4 cm) of DEAE-Sephadex A-25 (Pharmacia) equilibrated with the same buffer. The sample was eluted with a linear gradient of 0–1M NaCl in 0.01M potassium phosphate, pH 7.8, 3 L total volume, at a flow rate of 60 $mL.h^{-1}$. The eluate was monitored continuously at 206 nm, and fractions were collected at 18 min intervals and analyzed for hexosamine[10], neutral carbohydrate[7], and serological activity by i.d.[19] (see Figs. 1 and 2). Appropriate column fractions were pooled, dialyzed, and lyophilized. The major fractions from the chromatography of **A** and **B** that were serologically active were designated as **A-A2** and **B-A4** respectively.

(*f*) *Purified species-specific antigen* (**G**); *gel-filtration chromatography*. A column (87 × 2.6 cm) of Sephacryl S-200 (Pharmacia) equilibrated with 0.05M Tris–0.1M NaCl buffer, pH 7.6, was calibrated with the standard proteins ribonuclease (mol. wt. 13 700), chymotrypsinogen A (25 000), and ovine albumin (45 000). **B-A4** (100 mg) was dissolved in the equilibration buffer (5 mL), applied to the column, and eluted at a flow rate of 54 $mL.h^{-1}$. The eluate was monitored at 206 nm. Fractions were analyzed as described in the previous paragraph (*e*). Serologically active fractions were combined, dialyzed exhaustively, and lyophilized (**G**, yield 74%).

(*g*) *The oxidized derivatives of* **G** (**GPS** *and* **GS**); *Smith degradation*[25]. **G** in freshly prepared 0.04M $NaIO_4$ (7 mg per mL) was maintained for 96 h at 4° in the dark. 1,2-Ethanediol was then added and the reaction flask was stored for 1 h at 23°. The resulting polyaldehyde was reduced with $NaBH_4$ (40 mg per mg of **G**) overnight at 4°. The pH was adjusted to 5 by the addition of 2M acetic acid, and the resulting polyalcohol (**GPS**) was concentrated, dialyzed, and lyophilized (yield 75%). **GPS** was hydrolyzed for 24 h at 37° in aqueous 0.15M trifluoroacetic acid (TFA), since the conditions usually used for the acid catalyzed hydrolysis of non-hexosamine-containing polyalcohols (0.1M HCl, 37°, 16 h)[25] did not completely cleave the acyclic acetals derived from GlcNAc. TFA was removed by three ether extractions[26], and the solution was dialyzed (Spectrapore 6 dialysis tubing, mol.-wt. cutoff 1000). The retentate (26%) and the dialyzate (**GS**, 69%) were recovered by lyophilization.

(*h*) *Dephosphorylated derivatives* (**GF** *and* **GSF**); *hydrofluoric acid treatment*[27]. **GS** (28.7 mg) was dissolved in 48% HF (3 mL) and stirred overnight at 4°. The reaction was quenched by adding cold water and ether. The bulk of the HF was removed by four extractions with cold ether and the aqueous phase was then neutralized with solid calcium hydroxide (100 mg). The precipitate was removed by centrifugation, and the aqueous phase was flushed with N_2 and lyophilized to give a mixture of **GSF** and glycerol (combined mass 21.7 mg) that was used without purification. **G** was treated similarly, except the ether extraction was omitted, excess $Ca(OH)_2$ was employed to remove residual HF, and the dephosphorylated derivative **GF** was recovered by dialysis (mol.-wt. cutoff 1000, yield 56%).

Mild acid hydrolysis. — **G** was dissolved in 0.01M TFA (1 mg per mL) and heated at 85°. Aliquots were taken at timed intervals for the determination of reducing sugar[17].

Alkaline phosphatase treatment. — **G** (0.5 mg) in 0.1M HCl (2 mL) was heated for 15 min at 100°. The HCl was removed by perevaporation with ethanol. The partially

hydrolyzed antigen was dissolved in 0.1M glycine buffer, pH 10.5 (1 mL); alkaline phosphatase (EC 3.1.3.1, Sigma P-4252, 10 μL containing 180 μg of protein) was added, and the reaction mixture was incubated for 24 h at 37°. The release of inorganic phosphate[13] was used to follow the course of the reaction.

Equivalent weight of **G** *by potentiometric titration.* — The titration was conducted as described previously[24], in a special 2-mL cell that was designed to minimize the exposure of the sample to the atmosphere. **G** was dissolved in 2 mL of 0.2M KCl and titrated with 0.5016M KOH in CO_2-free water at 25°.

Determination of the configuration of the glyceric acid. — **G** (8 mg) in 2M TFA (1 mL) was heated for 5 h at 120°. The TFA was removed by evaporation *in vacuo*, and the residue was dissolved in water (1 mL). The hydrolyzate was applied to a column (4 × 0.5 cm) of Dowex-50 X2-200 (H^+) ion-exchange resin, and the column was washed with water (5 mL). The effluent and washings were then applied to a column (4 × 0.5 cm) of Dowex-1 X8-400 (acetate) ion-exchange resin, and the column was washed with water (10 mL). The glyceric acid was eluted from the column with M acetic acid (5 mL), the eluate was evaporated to dryness, and the residue was dried over H_2SO_4 *in vacuo*. The dried residue was treated with (*S*)-2-butanol (200 μL; Aldrich Chemical Co., 23769-8) and TFA (10 μL) for 16 h at 100°, and the mixture was evaporated to dryness. Pyridine–acetic anhydride (1:1, 500 μL) was added to the sample, which was then heated for 1 h at 100°. Chloroform was added and the sample was prepared for g.l.c. analysis as described for the PAAN derivatives of sugars[18]. The derivative was dissolved in a minimum amount of $CHCl_3$ and analyzed by g.l.c. (program *d*). Authentic standards were prepared from D- and L-glyceric acid in a similar manner. The retention times of the (*S*)-*sec*-butyl D-glycerate and (*S*)-*sec*-butyl L-glycerate acetates were 17.58 and 17.80 min, respectively.

RESULTS

Isolation and purification of the polysaccharides and antigens. — Table I summarizes the serological activity, yield, and composition of the separated fractions. The total wet weight of cells recovered from four 100-L batches of culture was 709 g. These cells gave two serologically active fractions: **PA**, from heating whole cells in an autoclave, and **PB**, from treatment of the cell residue with base. Nucleic acid and protein constituents were removed from the extracts by enzymatic treatment; ribonuclease and pronase gave **A** from **PA** and pronase gave **B** from **PB**.

Figures 1 and 2 show the results of the fractionation of **A** and **B** respectively by ion-exchange chromatography. The initital fractions were rich in both hexosamines and neutral sugars but they were serologically inactive. These carbohydrate-rich fractions are indicated in Figs. 1 and 2 and in Table I as **A-C1–4** and **B-C1**. Several serologically active fractions, eluted later, are designated as **A–A1,2** and **B-A1–4**. Chromatographic fractions that exhibited serological activity contained only small amounts of neutral sugars but remained rich in hexosamines. Of these fractions, **A-A2** and **B-A4** were eluted from the ion-exchange columns under similar conditions and had similar compositions,

TABLE I

Serological activity, yield, and composition of fractions isolated from *Peptostreptococcus anaerobius* cells

Fraction[a]	*Activity*[b]	*Yield (%)*[c]	*Composition (% by weight)*			
			Hexosamine[d]	*Phosphate*[e]	*Neutral sugar*[f]	*Protein*[g]
PA	+	3	10	16.0	14	6
A	+	21	23	7.5	22	5
A-C1	−	3	26	7.0	32	tr.[h]
A-C2	−	8	25	6.9	28	tr.
A-C3	−	5				
A-C4	−	14	40	6.3	25	tr.
A-A1	+					
A-A2	+	5	18	16.3	4	tr.
PB	+	7	6	4.2	2	71
B	+	16	11	1.4	6	10
B-C1	−	1	30	5.7	30	tr.
B-A1	+	1	13	8.2	4	tr.
B-A2	+	2	25	9.9	5	tr.
B-A3	+	1	13	9.5	3	tr.
B-A4	+	20	18	13.6	3	tr.
G	+	74	23	14.0	3	tr.

[a] See Figs. 1 and 2 for identification of fractions. [b] Serological activity was determined by double immunodiffusion in agarose gels. [c] By weight, based on the final step in the isolation of the fraction. [d] Determined by the Smith–Gilkerson method[10]. [e] Expressed as PO_4. [f] Determined by the phenol–sulfuric acid method[7]. [g] Determined by a Coomassie Blue dye-binding assay[12]. [h] Trace.

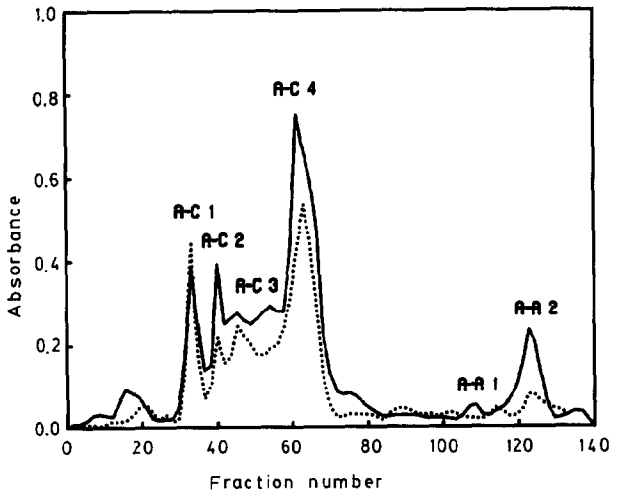


Fig. 1. Ion-exchange chromatography (DEAE-Sephadex A-25) of the antigen, **A**, from the autoclave extract. Absorbance measurements obtained at 650 nm (—) and 490 nm (...). Fractions **A-C1** through **A-C4** were carbohydrate-rich but serologically inactive while fractions **A-A1** and **A-A2** were serologically active.

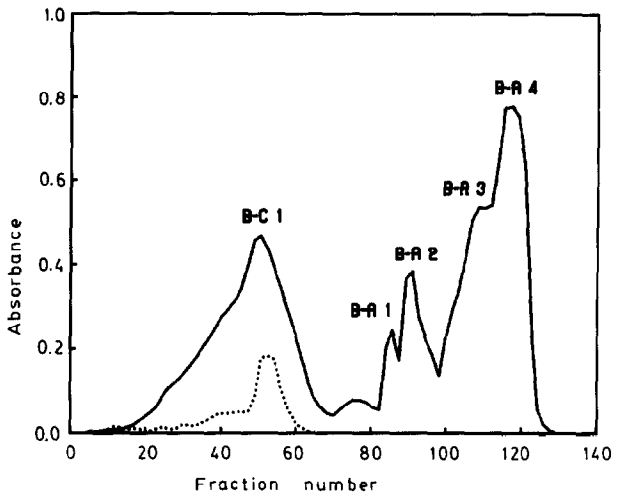


Fig. 2. Ion-exchange chromatography (DEAE-Sephadex A-25) of the antigen, **B**, from the base extract. Absorbance measurements were obtained at 650 nm (—) and 490 nm (...). Fraction **B-C1** was carbohydrate-rich but serologically inactive while fractions **B-A1** through **B-A4** were serologically active.

but **B-A4** was obtained in greatest yield (see Table I). For this reason, **B-A4** was chosen for further fractionation by gel-filtration chromatography. The remaining fractions as well as the final cell residue were reserved for future study.

Gel-filtration chromatography of **B-A4** gave the species-specific antigen (**G**) in one symmetrical peak at an elution volume corresponding to an apparent mol. wt. of 30 000. Increased serological activity accompanied the isolation and purification of this fraction. Enzymatic digestion improved the specific activity of both **A** and **B** (94 μg per mL) compared to that of their precursors **PA** and **PB** (960 μg per mL). **G** had greater serological activity (10 μg per mL) than any of its precursors, and it was serologically identical to the species-specific antigen described by Graham and Falkler[5]. When compared to each other in immunodiffusion, G and all of the serologically active fractions from ion-exchange chromatography gave immunoprecipitin lines of identity.

Chemical analysis of **G**. — **G** was composed of 23% hexosamine, 14% phosphate, 3% neutral sugar, and a trace of protein (see Table I). Measurement of the hexosamine content by a timed-release experiment and by an extended hydrolysis gave virtually identical results. Sixty eight percent of **G** was released as reducing sugars (expressed as glucose) by total acid hydrolysis. Analysis by g.l.c., which required total hydrolysis, showed the presence of only 17% 2-amino-2-deoxyglucose (glucosamine), and 2% glucose. Ninhydrin degradation of the isolated hexosamine component of **G** gave arabinose as the sole product, thus confirming the presence of glucosamine. Amino acid analysis of **G** gave 0.3% peptide and further confirmed the presence of glucosamine; no other nitrogen containing compounds were detected. No uronic acid was detected. No formaldehyde was released by $NaIO_4$ treatment of **G**. The only other component identified was D-glyceric acid. The occurrence of glyceric acid was confirmed by reduction of the carboxylic acid groups of **G**, acid hydrolysis, and identifcation of the

derived glycerol as its triacetate ester by g.l.c.–m.s. Without prior reduction, **G** gave no low mol. wt. volatile compounds (for **G**, the experimental molar ratio of glyceric acid to phosphorus was 0.8:1, based on 11.3% glyceric acid found). G.l.c. of the acetylated (*S*)-*sec*-butyl ester of the glyceric acid derived from **G** showed that it belonged to the D-series by comparison of its retention time with those of samples prepared from the authentic D- and L-enantiomers. Both independent injections and coinjection of the three esters led to the same conclusion.

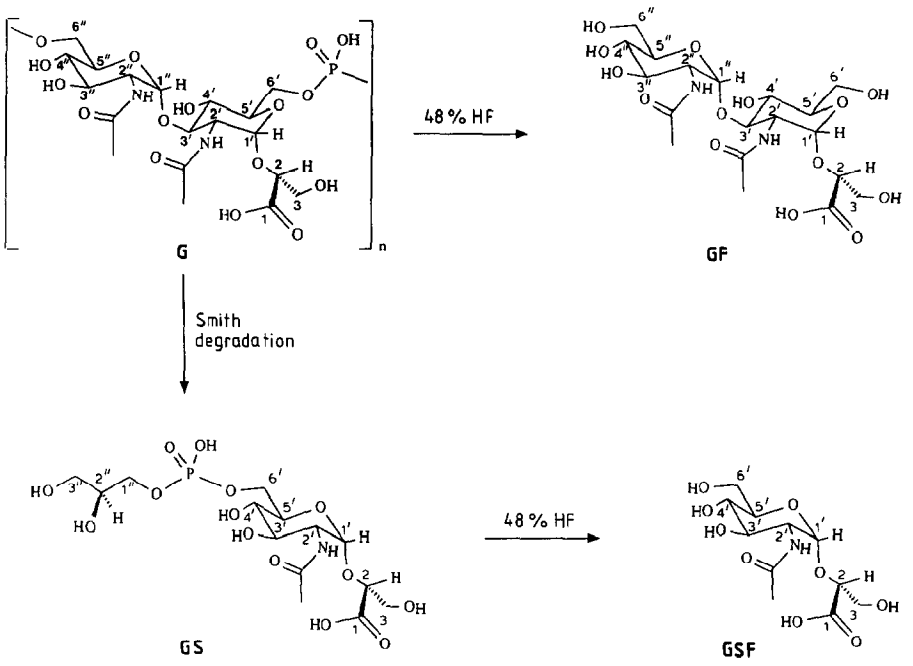
Titration of **G**. — The titration of the free acid form of **G** with KOH indicated the presence of two types of acidic functions. The two pK_a values estimated from the titration data were approximately 2.2 and 3.8. The apparent formula weight of **G** was 678, calculated on the basis of its titration to pH 8 by two equivalents of KOH. The experimental value for the apprent formula weight decreased to 644 with more stringent drying of the sample prior to titration.

Elemental analysis of **G**. — The analyst was instructed to dry the sample extensively *in vacuo* at 78°. The results reported were 38.52% C, 5.95% H, and 4.64% N.

Smith degradation of **G**. — Smith–Gilkerson analysis showed that the hexosamine content of **GPS** was reduced to 11% from 23% originally present in **G**. Further treatment of **GPS** by mild acid hydrolysis (TFA) gave **GS**, which on dialysis appeared in both the dialyzate and retentate. The dialyzate and the retentate gave identical ^{13}C-n.m.r. spectra, and neither fraction gave a positive test for reducing sugar, even though **GS** is evidently of low molecular weight (see Scheme 1).

Removal of phosphorus from **G** *and* **GS**. — The hydrolysis of **G** by mild acid treatment, under conditions that preferentially cleave glycosyl-phosphate linkages, produced no new reducing end groups. Further treatment of the hydrolyzate with alkaline phosphatase also produced no reducing end groups, or inorganic phosphate. Treatment[27] with 48% HF gave a phosphate-free product (**GF**), having no new reducing end groups, but of drastically reduced mol. wt. by comparison to **G** (slow diffusion of **GF** through a dialysis membrane having mol.-wt. cutoff 1000 was observed). **GF** had no serological activity in i.d. An analogous treatment of **GS** with HF gave another phosphate-free derivative (**GSF**, Scheme 1).

Methylation analysis of **GF** *and* **GS**. — G.l.c. of the alditol acetates derived from methylated **GF** gave four main peaks. The retention times, relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, and the relative quantities of these peaks, identified by m.s. and designated according to the parent methylated sugar, were as follows: (*a*) 2-amino-2-deoxy-3,4,6-tri-*O*-methylglucose, 1.64 min, 34.5%; (*b*) 2-deoxy-3,4,6-tri-*O*-methyl-2-methylaminoglucose, 1.90 min, 20.8%; (*c*) 2-amino-2-deoxy-4,6-di-*O*-methylglucose, 2.09 min, 36.3%; and (*d*) 2-deoxy-4,6-di-*O*-methyl-2-methylaminoglucose, 2.46 min, 8.44%. Two minor peaks were tentatively identified as the alditol acetates of 2,3,4,6-tetra-*O*-methylglucose and 3,4,6-tri-*O*-methylglucose. Similar analysis of **GS** gave only the two peaks corresponding to *a* and *b*, showing that the phosphate was probably removed in the strongly alkaline methylation medium, thus allowing the methylation of O-6′. Although *N*-methylation of all of the GlcNAc residues was expected[23], less than half of the glucosamine was detected as *N*-methylated derivatives, a



Scheme 1.

methylation of O-6′. Although *N*-methylation of all of the GlcNAc residues was expected[23], less than half of the glucosamine was detected as *N*-methylated derivatives, a finding similar to that of Dengler *et al.*[28]. Whatever the cause of the incomplete *N*-methylation, a pair of *N*-methylated and a pair of non-*N*-methylated derivatives were identified, and the data support the the conclusion that equal amounts of two types of GlcNAc residues are present in **GF**.

^{31}*P-N.m.r. spectroscopy.* — ^{31}P-N.m.r. spectra of **G** were determined at 6 pD values ranging from 1.66 to 12.11, and the spectra of **GPS** and **GS** were determined at pD 3.42 and 2.55, respectively. A small displacement of the ^{31}P chemical shift (<0.40 p.p.m.) of **G** was observed as the pD was raised from its lower to its upper limit. This observation is consistent with the presence in **G** of a phosphate diester, because a larger displacement of the chemical shift (~4 p.p.m.) with change in pD would be expected if a phosphate monoester were present[29]. The spectra showed the presence of phosphorus in **GPS** and **GS** with chemical shifts similar to that observed in **G**. In contrast, **GF** exhibited no ^{31}P-n.m.r. signal. The observed chemical shifts in p.p.m. relative to 25% phosphoric acid (pD values in parentheses) were: for **G**, 1.14 (1.66), 1.31 (3.18), 1.38 (3.47), 1.49 (5.51), 1.51 (9.01), 1.50 (12.11); for **GPS**, 1.02 (3.42); for **GS**, 1.04 (2.55).

^{13}*C-N.m.r. spectroscopy.* — The spectra for **GSF**, **GS**, **GF**, and **G** were each determined at an acidic and a basic or neutral pD value. The tentatively assigned chemical shifts are given in Table II, and six partial spectra are illustrated in Fig. 3. In the region where acetyl-methyl carbon atoms absorb (~25 p.p.m., not shown in Fig. 3) a single resonance occurred in the spectra of **GSF**, while two such signals of approximately equal intensity occurred in the spectra of both **GF** and **G**. In the range where C=O

carbon atoms resonate, signals of relatively low intensity occurred in all of the spectra. Two C=O signals were observed in the spectra of **GSF** while three each occurred in those of **GF** and **G** (see insert for **G**, Fig. 3). For all three compounds, some of the signals were sensitive to changes in pD. Most notable were three signals that increased in chemical shift at higher pD values. One C=O signal changed from ~177 to ~179 p.p.m. A second signal changed from ~79.5 to ~82 p.p.m. (see Fig. 3), and a third shifted downfield by a small increment to ~66 p.p.m. at the higher values of pD. In the spectra of **G**, two signals that were pD sensitive were observed at ~63 p.p.m. These

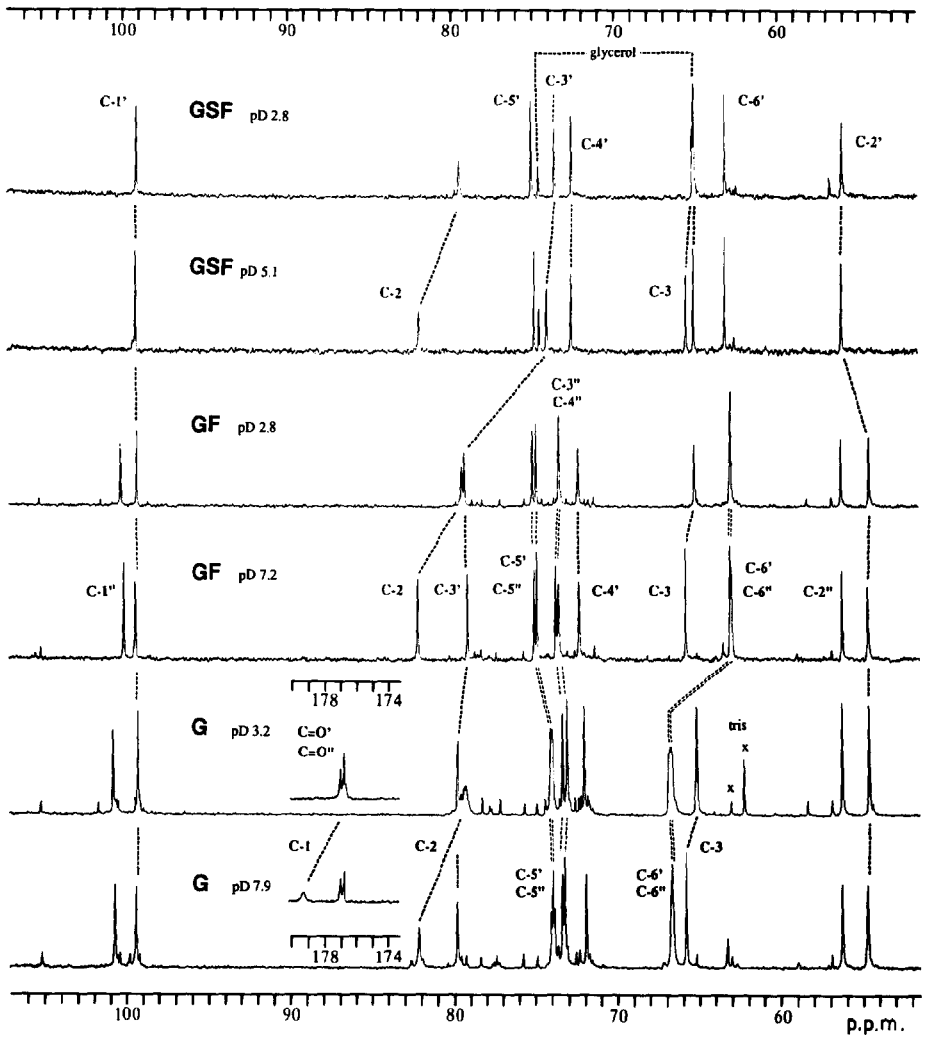


Fig. 3. Proton-decoupled ^{13}C-n.m.r. spectra of the gel-filtered antigen, **G**, and the chemical derivatives **GF** and **GSF** in acidic and neutralized D_2O solutions.

coincided with separately determined, pD-sensitive signals of Tris and thus resulted from a small residue of Tris buffer in this sample of **G**.

Several other signals of minor intensity were observed in the spectra of **GF** and **G**. One of these signals (56.9 p.p.m.) is due to the internal reference, DSS (~5% by wt.) and can be seen in most of the spectra. The other minor signals are undoubtedly due in part to to a small amount of glucose that was bonded to **G** and thus not removed by the purification steps. These signals are not specifically assigned here, but from the spectrum and the g.l.c.–m.s. results, they may have their origin in isolated branches containing the Glc-(1→2)-Glc-(1→?) disaccharide unit, with one of the residues in the β-anomeric configuration (~106 p.p.m.). Glucose was shown to be present by analysis (see Table I). Most of the minor signals vanished following the Smith degradation of **G**, and so were not present in the spectra of either **GS** or **GSF**.

In addition to the ^{13}C chemical shifts, selected coupling constants and chemical-shift differences for the major signals of **G**, its derivatives, and related compounds are given in Table II. Three categories of chemical shift differences ($\Delta\delta$) are tabulated. The first is differences in the δ_C values for a given glycosyl group resulting from a change in the attached aglycon. For example, for α-D-Glc*p* the change from methyl (compound **1**) to a glyceric acid residue (**2**) causes an upfield shift of 1.7 p.p.m. in $\delta_{C\text{-}1}$. A second category is the differences resulting from phosphate-ester substitution or *O*-glycosylation. Thus substitution at O-6′ of **GSF** by a glycerol phosphate group, to give **GS**, causes the signal for C-6′ to shift 3.7 p.p.m. downfield. Finally, shifts in the spectra of specific compounds resulting from changes in pD are listed.

The ^{13}C chemical shifts of **GSF** were assigned by comparison with related values reported by Hunter and co-workers[30]. Table II gives their results for methyl α-D-glucopyranoside (**1**) and for the glucose analog of **GSF**, *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid (**2**), with our reversal of their tentative assignments for C-2′ and C-5′ of **2**. Table II also includes our measured data for glycerol (**3**), DL-glyceric acid calcium salt hydrate (the "hemicalcium salt" of glyceric acid, **4**), and methyl 2-acetamido-2-deoxy-α-D-glucopyranoside (**5**). The two resonances for **3** were also observed as pD-insensitive signals in the spectrum of **GSF** (see Fig. 3). The hydrolysis of **GS** to produce **GSF** (see Scheme 1) was accompanied by formation of **3**, which was not removed before measurement of the spectrum. Except for the signals for **3**, the resonances in the spectra of **GSF** from 56 to 76 p.p.m. were assigned (see Table II) by comparison to those of **5**, and by similarity between the chemical-shift differences due to substituent effects and to changes in pD. For **GSF**, there is no question about the assignment of the signal of C-2′ and little question about that of C-5′. The previous assignments[30] were tentative. On reversing the literature assignments for these two carbon atoms, as has been done in Table II, their [$\Delta\delta$] values become very similar to those for C-2′ and C-5′, respectively, of **GSF**. The [$\Delta\delta$] values for the carbon atoms of the glyceric acid residue in **GSF** indicate that this residue is *O*-2-substituted. The $<\Delta\delta>$ values (induced by changes in pD) for the glyceric acid carbons in **2** and **GSF** are characteristically large[31], but relatively small for most of the carbon atoms of the sugar residue. Noteworthy, however, is the large value of $<\Delta\delta>$ for C-3′ of the three compounds, **2**, **GSF**, and **GS**; these compounds

TABLE II

^{13}C-N.m.r. data for the species-specific antigen of *Peptostreptococcus anaerobius* and related compounds

Compound, shift difference[b]	*pD*	*Chemical shifts (p.p.m.)*[a]; $^{1}J_{C,H}$ *values (Hz), in ( ); and chemical-shift differences (p.p.m.), in [ ] or < >*									
		C-1	*C-2*	*C-3*	*C-1'*	*C-2'*	*C-3'*	*C-4'*	*C-5'*	*C-6'*	*CH$_3$*	*C=O'*
1 (α-D-Glc*p*-OCH$_3$)					100.7	73.0	74.7	71.1	72.7	62.1		
2 [α-D-Glc*p*)-(1→2)-D-glycerate]	3.0[c]		175.5	77.6	64.1	99.0	72.9[e]	74.4	70.9	73.8[e]	62.0	
	8.5[d]	178.5	80.6	64.7	99.0	73.2[f]	74.9	71.0	73.7[f]	62.1		
$\delta_2 - \delta_1$	3.0				[−1.7]	[−0.1]	[−0.3]	[−0.2]	[1.1]	[−0.1]		
$\delta_2 - \delta_1$	8.5				[−1.7]	[0.2]	[0.2]	[−0.1]	[1.0]	[0.0]		
$\delta_{8.5} - \delta_{3.0}$		<3.0>	<3.0>	<0.6>	<0.0>	<0.3>	<0.5>	<0.1>	<−0.1>	<0.1>		
3 (Glycerol)		65.3	74.7	65.3								
4 (Calcium DL-glycerate hydrate)	6.6	181.3	76.2	66.7								
5 (α-D-Glc*p*NAc-OCH$_3$)	8.8	57.8			100.7	56.3	73.9	72.8	74.3	63.4	24.5	176.9
		(144)			(173)	(142)	(148)	(147)	(146)	(145)	(129)	
GSF	2.8	177.0	79.6	65.3	99.3	56.2	73.8	72.8	75.2	63.4	24.7	177.2
	5.0	179.2	82.1	65.8	99.5	56.3	74.3	72.8	75.1	63.4	24.8	177.3
			(144)	(145)	(172)	(141)	(146)	(144)	(144)	(143)	(129)	
$\delta_{GSF} - \delta_5$	2.8				[−1.4]	[−0.1]	[0.1]	[0.0]	[0.9]	[0.0]	[0.2]	[0.3]
$\delta_{GSF} - \delta_4$	5.0	[−2.1]	[5.9]	[−0.9]								
$\delta_{GSF} - \delta_5$					[−1.2]	[0.0]	[0.5]	[0.0]	[0.8]	[0.1]	[0.3]	[0.4]
$\delta_{5.0} - \delta_{2.8}$		<2.2>	<2.5>	<0.5>	<0.2>	<0.1>	<0.5>	<0.0>	<−0.1>	<0.0>	<0.1>	<0.1>
		C-1	*C-2*	*C-3*	*C-1'*	*C-2'*	*C-3'*	*C-4'*	*C-5'*	*C-6'*	*CH$_3$*	*C=O'*
GS	2.6	176.5	79.3	65.2	99.4	56.2	73.6	72.4	74.2	67.1	24.7	177.2
	9.2	179.4	82.6	65.9	99.7	56.3	74.3	72.4	74.0	67.0	24.8	177.2
$\delta_{GS} - \delta_{GSF}$	2.6	[−0.5]	[−0.3]	[−0.1]	[0.1]	[0.0]	[−0.2]	[−0.4]	[−01.0]	[3.7]	[0.0]	[0.0]
$\delta_{GS} - \delta_{GSF}$	9.2	[0.2]	[0.5]	[0.1]	[0.2]	[0.0]	[0.0]	[−0.4]	[−1.1]	[3.6]	[0.0]	[−0.1]
$\delta_{9.2} - \delta_{2.6}$		<2.9>	<3.3>	<0.7>	<0.3>	<0.1>	<0.7>	<0.0>	<−0.2>	<−0.1>	<0.1>	<0.0>

								C-3″	*C-2″*	*C-1″*		
GS *(cont'd)*	2.6							65.0	73.5	69.2		
	9.2							65.0	73.5	69.2		
$\delta_{GS} - \delta_3$	2.6							[−0.3]	[−1.2]	[3.9]		
$\delta_{GS} - \delta_3$	9.2							[−0.3]	[−1.2]	[3.9]		
$\delta_{9.2} - \delta_{2.6}$								<0.0>	<0.0>	<0.0>		
		C-1	*C-2*	*C-3*	*C-1′*	*C-2′*	*C-3′*	*C-4′*	*C-5′*	*C-6′*	CH_3'	C=O′
GF	2.8	177.0	79.5	65.3	99.4	54.7	79.4	72.4[g]	75.0[h]	63.1	24.9	176.8
	7.3	179.3	82.3	65.9	99.5	54.8	79.2	72.4[i]	74.9[j]	63.1	25.0	176.8
			(144)	(143)	(173)	(141)	(144)		(145)	(142)	(144)	(128)
$\delta_{GF} - \delta_{GSF}$	2.8	[0.0]	[−0.1]	[0.0]	[0.1]	[−1.5]	[5.6]	[−0.4]	[−0.2]	[−0.3]	[0.2]	[−0.4]
$\delta_{GF} - \delta_{GSF}$	7.3	[0.1]	[0.2]	[0.1]	[0.0]	[−1.5]	[4.9]	[−0.4]	[−0.2]	[−0.3]	[0.2]	[−0.5]
$\delta_{7.3} - \delta_{2.8}$		<2.3>	<2.8>	<0.6>	<0.1>	<0.1>	<−0.2>	<0.0>	<−0.1>	<0.0>	<0.0>	<0.0>
					C-1″	*C-2″*	*C-3″*	*C-4″*	*C-5″*	*C-6″*	CH_3''	C=O″
GF *(cont'd)*	2.8				100.3	56.4	73.6	73.6[g]	75.2[h]	63.1	24.7	176.9
	7.3				100.2	56.3	73.8[k]	73.6[i,k]	75.1[j]	63.1	24.7	176.9
					(174)	(135)	(142)	(139)	(142)	(144)	(128)	
$\delta_{GF} - \delta_5$	2.8				[−0.4]	[0.1]	[−0.3]	[0.8]	[0.9]	[−0.3]	[0.2]	[0.0]
$\delta_{GF} - \delta_5$	7.3				[−0.5]	[0.0]	[−0.0]	[0.8]	[0.8]	[−0.3]	[0.2]	[0.0]
$\delta_{7.3} - \delta_{2.8}$					<−0.1>	<−0.1>	<0.2>	<0.0>	<−0.1>	<0.0>	<0.0>	<0.0>
		C-1	*C-2*	*C-3*	*C-1′*	*C-2′*	*C-3′*	*C-4′*	*C-5′*	*C-6′*	CH_3'	C=O′
G	3.2	176.7	79.4	65.2	99.4	54.7	79.8	72.1	74.1	66.7[l]	25.0	176.7
	7.9	179.3	82.2	65.9	99.5	54.8	79.9	72.0	74.0[m]	66.7[n]	25.1	176.7
				(145)	(171)	(142)	(~142)	(~140)	(~138)	(~154)	(128)	
$\delta_G - \delta_{GF}$	3.2	[−0.3]	[−0.1]	[−0.1]	[0.0]	[0.0]	[0.4]	[−0.3]	[−0.9]	[3.6]	[0.1]	[−0.1]
$\delta_G - \delta_{GF}$	7.9	[0.0]	[−0.1]	[0.0]	[0.0]	[0.0]	[0.7]	[−0.4]	[−0.9]	[3.6]	[0.1]	[−0.1]
$\delta_{7.9} - \delta_{3.2}$		<2.6>	<2.8>	<0.7>	<0.1>	<0.1>	<0.1>	<−0.1>	<−0.1>	<0.0>	<0.1>	<0.0>

		C-1″	*C-2″*	*C-3″*	*C-4″*	*C-5″*	*C-6″*	CH_3'	C=*O″*
G *(cont'd)*	3.2	100.9	56.3	73.4[o]	73.1[o]	74.1	66.9[l]	24.8	177.0
	7.9	100.8	56.3	73.4[p]	73.3[p]	73.9[m]	66.8[n]	24.8	177.0
		(173)		(136)	(142)	(147)	(~138)	(~154)	(129)
$\delta_G - \delta_{GF}$	3.2	[0.6]	[−0.1]	[−0.2]	[−0.5]	[−1.1]	[3.8]	[0.1]	[0.1]
$\delta_G - \delta_{GF}$	7.9	[0.6]	[0.0]	[−0.4]	[−0.3]	[−1.2]	[3.7]	[0.1]	[0.1]
$\delta_{7.9} - \delta_{3.2}$		<−0.1>	<0.0>	<0.0>	<0.2>	<−0.2>	<−0.1>	<0.0>	<0.0>

[a] Relative to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate at 70° except for compound **2** from ref. 30. [b] The entries $\delta_x - \delta_y$, where x and y are letter symbols or bold-faced integers, record chemical-shift differences between corresponding atoms in the compounds designated by the subscripts. Letter designations are for the compounds shown in Scheme 1, numbers for the comparison substances **1**–**5** listed in this table. Entries of the form $\delta_{8.5} - \delta_{3.0}$ record shift differences for the designated compound at the pD-values given in the subscripts. [c] Free acid. [d] Ammonium salt. [e,f] Here values are reversed from the listing in ref. 30 because of $[\Delta\delta]$ for C-2′ and C-5′ and because of $<\Delta\delta>$ for **2** (see text). [g–p] Assignments may be reversed.

have no O-3′ substituent. By contrast, in **GF** and **G**, which are glycosylated at O-3′, the $<\Delta\delta>$ values for C-3′ are small.

The assignments of signals for **GF** (Table II) are based on comparisons among the spectra of **GF** and all other derivatives available, including the precursor polyalcohol, **GPS**. The shift differences between **GF** and **GSF** for C-1 through $C=O'$ show the effects of glycosylation at O-3′ in **GSF**. Thus the $[\Delta\delta]$ values of ~ 5, -1.5 and -0.4 p.p.m. for C-3′, C-2′, and C-4′, respectively, are comparable to the corresponding shift differences (8.9, -1.8, and -1 p.p.m.) between methyl 2-acetamido-2-deoxy-3-*O*-methyl-α-D-glucopyranoside and **5** (ref. 32). The differences between **GF** and **5** for C-1″through $C=O''$ reflect the change in aglycon from methyl (**5**) to the 3-linked **GSF** group. Identical shifts of 0.9 p.p.m. are observed for C-5″ in the comparison of **GF** and **GSF** with **5**. Despite the similarities among the chemical-shift differences for **GSF** and those for the pyranoside-ring carbon atoms of **GF**, some assignments in this region of the spectrum may be reversed, and their tentativeness is noted in Table II. Additional assignments that may be reversed are obvious, including the signals of the acetyl groups. However, the only assignments for **GF** that are completely arbitrary are those of the acetyl carbonyl-carbon atoms.

The ^{13}C chemical shifts and chemical-shift differences for **GS** are also given in Table II. **GS** differs from **GSF** in that it has a glycerol 1-phosphate ester group attached to C-6′ of the *N*-acetyl-D-glucosamine unit (see Scheme 1). This difference was reflected in three ways in the spectrum of **GS**. The first was the presence of the three signals of the glycerol substituent; those of C-1″ and C-2″ were identified on the basis of their doublet from (^{31}P coupling) in the ^{1}H-decoupled spectrum. The second was the manifestation of ^{31}P–^{13}C coupling not only in these two signals, but also those for C-5′ and C-6′. The third was the changes in the chemical shifts of **GS** from those in **GSF** and in **3** due to the phosphorylation of C-6′ and C-1″ of **GS**. The phosphorylation shifts for **GS** (Table II) show the expected values of ~ 4 p.p.m. for the resonances of C-6′ and C-1″, and also the expected values for the next neighboring carbon atoms, ~ -1 p.p.m. for C-5′ and C-2″; and ~ -0.4 p.p.m. for C-4′ and C-3″. The $<\Delta\delta>$ values for **GS** (induced by changes in pD) were minimal except for the signals of the glyceric acid unit, and that for C-3′ of the *N*-acetyl-D-glucosamine unit.

The similarities in the spectra of **G** and **GF** are self-evident (see Fig. 3). The most notable differences are, in the spectra of **G**, the absence of major signals at ~ 63 p.p.m., and the appearance of two sets of signals at ~ 66 p.p.m. and at ~ 74 p.p.m., all exhibiting doublet form in the ^{1}H-decoupled spectrum, attributable to scalar coupling to ^{31}P. The signals at ~ 66 p.p.m. are assigned to C-6′ and C-6″, and those at ~ 74 p.p.m. are due to C-5′ and C-5″, shifted from the corresponding values in the spectra of **GF** by proximity to phosphorylated C-6′ and C-6″.

DISCUSSION

The proposed structures of **G** and its derivatives obtained by chemical modification, **GF**, **GS**, and **GSF**, are shown in Scheme 1. The structures are supported by

evidence from elemental and chemical analysis, titration with KOH, g.l.c.–m.s., ^{13}C-n.m.r., and ^{31}P-n.m.r., as well as by the resistance of **G** to enzymatic dephosphorylation and by the chemical relationships between the derivatives.

Elemental analysis of **G** gave results in reasonable agreement with the proposed structure. If the 3% glucose known to be present is taken into account as 2.7% anhydroglucose and the remainder of the sample were **G** as $C_{19}H_{31}N_2O_{16}P$, then the analytical values would be slightly lower than the theoretical in percentage carbon and nitrogen and higher in percentage hydrogen and apparent formula weight. [Calc.: f.w. 590; C, 39.85; H, 5.46; N. 4.75. Found: f.w. (titration) 644; C, 38.52; H, 5.95; N, 4.64].

Preliminary chemical analysis of **G** revealed the presence of substantial amounts of hexosamine and phosphate (see Table I). Analysis for hexosamine by a number of common methods (Smith–Gilkerson[10], Elson–Morgan[11], g.l.c., timed release, etc.) did not provide an accurate estimation of the amount present, but consistently gave values that accounted for one third of the reducing power found for an HCl hydrolyzate of **G**. Because significant amounts of other carbohydrates were not detected, and because results from both elemental analysis (including N) and ^{13}C-n.m.r. spectroscopy were consistent with *N*-acetylglucosamine being the preponderant carbon containing species present, the discrepancies in the analytical results can be attributed to the colorimetric methods themselves. These discrepancies show that care must be taken in the assessment of the hexosamine content of polysaccharides by the usual colorimetric methods; structural factors may be present that lead to significant underestimation. Half of the glucosamine was destroyed on Smith degradation of **G**, as followed by chemical methods and by ^{13}C-n.m.r. spectroscopy.

The titration curve of **G** was consistent with the presence in a 1:1 ratio of a phosphate diester and a carboxylic acid of slightly higher pK_a value. It was not possible to dephosphorylate **G** with alkaline phosphatase. Furthermore, no reducing sugars were formed on hydrolysis of **G** by mild acid treatment. These findings show that the phosphate group is not a monoester and is not linked to an anomeric carbon atom. Treatment with aqueous HF removed all of the phosphate residues from **G** and **GS** to form **GF** and **GSF**, respectively. In both cases no reducing sugars were formed. The absence of phosphorus in **GF** and its presence in **G**, **GPS** (the polyalcohol precursor of **GS**), and **GS** were confirmed by ^{31}P-n.m.r. spectroscopy. The ^{31}P chemical shifts revealed the similarity between the phosphorus in **GPS** and **GS** and the difference of these two species from **G**. In addition, the insensitivity of the phosphorus resonance of **G** to changes in pD further confirmed the absence of a phosphate monoester. In summary, the resistance to hydrolysis by akaline phosphatase, the ^{31}P chemical-shift data, and the titration data are all consistent with the presence of a nonglycosidic phosphate diester group in **G**. In turn, the ^{13}C-n.m.r. spectra of **G** and all of its derivatives provided further evidence of the presence of a carboxyl group. All of the ^{13}C spectra had three resonances, including a carbonyl resonance, that were particularly sensitive to changes in pD, typical of the resonances of carboxylic acids[31]. These findings prompted further analysis, and the discovery of the presence of D-glyceric acid as a major part of the structure of **G**.

^{13}C-N.m.r. spectra of **G** and the derivatives indicated that the sugar residues were in the pyranose ring form. The structural relationship between the GlcNAc residues was determined by per-*O*-methylation of **GF** followed by hydrolysis, reduction, per-*O*-acetylation, and g.l.c.–m.s. of the derivatives. The results indicated that **GF** contained a 1:1 ratio of two types of *N*-acetylglucosamine residues, one unsubstituted (thus periodate sensitive), the other 3-*O*-substituted (periodate resistant). The anomeric configurations of the *N*-acetylglucosamine residues and the order of attachment of the four constituent residues (phosphate, glyceric acid, Glc*p*NAc, and 3-*O*-substituted Glc*p*NAc) were deduced from the chemical relationships between **G** and its derivatives, and from the ^{13}C-n.m.r. spectra. The simpler spectra were those of **GSF**, parts of which are shown at two pD values in Fig. 3. The resonance at ~80 p.p.m. of an *O*-substituted carbon atom was assigned to C-2 of glyceric acid because of the pD sensitivity of the signal. The chemical shift indicated substitution of the oxygen attached to this carbon atom, and thus glycosylation by the glucosamine residue. The chemical shift (99.5 p.p.m.) and coupling constant ($^1J_{C,H}$, 172 Hz) for the single anomeric carbon atom were consistent with those of an α-glycoside of *N*-acetylglucosamine. Therefore, **GSF** was *O*-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-(1→2)-D-glyceric acid (see Scheme 1).

The partial spectrum of **GF** is shown in Fig. 3. This compound contained one glyceric acid and two *N*-acetylglucosamine residues but no phosphorus (see Scheme 1). The g.l.c.–m.s. data for the per-*O*-methylated derivatives indicated that the two glucosamine residues were linked through a (1→3) glycosidic bond. Glyceric acid was the aglycon at the anomeric carbon atom of the first glucosamine residue. The pair of anomeric carbon resonances at ~100 p.p.m. were consistent with this, and the $^1J_{C,H}$ values (~173 Hz) for these signals indicated both carbons were in the α-anomeric configuration. Therefore, **GF** was *O*-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-(1→3)-*O*-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-(1→2)-D-glyceric acid.

The ^{13}C-n.m.r. spectra of **G** and **GF** are virtually identical, except for the effects of phosphorylation at C-6′ and C-6″ in **G**. The most notable of these effects is the ^{31}P–^{13}C coupling observed in two major signal multiplets of the **G**-spectrum, and the differences in the chemical shifts of these same signals in the two spectra. The signals are for C-5′, C-5″, C-6′, and C-6″; they appear as pairs of overlapping, coupled, and doubled signals at ~74.1, and ~66.8 p.p.m. in the spectra of **G** in Fig. 3. At acidic values of of pD these pairs of signals are almost resolved, but at neutral pD they are more closely superimposed. The $\delta_G - \delta_{GF}$ values of ~4 p.p.m. for C-6′ and C-6″ are close to the $\delta_{GS} - \delta_{GSF}$ values for C-6′ and C-1″ of **GS**, which also represent changes due to phosphorylation (see Table II). The absence of signals at ~63 p.p.m. in the spectra of **G** indicates that some form of *O*-substitution must exist at C-6 of both glucosamine residues. The only resonance of **G** near this value is that at ~65 p.p.m., which in view of its pD-sensitivity is assignable to C-3 of the glyceric acid residue. The absence of signals at ~63 p.p.m. and the presence of the doubled signals of phosphorus-coupled carbon atoms at ~67 p.p.m. that are also triplets in the proton-coupled spectrum provide strong evidence for the location of the phosphate diester linkage, and show that there are no glucosamine residues in **G** that are not phosphorylated. This is the center piece of the evidence for the

proposed structure of **G**, revealing **G** to be a novel substance with a C-6′ to C-6″ phosphate diester linkage.

To our knowledge the only reported structure (excluding the nucleic acids) having a phosphate diester bridge between two nonanomeric glycose carbons is agrocinopine A. This molecule, isolated from plant tumors induced by *Agrobacterium tumefaciens*[33], had a phosphate diester linkage between C-2 of L-arabinose and C-4 of the fructose moiety of sucrose. Other examples of *N*-acetylglucosamine polymers with phosphate diester bridges are known[29,34], but in these cases the phosphate diester groups link anomeric carbons to C-4 or C-6 of other saccharide residues in the repeating structures. A second novel feature of **G** is the presence of an unsubstituted glyceric acid aglycon in the polymer repeating structure. Glyceric acid has been previously identified as the single aglyconic terminus in molecules of the 6-*O*-methylglucose polysaccharide of *Mycobacterium smegmatis*[30,35] and *M. phlei*[36], in low molecualr weight glycosylglyceric acids in certain algae[37,38,39], and as an acyl substituent in glycolipids[40,41].

The failure of Smith *et al.*[2] to obtain a fraction analogous to **G** in serological activity may be explainable by their choice of a single analytical procedure, total carbohydrate[7], for monitoring the eluate obtained during ion-exchange column chromatography, or by the possibility that the serologically important fraction was lost during the preparation of cell walls. The absence of **G** cannot be attributed to their use of sodium hydroxide, since a much higher yield of **G** was obtained in the present study by extraction with alkali than by the autoclave procedure. The cell-wall constituents isolated[2] by Smith *et al.* may correspond to the **A–C** and **B–C** fractions described herein, because their saccharide composition is similar.

ACKNOWLEDGMENTS

The instrumentation for performing the g.l.c.–m.s. analysis was funded in part by Public Health Service Grant AI 18877 from the National Institutes of Health.

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