

Multiantennary Group-Specific Polysaccharide of Group B *Streptococcus*[†]

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ABSTRACT: The group-specific antigen of group B *Streptococcus* is composed of four different oligosaccharide units of M_w 766 (III), 1277 (II), 1462 (IV), and 1788 (I). The major constituent sugars of the oligosaccharides are α -L-rhamnopyranose, α -D-galactopyranose, 2-acetamido-2-deoxy- β -D-glucopyranose, and D-glucitol except that III does not contain α -D-galactopyranosyl or 2-acetamido-2-deoxy- β -D-glucopyranosyl residues and IV contains no D-glucitol but has one additional β -L-rhamnopyranosyl residue. The structures of II and III have been previously elucidated [Michon, F., Katzenellenbogen, E., Kasper, D. L., & Jennings, H. J. (1987) *Biochemistry* 26, 476-486]. In the group B antigen all the oligosaccharides are linked by one type of phosphodiester bond from O6 of the D-glucitol residue of one oligosaccharide to O6 of the α -D-galactopyranosyl residue of the next to form a complex and highly branched multiantennary structure. However, despite the heterogeneous nature of its component oligosaccharides, some order has been identified in the biosynthesis of the group B antigen from chemical and enzymatic sequence studies. Because III lacks an α -D-galactopyranosyl residue but has a D-glucitol residue, it is situated at the nonreducing terminus of all the branches of the group B antigen where it is always adjacent to a II moiety. Conversely, IV has an α -D-galactopyranosyl residue but has no D-glucitol and is therefore located at the reducing terminus of the group B antigen where it probably functions as a linker molecule between the group B polysaccharide and the cell wall peptidoglycan of the group B streptococcal organisms. Oligosaccharide I contains two α -D-galactopyranosyl residues and one D-glucitol residue and thus constitutes the branch point in the group B antigen, whereas II contains one of each of the above residues and therefore is situated in linear interchain positions. The group B antigen is highly branched and probably has a unique multiantennary structure.

Group B streptococci can be differentiated from other streptococci by the presence of a group-specific polysaccharide antigen ("C" substance) (Lancefield, 1933, 1934, 1938). The group B antigen contains rhamnose, galactose, and N-acetylglucosamine (Heidelberger et al., 1967; Kane & Karakawa, 1977; Carey et al., 1980) and, on the basis of its cross-reaction with the group G streptococcal and type 23 pneumococcal antisera, was predicted to have terminal rhamnose residues (Heidelberger et al., 1967; Carey et al., 1980). Recently Pritchard et al. (1981, 1984) have confirmed the above findings and have also identified glucitol phosphate as a component of the group B antigen. Pritchard et al. (1984) also proposed a structure for the repeating unit of the group B polysaccharide and suggested that the repeating units were linked by phosphodiester bonds attached to the glucitol residues, a structural feature normally associated with teichoic acids. Michon et al. (1987) confirmed much of this preliminary structural information but also demonstrated that the structure of the group B antigen was more complex than had been supposed. Three different but structurally related oligosaccharides, two of which were structurally elucidated, were identified as components of the group B antigen. However, definite evidence as to the possibility of heterogeneity in the structure or the presence of a mixture of polysaccharides was not obtained. A total of four oligosaccharides have now been identified and structurally elucidated. All of these oligosaccharides are components of the group B antigen, and their precise mode of linkage to each other has been determined.

While the group B antigen is a complex, highly branched polysaccharide, some order in its biosynthesis has been identified.

EXPERIMENTAL PROCEDURES

Growth of the Organism and Purification of the Group Antigen. Prototype strain 090, type Ia, group B *Streptococcus* was originally obtained from Dr. Rebecca Lancefield and was grown in a fermenter at controlled pH (pH 7.2) as previously described (Michon et al., 1987). The group antigen was obtained from the culture supernatant and purified by previously described procedures (Michon et al., 1987).

Analytical Methods. Solutions were concentrated under reduced pressure below 40 °C. Phosphorus was determined by the method of Chen et al. (1956). Analysis for glycoside constituents was carried out essentially by the method of Dimitriev et al. (1975) as previously described (Jennings et al., 1980). Following derivatization the individual glycoside constituents were identified by gas-liquid chromatography-mass spectrometry (GLC-MS) using column i. All other analytical techniques including methylation analysis and determination of the (D or L) configuration of the monosaccharide components have been described in a previous paper (Michon et al., 1987).

Instrumental Methods. Gas-liquid chromatography (GLC) was performed on a Hewlett-Packard 5710A instrument equipped with a flame ionization detector and a Model 3380A electronic integrator using the following columns: (i) a glass column (180 × 0.15 cm) containing 3% SP2340 on Supelcoport (80-100 mesh) operated at 180 °C (delay 2 min) to 240 °C at 4 °C/min; (ii) a fused silica capillary column (0.3 mm × 25 cm) containing 3% OV17 operated at 180-230 °C at 2 °C/min and held for 10 min at 230 °C. Combined gas-

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liquid chromatography-mass spectrometry (GLC-MS) was performed on a Hewlett-Packard 2985B instrument equipped with a dual EI/CI source, using the above columns and an ionization potential of 70 eV. Permethylated and acetylated oligosaccharides were analyzed on a Dexil 300 glass column (iii) operated at 150–330 °C at 10 °C/min. Chemical ionization (CI) spectra were obtained with methane or isobutane as the carrier and reagent gas at a source temperature of 150 °C and an ionization voltage of 230 eV.

Fast atom bombardment (FAB) spectrometry was performed with a VG Analytical ZAB HF high-field magnet mass spectrometer equipped with an FAB source and an M-scan FAB gun. Mass spectra were obtained according to published procedures (Dell & Ballou, 1983a,b; Tsai et al., 1986).

^{13}C and ^1H NMR spectra were recorded on a Bruker AM500 spectrometer. Acetone was used as an internal chemical shift reference for ^1H NMR (2.225 ppm) and for ^{13}C NMR (31.07 ppm). ^{13}C NMR spectra were recorded at 300 K in 5-mm tubes at concentrations of 20–30 mg in 0.5 mL of D_2O at 125.0 MHz. DEPT experiments (Doddrell et al., 1982) were performed for a 12.5-kHz spectra width with a $3\pi/2$ proton pulse to distinguish between CH and CH_2 resonances.

^{31}P NMR spectra at 202 MHz were recorded at 300 K on a Bruker AM500 spectrometer, in 10-mm tubes containing 10–20 mg of oligosaccharide in 2.5 mL of D_2O . Paramagnetic contaminants were removed from the samples by chromatography on Chelex-100 (Bio-Rad Laboratories). The sampling conditions at 202 MHz were as follows: 48- μs $\pi/2$ pulse; 10-kHz spectral window; 16 384 data points. Chemical shifts are given in parts per million and are relative to external 25% H_3PO_4 .

The proton homonuclear shift correlated COSY (Bax et al., 1981) and the two-step relay COSY (Bax & Drobny, 1985) were performed at 305 K with the standard software provided by Bruker (DISNMR). Quadrature detection in both dimensions was employed. The initial (t_1 , t_2) matrix of 512×2 K data points was zero-filled to 1024×2048 points to provide 2 Hz per point digital resolution in both domains. Resolution enhancement in both domains was done by an unshifted sine bell function prior to Fourier transformation. Magnitude spectra symmetrized about the diagonal were used to represent the data.

The ^{13}C – ^1H shift correlation experiment with proton decoupling in the F_1 domain was done according to Bax and Freeman (1981). The initial (t_1 , t_2) matrix of 64×1024 points corresponded to a digital resolution of 23 Hz per point in the F_2 domain and 15 Hz per point in the F_1 domain. The CHORTLE experiments (carbon-hydrogen correlations from one-dimensional polarization-transfer spectra by least-squares analysis) were performed according to Pearson (1985).

The nuclear Overhauser enhancement experiments were performed in the difference mode with sequential irradiation of each line in a multiplet (Neuhaus, 1983; Kinns & Sanders, 1984). Total irradiation time per multiplet was 1 s.

Partial Alkaline Hydrolysis of the Group B Polysaccharide. The group B polysaccharide (100 mg) was treated with 0.5 M NaOH (3.0 mL) for 5 h at 50 °C, and the solution was then neutralized by passage through Rexyn 101 (H^+) ion-exchange resin (Fisher Scientific Co.) and lyophilized. The residue was dissolved in 0.2 M $(\text{NH}_4)_2\text{SO}_4$ at pH 8.8 and treated with alkaline phosphatase (EC 3.1.3.1) (Millipore Corp., Freehold, NJ) for 16 h at room temperature, and the solution was lyophilized. The residue was dissolved in 0.02 M pyridinium acetate (pH 5.4) and fractionated by passage

through a Bio-Gel P-4 (Bio-Rad Laboratories, ~400 mesh) column using the above buffer as eluant. The eluate was monitored for the appearance of the resultant oligosaccharides by use of a Waters R403 differential refractometer. The fractions were numbered (1–5) in order of their elution from the column and fractions 3–5 had identical K_a values with the fractions containing oligosaccharides I, II, and III previously isolated from the hydrolysate of the complete alkaline hydrolysis of the group B polysaccharide (Michon et al., 1987). Fractions 1 and 2 were individually rechromatographed on the aforementioned column.

The products obtained from fractions 1 and 2, the latter consisting entirely of oligosaccharide V, were fully hydrolyzed with 0.5 M NaOH for 48 h at 50 °C and following treatment with alkaline phosphatase were each fractionated on a Bio-Gel P-4 column as previously described (Michon et al., 1987).

Treatment of V with an Exorhamnosidase. Oligosaccharide V (15 mg) was dissolved in 100 mM sodium acetate buffer and treated with 4 units of naringinase from *Penicillium* species (EC 3.2.1.40, 400 units/g) (Sigma, St. Louis, MO). After 16 h at room temperature (rt) the degradation products were separated on a Bio-Gel P-4 column as described above. It yielded oligosaccharide VI which was analyzed by ^1H NMR and methylation.

Location of Phosphodiester Group in V. Oligosaccharide V (4 mg) was oxidized with 0.05 M NaIO_4 (1 mL) for 72 h at 16 °C in the dark. Excess NaIO_4 was precipitated from the solution with barium carbonate, and the polyaldehyde was reduced with NaBH_4 . Excess NaBH_4 was destroyed by the addition of acetic acid to pH 7.0, and after concentration of the solution it was desalted on a Sephadex G-10 column (Pharmacia). The polyol was then permethylated (Hakomori, 1964) and hydrolyzed to its individual methylated fragments with 0.5 M H_2SO_4 in 95% acetic acid for 18 h at 80 °C. A portion of this material was reduced (NaBH_4) and acetylated, and the alditol acetate derivatives were analyzed by GC-MS (Lindberg, 1972) using column ii. The remainder was dephosphorylated with 48% HF for 3 days at 4 °C (Iipkin et al., 1969), and following evaporation of the HF in vacuo at 4 °C over solid NaOH, the dephosphorylated methylated components were reacylated and analyzed by GC-MS using column ii.

Partial Hydrolysis of Permethylated I. Oligosaccharide I (2.0 mg) was permethylated (Hakomori, 1964), and the product was subjected to partial hydrolysis with 90% formic acid for 10 min at 100 °C. Formic acid was removed by repeated evaporations with water, and the residue was reduced with NaBD_4 , acetylated with pyridine-acetic anhydride (1:1 v/v) for 1 h at 100 °C, and subjected to GLC-MS analysis using column iii.

Deamination of Oligosaccharide I. Prior to deamination, I (20.0 mg) was N-deacetylated with 2 M NaOH for 10 h at 100 °C. The solution was neutralized with 2 M HCl, desalted on a Sephadex G-15 column (Pharmacia) using water as the eluant, and lyophilized. The N-deacetylated I was then deaminated by the method of Dmitriev et al. (1975) as described by Jennings et al. (1983). The products of deamination were fractionated on a Bio-Gel P-4 column (Bio-Rad Laboratories) with 0.02 M pyridinium acetate buffer at pH 5.4 and with a Waters R403 differential refractometer to monitor the eluate. Five major fractions were identified by ^1H NMR and GC-MS analysis as oligosaccharides I, (II and IX), III, and X.

Smith Degradation of the Group B Polysaccharide. The group B polysaccharide was subjected to a Smith degradation

(Goldstein et al., 1965) using the following procedures. The group B polysaccharide (150 mg) was oxidized with 0.05 M NaIO_4 (15 mL), and the mixture was kept at room temperature in the dark for 72 h. Excess periodate was precipitated from the solution with BaCO_3 , and the product of the oxidation was reduced with NaBH_4 (50 mg). Excess NaBH_4 was destroyed by the addition of acetic acid to pH 7.0, and following lyophilization of the solution, the residue was desalted on a Sephadex G-10 column (Pharmacia) using water as the eluant. The solution was lyophilized again, the intermediate Smith degradation product was hydrolyzed with 2% acetic acid for 1 h at 100 °C, and following lyophilization the hydrolysis products were reduced with NaBD_4 . After usual workup the Smith products were purified by gel filtration on Bio-Gel P-4. Two major fractions were collected. The first eluted fraction had a K_d very similar to that of I and was referred as oligosaccharide XI, and the second retarded fraction corresponded to a component oligosaccharide referred as XIII. Both oligosaccharides were structurally defined by glucose, methylation, FAB-MS, and 1-D and 2-D ^1H and ^{13}C NMR analyses.

Treatment of the Group B Polysaccharide with Naringinase.

The group B polysaccharide (50 mg) was dissolved in 100 mM sodium acetate buffer (5 mL) at pH 4.0 and treated with 50 units of naringinase from *Penicillium* species (EC 3.2.1.40, 400 units per 1 g) (Sigma, St. Louis, MO) as previously described (Michon et al., 1987). After 16 h at rt 90% cold phenol (5.0 mL) was added to the cooled solution (4 °C), and the mixture was stirred for 3 h. Following centrifugation for 15 min at 10000 rpm the aqueous phase was separated, exhaustively dialyzed against distilled water, and lyophilized. The residue was subjected to complete alkaline hydrolysis, and the resultant oligosaccharides were treated with alkaline phosphatase and separated on a Bio-Gel P-4 column as previously described (Michon et al., 1987). The naringinase-treated group B polysaccharide yielded oligosaccharides I, IV, II, and XII.

Treatment of Oligosaccharide I with Naringinase. Oligosaccharide I (10.0 mg) was treated with 10 units of naringinase as described above. After 2 days at 37 °C the resultant oligosaccharides were separated on a Bio-Gel P-4 column as previously described. Three not well resolved fractions corresponding to oligosaccharides I, VII, and VIII were yielded, each one was rechromatographed on the aforementioned column, and each component was then analyzed by methylation and ^1H NMR.

RESULTS

Partial Hydrolysis of the Group B Polysaccharide. The heterogeneous nature of the group B antigen was confirmed when its complete alkaline hydrolysis, and molecular exclusion chromatography of the hydrolyzate, yielded three distinct products, designated oligosaccharides I, II, and III in order of decreasing size (Michon et al., 1987). To obtain further sequence and linkage information, the group B antigen was subjected to a partial alkaline hydrolysis in order to generate larger oligosaccharide fragments. Following dephosphorylation (Michon et al., 1987) of the fragments they were separated on the same Bio-Gel P-4 column as was used in the separation of the products of the complete alkaline hydrolysis (Michon et al., 1987). The elution profile of the separation is shown in Figure 1a, and five fractions can be clearly seen which were designated 1–5 in order of their elution from the column. Fractions 3–5 had identical exclusion volumes to those of the three oligosaccharides (I, II, and III, respectively) obtained in the complete alkaline hydrolysis of the group B antigen (Michon et al., 1987), and the presence of oligosaccharides

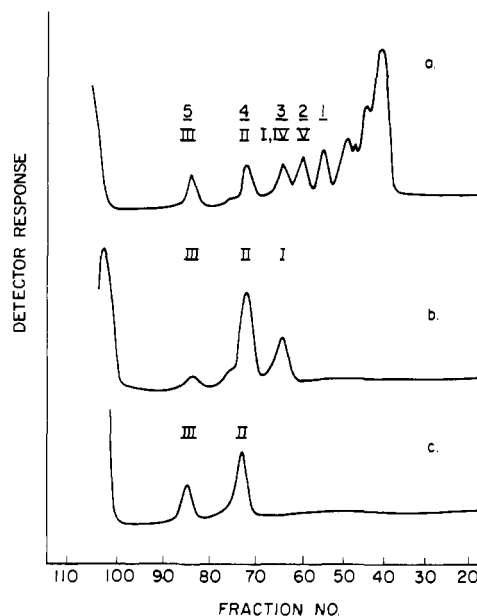


FIGURE 1: Elution profiles (Bio-Gel P-4) of (a) partially base treated group B antigen, (b) totally alkali treated fraction 1, and (c) totally alkali treated fraction 2.

II and III in fractions 4 and 5 from both hydrolyses was established by virtue of their respective indistinguishable methylation analyses and ^1H NMR spectra (Michon et al., 1987) (Table II). It has now been established that oligosaccharide I obtained from fraction 3 in both the partial and complete hydrolyses is in fact a mixture of two oligosaccharides which have been designated I and IV (for details see structural information on I and IV).

The components contained in fractions 1 and 2 obtained from the partial alkaline hydrolysis of the group B antigen were further purified by their individual rechromatography on the same column as was used for the first separation. Fraction 2 was found to contain a pure oligosaccharide (V), and fraction 1 contained a large fragment of the group B antigen which on complete alkaline hydrolysis gave the same three fractions (3–5) as were obtained from the group B antigen (Figure 1b). Fractions 4 and 5 contained II and III, respectively, but in this case fraction 3 contained oligosaccharide I uncontaminated by IV. The structures of II and III (structures b and a of Figures 2, respectively) have been elucidated (Michon et al., 1987), and in order to complete the structure of the group B antigen, the initial task was to determine the structures of I, IV, and V.

Structure of I. Glycose analysis of I, obtained from fraction 1 of the above partial alkaline hydrolyzate, indicated that it contained L-rhamnose, D-galactose, 2-acetamido-2-deoxy-D-glucose, and D-glucitol in the molar ratio of 6.0:2.0:2.0:1.0. Thus in comparison to II it contains an additional residue of each of the three former components. This, together with similar methylation analysis for both I and II (Table I), suggested that I contained two similarly linked trisaccharide ($\text{Rhap1} \rightarrow 3\text{Galp1} \rightarrow 3\text{GlcPNAc-}$) moieties in contrast to II which contains only one (Michon et al., 1987). The unique presence of 2,3-di-O-methylrhamnose in the methylation analysis of I also indicates that I is structurally related to II by the addition of the second trisaccharide to O4 of one of the terminal rhamnopyranosyl residues of II. The structure of I is shown together with those of II and III in Figure 2 and was deduced from the following collective evidence.

The sequence of the glycose constituents in I was consistent with the pattern of fragment ions produced when it was sub-

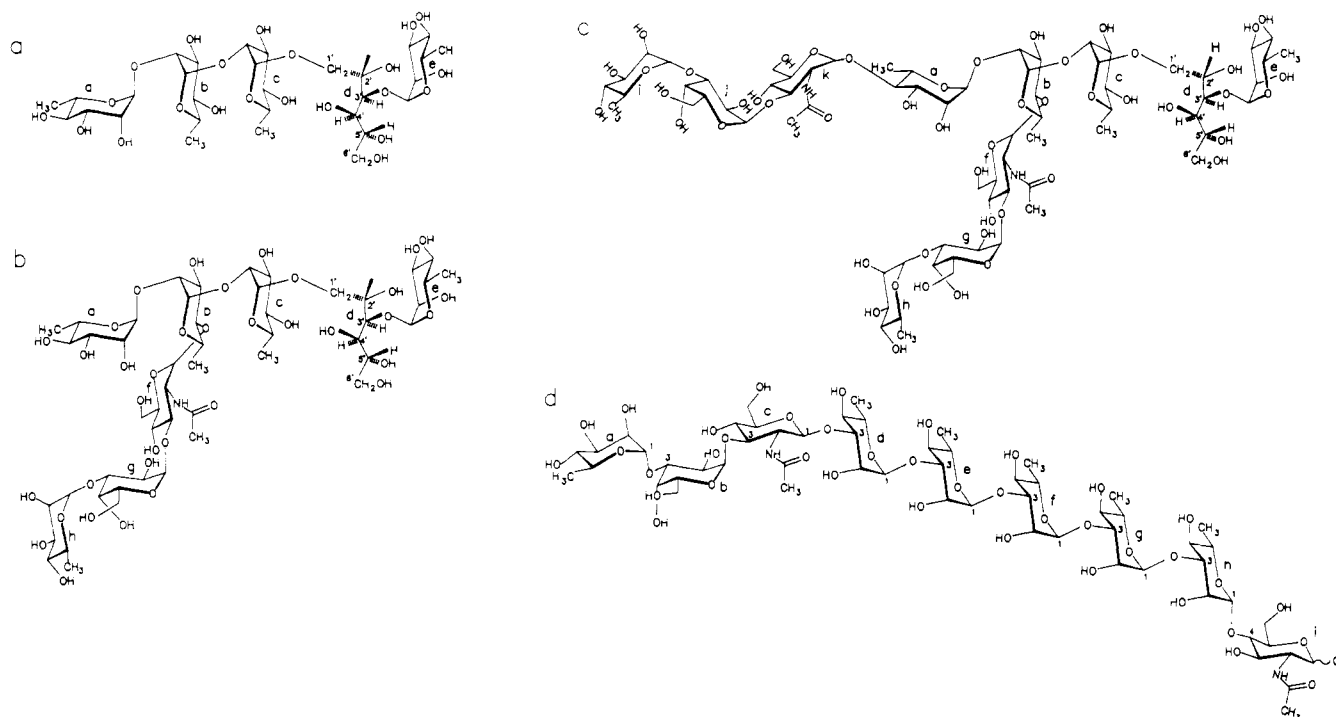


FIGURE 2: Structure of oligosaccharides (a) III, (b) II, (c) I, and (d) IV. The conformation of the oligosaccharides has not yet been confirmed.

Table I: Methylation Analysis of Oligosaccharides Obtained by Selective Degradations of Group B Antigen^a

methylated sugar	detector response (%)								
	I	II	V	VII	VIII	IX	XI	XII	XIII
2,3,4-Me ₃ Rha ^b	24.5	28.7	32.0	15.0	7.0	23.0	—	60.0	—
3,4-Me ₂ Rha ^b	9.5	10.6	22.0	9.5	9.5	25.0	—	—	—
2,3-Me ₂ Rha ^b	9.5	—	—	9.5	9.5	8.0	—	—	—
2,4-Me ₂ Rha ^b	—	—	—	—	—	—	58.0	—	16.0
2,4,5,6-Me ₄ -glucitol ^b	8.4	8.5	10.5	9.0	9.0	11.0	—	25.0	—
2,3,4,6-Me ₄ Gal ^b	—	—	—	11.0	18.0	—	2.0 ^d	—	5.0 ^d
3-Me Rha ^b	11.0	9.5	7.0	10.4	11.0	3.0	—	—	—
2,4,6-M ₃ Gal ^b	19.0	10.6	4.5 ^d	11.0	—	12.0	—	—	—
4,6-Me ₂ GlcNMeAc	++	+	+	++	++	+	+	—	++
1,3,5,6-Me ₄ GlcNMeAc ^c	—	—	—	—	—	—	+	—	—
1,3,5-Me ₃ Rha ^c	—	—	—	—	—	—	—	—	13.0

^a (+) Slight nonquantitative response; (—) not detected. ^b Identified as alditol acetates not deuteriated at C1, using column ii. ^c Identified as alditol acetates deuteriated at C1, using column ii. ^d Stoichiometric amounts detected after HF (48%) treatment of the corresponding permethylated oligosaccharide and remethylation.

jected to FAB-MS analysis (Figure 3a). Fragmentations were obtained in both the positive and negative modes. Pseudo molecular ions at m/z 1789 ($M + H$) and m/z 1787 ($M - H$) were obtained from underivatized I in both respective modes and were also obtained in the positive mode from peracetylated [m/z 2965 ($M + H$)] and permethylated [m/z 2209 ($M + H$)] I. That I has an even molecular weight (M , 1788) is consistent with the presence of two glucosamine residues in its structure. The positive ion fragmentation pattern of underivatized I (Figure 3a) shows a striking resemblance to that of II (Michon et al., 1987), and a series of common ions at m/z 512, 658, 804, and 950 is consistent with their close structural relationship (Figure 2b,c). The ion at m/z 512 was very intense due to glycosidic cleavage at a hexaminy residue. That it was the only intense ion in this intermediate mass range of the spectrum is consistent with the presence of two identical Rha-Gal-GlcNAc sequences in I. This was further confirmed by the identification of only one equivalent high-intensity ion (m/z 638) in the spectrum of permethylated I. The absence of an ion at m/z 606 in this latter spectrum, due to the loss of methanol from the ion at m/z 638 and the presence of an ion at m/z 228, are diagnostic of O3-linked glucosamine residues (Kochetkov & Chizov, 1966).

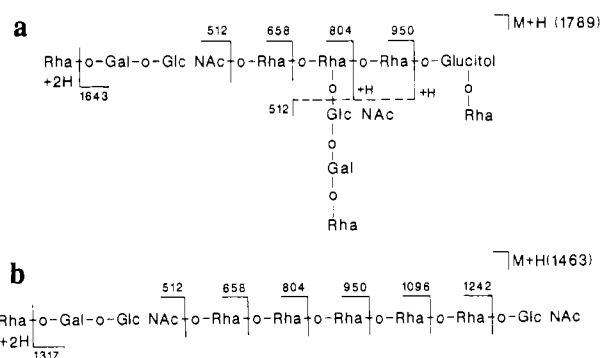


FIGURE 3: Pertinent primary ions from the positive FAB mass spectra of (a) oligosaccharide I and (b) oligosaccharide IV.

That I contained ten glucose residues was confirmed by examination of signals in the anomeric region of its ¹H NMR and ¹³C NMR spectra. The chemical shifts and coupling constants of the proton and carbon signals of I are listed in Tables II and III, respectively. Ten proton doublets, two of them coincident, were observed in the anomeric region of its ¹H NMR spectrum (Figure 5), and ten carbon signals, four of them coincident, were also observed in the equivalent region

Table II: ^1H Chemical Shifts^a of Anomeric Protons of Oligosaccharides Derived from Group B Antigen

proton ^b	I	II ^d	III ^d	V	VI	VII	VIII	IX	XI	XII	XIII
1-a	4.958	4.971	4.971	4.991	4.967	4.958	4.959	4.973			4.959
1-a'				4.978							
1-b	5.094	5.117	5.102	5.109	5.113	5.092	5.094	5.098	5.505		
1-b'				5.124							
1-c	4.864	4.869	4.871	4.874	4.872	4.863	4.864	4.880	4.776	4.804	
1-c'				4.890	4.814						
1-d									5.046		
1-e	5.035	5.038	5.030	5.032	5.028	5.036	5.025	5.030	5.036	5.020	
1-e'				5.032	5.028						
1-f	4.835 ^c	4.835		4.867	4.849	4.836 ^c	4.838 ^c		5.036		4.741
1-g	5.443	5.441		5.528	5.542	5.444	5.409		5.014		5.480
1-h	5.022	5.022		5.032	5.028	5.024			5.682		
1-i	5.035							5.035			
1-j	5.439					5.406	5.409	5.441			5.480
1-k	4.843 ^c					4.843 ^c	4.823 ^c	4.845			4.856

^a Measured at 343 K in D_2O with acetone (0.1%) as internal chemical shift reference (δ 2.225). ^b a-k letters refer to residues a-k of compounds reported in Figures 2, 7, and 8. ^c Assignments may be reversed. ^d Data taken from previous paper (Michon et al., 1987).

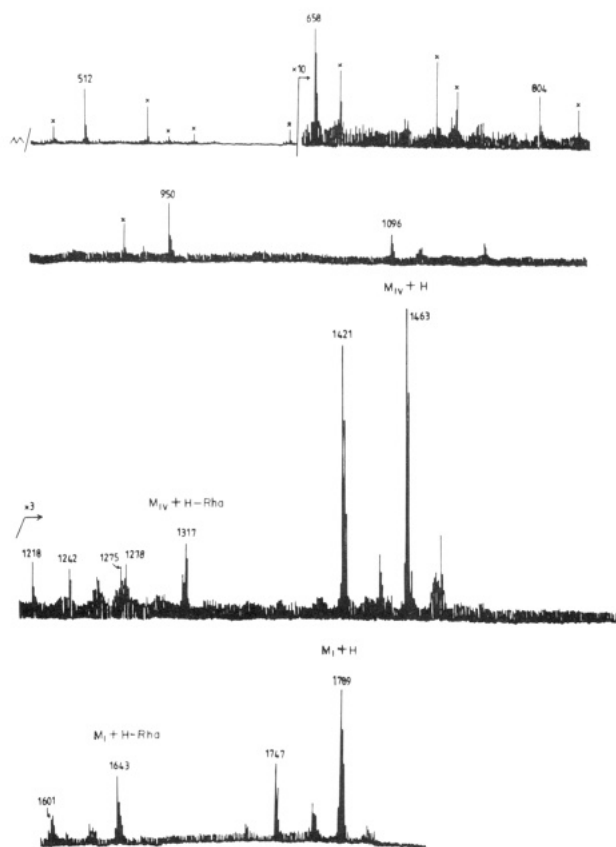
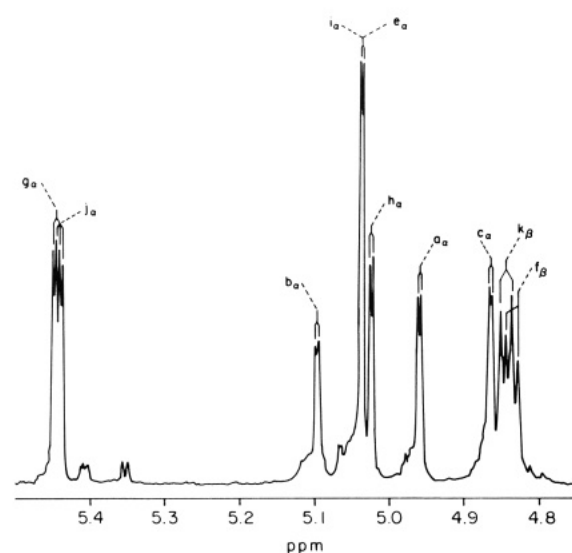
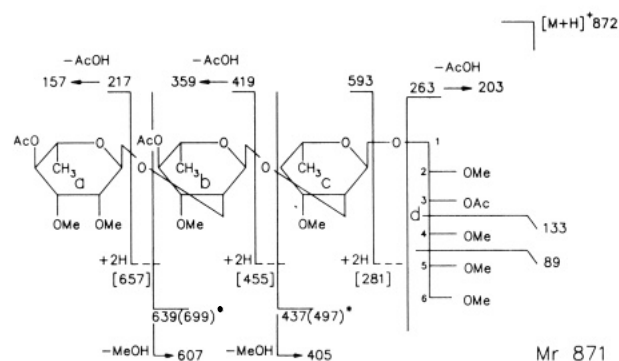


FIGURE 4: Positive FAB mass spectrum of the fraction containing the mixture of I and IV.

of its ^{13}C NMR spectrum (Table III). Assignments of the anomeric proton and carbon signals of the ^1H NMR and ^{13}C NMR spectra of I were made by use of the assignments made previously on II (Michon et al., 1987) and also by use of naringinase-treated I to establish the anomeric carbon and proton assignments of its terminal α -L-rhamnopyranosyl residues (h and i). The products of enzymatic treatment of I were oligosaccharides VII and VIII (Figure 7), the ^1H NMR spectra of which had one and two fewer anomeric signals, respectively, than those exhibited in the equivalent spectrum of I (Table III). It could be anticipated from the known pattern of cleavage of II with naringinase (Michon et al., 1987) that both residues h and i of I would be vulnerable to cleavage. The anomeric carbon assignments of I were confirmed by use of a proton heteronuclear shift correlated 2-D NMR (^{13}C ,H COSY) experiment (Bax et al., 1981; Michon et al., 1985a,b).

FIGURE 5: Partial ^1H NMR spectrum (anomeric region) of oligosaccharide I in D_2O at 343 K.FIGURE 6: Fragmentation pattern of the EI mass spectrum of partially acetylated and methylated oligosaccharide resulting from partial formolysis of permethylated I. Some diagnostic CI (isobutane) fragments are in brackets. J_1 fragment ions are shown in parentheses marked with an asterisk.

Further structural proof of I was provided by degradation techniques. Permethylated I was partially hydrolyzed with formic acid and reduced with sodium borodeuteride, and following acetylation the fragments were detected by GC-MS analysis using column iii. One of these fragments yielded valuable confirmatory sequence information in its EI spectrum, and its fragmentation pattern is shown in Figure 6. A pseudo molecular ion was observed at m/z 872 $[(M+H)^+]$ and primary fragment ions at m/z 217 (aA1), 419 (abA1), and

Table III: ^{13}C Chemical Shifts^a and $^1J_{\text{C,H}}$ Coupling Constants^b (Hz) of Signals of Oligosaccharides Obtained from Group B Antigen

carbons	I ^c	II ^d	III ^d	V	VI ^e	XI ^f	XII	XIII ^f
C1a	102.77 (172.0)	102.95 (172.0)	102.92 (169.5)	103.12	103.10			99.98
C1a'				103.00				
C1b	101.24 (171.5)	101.17 (172.0)	101.59 (170.6)	101.27	101.29	99.50 (172.0)		
C1b'				101.67				
C1c	99.52 (171.0)	99.56 (171.0)	99.57 (167.3)	99.59	99.62	103.13 (163.0)	100.94	
C1c'				99.59	100.87			
C1d						102.80 (174.0)		
C1e	102.51 (171.0)	102.42 (172.0)		102.51	102.52	103.08 (170.0)	102.42	
C1e'				102.12	102.15			
C1f	102.06 (164.0)	101.99 (164.0)		102.12	102.00	103.08 (170.0)		101.68
C1g	99.62 (172.0)	99.56 (172.0)		99.14	99.19	103.13 (170.0)		99.51
C1h	103.17 (171.0)	103.02 (170.0)		103.12	103.10	101.20 (161.0)		
C1i	103.17 (171.0)							
C1j	99.52 (172.0)							99.51
C1k	102.16 (163.0)							102.12
C6g	61.30			64.88 (4.0) ^g	64.91 (4.0) ^g			64.88 (4.5) ^g
C6d	63.70	63.70	63.63	63.70	63.74		63.70	
C6d'				67.81 (5.7) ^g	67.79 (5.6) ^g			
C6b						64.94 (4.0) ^g		
C6j								64.88 (4.5) ^g
C2EG						67.79 (5.4) ^g		67.78 (5.6) ^g
C1EG						62.01 (7.5) ^h		62.05 (7.7) ^h

^a Measured at 310 K in D₂O with acetone as internal chemical shift reference (δ 31.07). ^b In parentheses (Hz) obtained by the gated decoupling technique. ^c Assignments made by heteronuclear 2D shift correlated (C,H) COSY. ^d Data from previous paper (Michon et al., 1987). ^e V following treatment with naringinase. ^f Assignments made by CHORTLE. ^g $^1J_{\text{C,H}}$ coupling constant value in hertz. ^h $^1J_{\text{C,P}}$ coupling value in hertz.

593 (abcA1) could be assigned to residues a, b, and c of the fragment. That residues a and b are O-acetylated indicates that a was originally monosubstituted and b was a branch point (disubstituted) in I. Primary ions at m/z 263 (dA1), 437 (cdA1), and 639 (bcdA1) confirm this analysis. The fragment ions at m/z 89 and 133 and the absence of an ion at m/z 177 demonstrated that O3 of residue d is O-acetylated, indicating that this position was also originally a linkage point in I.

That an α -L-rhamnopyranosyl residue was linked to O3 of the D-glucitol residue of I was deduced by deamination (Williams, 1975) of I which also showed that both II and III were an integral part of I. In preparation for deamination I was first N-deacetylated. This was only achieved with difficulty as had been the experience with the N-deacetylation of II (Michon et al., 1987), and only 60% N-deacetylation was achieved. Partially N-deacetylated I was then deaminated (Dmitriev et al., 1973), and three major products were isolated by gel filtration. The largest fraction consisted of two isomeric oligosaccharides which were produced by deamination of I at either residue f or residue k (Figure 2c). Deamination at residue k yielded oligosaccharide II (Figure 2b), the structure of which has been previously determined (Michon et al., 1987), and at residue f it yielded IX (Figure 7), the linear isomer of II. These oligosaccharides could not be separated, but their presence in the mixture could be ascertained by ^1H NMR and methylation analysis of the mixture. Because II and VII are isomeric, the ^1H NMR spectra of II and VII are very similar. However, the major difference is in residue b because it is branched in II and unbranched in VII. The proton anomeric signal of b in II is at δ 5.117 (Table II), and the spectrum of the mixture exhibited both this signal and another signal at δ 5.098 not present in the spectrum of II. These signals were in the ratio of 7:3, respectively, indicating that VII and II were in these ratios in the mixture. The above evidence was also consistent with the methylation analysis of the mixture in which both 2,3-di-O-methylrhamnose and 3-O-methylrhamnose were detected in the ratio of 8:3 (Table I).

The second fraction of the products of deamination was shown to be III (Figure 2a) by comparison of its ^1H NMR spectra with that of authentic III (Michon et al., 1987) and was formed by the simultaneous deamination of residues f and

k of I. The third fraction contained oligosaccharide X (Figure 7) which was also a product of the deamination of II (Michon et al., 1987), and this was confirmed by the fact that the permethylated contents of the third fraction gave an identical fragmentation pattern in its EI mass spectrum to that previously observed in the EI spectrum of authentic X (Michon et al., 1987).

Oligosaccharide IV. Oligosaccharide IV, the structure of which is shown in Figure 2d, could not be isolated in a pure state because, although it was a product of the basic hydrolysis of the group B antigen, it was inseparable from I by both ion-exchange and molecular exclusion (Figure 1a) chromatography. However, due to its stability to periodate, a large part of IV could be recovered as one of the two major products of the Smith degradation of the group B antigen. In fact, the product, oligosaccharide XI (Figure 7), retained all the constituent sugars of IV except for its nonreducing terminal α -L-rhamnopyranosyl residue. Oligosaccharide XI contained L-rhamnose, D-galactose, 2-acetamido-2-deoxy-D-glucose, and phosphate in the molar ratio of 5:1:2:1, respectively, and its structure was deduced in the following way.

The identification of 2,4-di-O-methylrhamnose as the only methylated rhamnose derivative in the methylation analysis of XI (Table I) indicated that all five rhamnopyranosyl residues in XI were O3-linked. The two terminal pyranosyl residues of XI were also identified in the methylation analysis. The detection of a 2,3,5,6-tetra-O-methylglucosamine derivative confirmed the presence of a terminal O4-linked glucosaminitol residue in XI, which, because it was deuterium labeled at C1, must have originated from a reducing terminal O4-linked 2-acetamido-2-deoxyglucopyranosyl residue in IV. The identification of a nonstoichiometric amount of a 2,3,4,6-tetra-O-methylgalactose derivative provided evidence that a galactopyranosyl residue was situated at the nonreducing terminal end of XI and that it was probably phosphorylated. This was confirmed when a repeat methylation analysis of XI, following its dephosphorylation (treatment with HF), yielded a stoichiometric amount of the 2,3,4,6-tetra-O-methylgalactose derivative.

The FAB-MS spectrum of XI in the positive mode gave pseudo molecular ions at m/z 1466 $[(M + H)^+]$ and 1488 $[(M$

Table IV: Proton Chemical Shifts^a of Some of the Signals of Phosphorylated Oligosaccharide XI

proton	residue							
	b	c	d	e	f	g	h	i
H-1	5.505	4.776	5.046	5.036	5.036	5.014	4.682	
H-2	3.800	3.868	4.295	4.158	4.158	4.160	4.130	
H-3	3.800	3.805	3.930	3.920	3.920	3.925	3.620	
H-4	4.160	3.720					3.470	3.820
H-5		3.490					3.440	

^a Measured at 500.0 MHz and 305 K, assignments made from data obtained by homonuclear 2D shift correlated (H,H) COSY with one- and two-step relays.

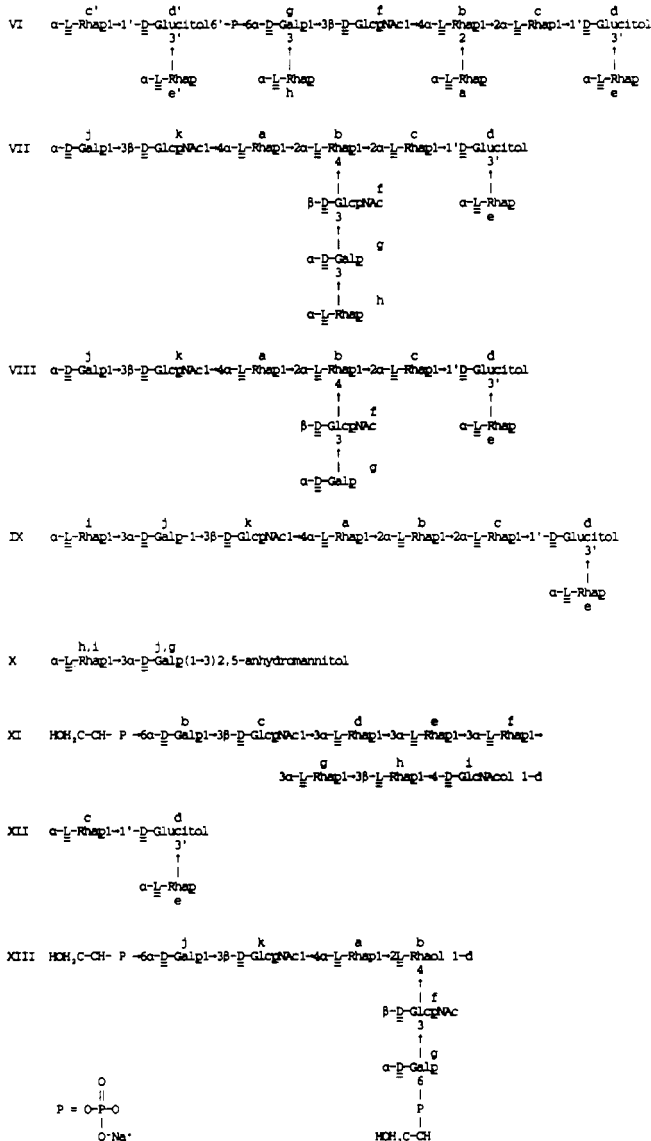


FIGURE 7: Structure of some of the oligosaccharides obtained by chemical and enzymatic degradations of the group B antigen: (VI) rhamnosidase-treated V; (VII and VIII) rhamnosidase-treated I; (IX and X) deamination of I; (XI and XIII) Smith degradation of the group B antigen; (XII) rhamnosidase-treated group B antigen.

+ Na⁺] which are consistent with its proposed structure (Figure 7). The presence of a phosphodiester linkage in XI was demonstrated by examination of its ³¹P NMR spectrum, which exhibited one complex signal at δ 0.6. The position of linkage of the phosphodiester was determined from the ¹³C NMR (DEPT) spectrum of XI in which three carbon-phosphorus coupled signals (doublets) were clearly discernible at δ 62.01, 64.94, and 67.79 (Table III). The signal at 62.01 ppm was assigned to the unsubstituted primary hydroxymethyl carbon of the ethylene glycol substituent (³J_{C,P} = 7.5) while the latter two signals were assigned to the phosphate-substi-

Table V: Nuclear Overhauser Enhancements for Phosphorylated XI

saturated signal ^b	observed negative NOE ^a			
	H ₂	H ₃	H ₄	H ₅
H1b	9 ^c (b)	9 ^c (c)		
H1d	7 (d)	19 (e)		
H1e,f	8 (e, f)	15 (f, g)		
H1g	7 (g)	8 (h)		
H1c		11 (d)		4 (c)
		2 (c)		
H1h	3 (h)	5 (h)	6 (i)	5 (h)

^a Assignments for the enhanced signals were made from data obtained by 2D (H, H) COSY with one- and two-step relays (Table IV). ^b b-i refer to residues b-i as shown in Figure 7. ^c Intensity observed is for the two overlapping signals.

tuted carbons of XI, i.e., C6 of its terminal galactopyranosyl residue (²J_{C,P} = 4.0) and the remaining hydroxymethyl carbon of the ethylene glycol substituent (²J_{C,P} = 5.4), respectively.

The sequence and anomeric configurations of the individual residues of XI were ascertained from ¹H NMR (NOE) data. The chemical shifts of some of the protons associated with the individual residues of XI are listed in Table IV, and they were assigned by 2D (H,H) COSY using both one-step (Bax et al., 1981) and two-step (Bax & Drobný, 1985) relayed coherent transfer. Following assignment the individual anomeric signals of each of the residues (b, c, d, e, f, g, h) in XI (Figure 7) were selectively irradiated, and the observed NOEs are listed in Table V. Irradiation of H1(b) gave enhancements only on H2(b) and H3(c) indicating that the terminal galactopyranosyl residue (b) of XI is in the α -D configuration and is linked to O3 of the adjacent 2-acetamido-2-deoxy-D-glucopyranosyl residue (c). When H1(c) was irradiated, it produced enhancements on H3(c) and H5(c) consistent with c being in the β -D configuration and on H3(d) indicating that c was linked to O3 of the next L-rhamnopyranosyl residue (d). Irradiation of H1 of the consecutive residues d, e, f, and g gave, respectively, enhancements on H2 of d, e, f, and g and on H3 of residues e, f, g, and h, thus demonstrating that following residue c there is in XI a sequence of four O3-linked α -L-rhamnopyranosyl residues. However, irradiation of H1 of the following L-rhamnopyranosyl residue (h) gave a different pattern of enhancements. In this case enhancements on H2(h), H3(h), and H5(h) indicate that, unlike all the other L-rhamnopyranosyl residues in XI, residue h is in the β -L configuration, and an enhancement on H4(i) demonstrates that h is linked to O4 of the terminal glucosaminitol residue (i). Confirmation of the configuration of h was also obtained with the anomeric one-bond ¹³C-¹H coupling constants (¹J_{13C,H}) which are listed in Table III. The magnitude of ¹J_{13C,H} has been demonstrated to be sensitive to change in the anomeric configuration (Perlin et al., 1970), and for h ¹J_{13C,H} = 161 Hz confirms that h is in the β -L configuration. The ¹J_{13C,H} couplings of all the other residues in XI were also consistent with the configurational assignments made on them by NOE experiments. Because IV was contaminated with I, its complete structural detail was determined from FAB-MS analyses

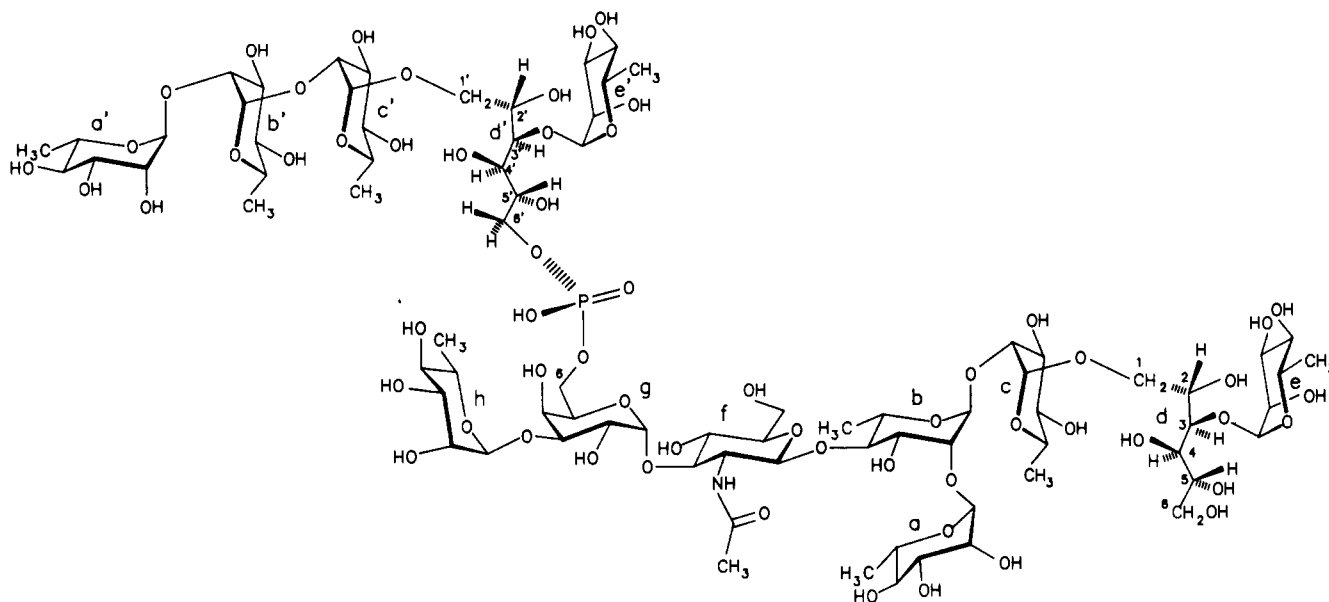


FIGURE 8: Structure of V.

(Figure 4) carried out on the mixture of I and IV obtained from the basic hydrolysis of the group B antigen. In this way it was possible to identify the ions associated with IV by difference, because the FAB-MS spectrum of pure I was available. The pseudo molecular ion m/z 1463 $[(M + H)^+]$ is consistent with a rhamnopyranosyl residue being at the nonreducing terminal end of IV, and this is confirmed by fragment ions at m/z 1317, 1242, and 1096 which are peculiar to IV and are consistent with the proposed fragmentation pattern shown in Figure 3b. Fragment ions in the spectrum of the mixture (Figure 4) at m/z 512, 658, 804, and 950 are probably common to both I and IV because except for some of the linkage positions they both have identical nonreducing terminal hexasaccharide moieties (structures c and d of Figures 2, respectively). The presence of a reducing 2-acetamido-2-deoxy-D-glucopyranose residue at the other terminal end of IV was confirmed by FAB-MS analysis of the reduced (NaBD₄) mixture of I and IV. In this spectrum the pseudo molecular ion m/z 1463 assigned to IV above (Figure 3b) was shifted by 3 m.u. to m/z 1466, consistent with the formation of a terminal monodeuterated glucosaminitol residue. The α -L configuration of the nonreducing terminal rhamnose residue (a) of IV (Figure 2d) was ascertained on the basis of its cleavage by the exo(α -L)rhamnosidase contained in the commercial preparation of naringinase (data not shown).

Structure of V. Oligosaccharide V was obtained from the partial alkaline hydrolysis of the group B antigen (Figure 1a), and its structure is shown in Figure 8. The structure is based on the following evidence. When V was subjected to complete alkaline hydrolysis, it yielded two smaller oligosaccharides (Figure 1c) which following dephosphorylation (alkaline phosphatase) were identified as II and III because of their identical ¹H NMR and ¹³C NMR spectra and methylation analysis with those of authentic II and III (Michon et al., 1987). As in the original group B polysaccharide, II and III were linked by a phosphodiester bond, and this was exemplified by the detection of a phosphorus signal at δ 0.97 in the ³¹P NMR spectrum of V identical in shape and chemical shift with the equivalent phosphorus signal previously detected in the ³¹P NMR spectrum of the group B antigen (Michon et al., 1987).

The sequence of II and III in V was determined by the treatment of V with an exorhamnosidase (naringinase). The resultant product (VI) of enzyme hydrolysis (Figure 7) was

purified by gel filtration and examined by both ¹H NMR and ¹³C NMR spectroscopy. Comparison of the chemical shifts of its anomeric proton signals and anomeric carbon signals with those of the equivalent assigned signals in the starting compound (V) (Tables II and III, respectively) indicated that the enzyme had only removed residues a' and b' from the III moiety of V (Figure 8). Because treatment of III alone with the exorhamnosidase gave an identical pattern of cleavage (Michon et al., 1987), it implies that III is a terminal residue in V and that the 1→2-linked α -L-rhamno-triose moiety of III is not involved in the linkage of III to II. Thus III cannot constitute the repeating unit of the group B polysaccharide, the possibility of which had been previously proposed (Michon et al., 1987). It is interesting to note that while the II moiety of V was not affected by the exorhamnosidase, the terminal α -L-rhamnopyranosyl residue (h) of the trisaccharide branch in oligosaccharide II is readily cleaved (Michon et al., 1987). The stability of residue h in V to the enzyme indicates either that it is the linkage point in V or that it is in close proximity to the linkage point. That the latter situation was correct was confirmed by ¹³C NMR spectroscopy and chemical techniques.

The position of the phosphodiester linkage between III and II in V was first deduced by ¹³C NMR spectroscopic analysis, and the chemical shifts and ³¹P-¹³C coupling constants associated with V are listed in Table III. The ¹³C NMR (DEPT) spectrum of V indicated that it contained two phosphorylated hydroxymethyl carbon signals (Jennings & Smith, 1978), which were doublets having ²J_{P,C} of 4.0 Hz and 5.7 Hz, respectively. The two signals were assigned to C6' and C6 of residues g and d' of V, respectively, by comparison with previous assignments made on model compounds. The signal at 67.81 ppm was easily assigned to C6' of the D-glucitol residue (d') because it had a chemical shift similar to that of the equivalent signal of III phosphorylated at C6 of its glucitol residue (δ 67.24) (Michon et al., 1987). The doublet at δ 64.66 could then be assigned to either residue g, residue f, or residue d of the II moiety of V. It was eventually assigned to C6 of the α -D-galactopyranosyl residue (g) of V on the following evidence.

Oligosaccharide V was oxidized with periodate and following reduction with sodium borohydride was permethylated and subjected to acid hydrolysis. A 4,6-di-O-methylglucosamine derivative was detected together with 3-O-methylrhamnose

(Table I), which is consistent with residues f and b of the II moiety of V being resistant to periodate oxidation. Failure to detect a stoichiometric amount of the 2,4,6-tri-*O*-methylgalactose derivative, when the O3-linked α -D-galactopyranosyl residue (g) of II (Figure 2b) should be periodate resistant, indicated that the phosphodiester linkage must be situated on residue g. This was confirmed when, following the dephosphorylation (48% aqueous HF) and reacetylation of the hydrolyzate of oxidized and reduced permethylated V, 2,4-di-*O*-methylgalactose was detected in GC-MS together with the three previously identified methylated glycose derivatives. Thus the phosphodiester bond in V is linked from O6' of the D-glucitol residue (d') of III to O6 of the α -D-galactopyranosyl residue (g) of II. Further evidence that the above linkage was present throughout the group B polysaccharide was obtained by exorhamnosidase treatment of the whole group B polysaccharide.

Rhamnosidase Treatment of the Group B Polysaccharide. Following treatment with the exorhamnosidase (naringinase), the group B antigen was subjected to complete alkaline hydrolysis (Michon et al., 1987), and the individual fragments were dephosphorylated and separated by gel filtration as previously described (Michon et al., 1987). The elution profile was only substantially different from that obtained by the complete alkaline hydrolysis of the native group B antigen in that a peak corresponding to III was not detected in this hydrolyzate. In this case the elution profile of the hydrolyzed rhamnosidase-treated group B antigen exhibited a unique peak corresponding to an oligosaccharide component (XII) smaller than III. The oligosaccharide (Figure 7) was recovered from the eluate, and ^1H NMR spectroscopic (Table II) and methylation analysis (Table I) indicated that it had originated by the consecutive loss of the two α -L-rhamnopyranosyl residues a and b from the III moiety of the group B antigen. Thus the pattern of enzymatic cleavage of the III moiety in the group B polysaccharide is identical with that previously reported for the free oligosaccharide III (Michon et al., 1987). Verification that I, II, and IV were intact following the above treatment was obtained by ^1H NMR spectroscopic analysis of the components contained in each of the other two fractions in the elution profile. Both spectra were identical with those of the equivalent fractions from the alkaline hydrolysis of the native group B antigen, corresponding to II in one fraction and a mixture of I and IV in the other.

Structure of XIII. Smith degradation of the group B antigen yielded, in addition to XI, oligosaccharide XIII as the other major periodate-stable fragment, and the structure of the latter is shown in Figure 7. Oligosaccharide XIII contained L-rhamnose, D-galactose, 2-acetamido-2-deoxy-D-glucose, and phosphate in the molar ratio of 2:2:2:2, and methylation analysis of XIII yielded the methylated glycose derivatives listed in Table I. The detection of 1,3,5-tri-*O*-methylrhamnose labeled at C1 with deuterium indicated that XIII originated from a branched structure in the group B antigen with a rhamnopyranosyl residue linked at both O2 and O4. This evidence plus the analytical data was sufficient to identify XIII as a degraded fragment of I, and this was further confirmed by the detection of 2,3-di-*O*-methylrhamnose and 4,6-di-*O*-methylglucosamine in the methylation analysis of XIII, these latter methylated derivatives being indicative of O4-linked rhamnopyranosyl and O3-linked 2-acetamido-2-deoxyglucopyranosyl residues.

The identification of a nonstoichiometric amount of 2,3,4,6-tetra-*O*-methylgalactose provided evidence that, as in the case of XI, oligosaccharide XIII contained terminal

phosphorylated galactopyranosyl residues, and this was substantiated by a further methylation analysis of XIII following its dephosphorylation (48% aqueous HF), in which a sufficient amount of the above methylated derivative was detected to establish that XIII contained two such residues whereas XI only contained one (Table I). That both the galactopyranosyl residues of XIII and that of XI were all similarly substituted at O6 with ethylene glycol phosphate was established by comparison of the ^{31}P NMR and ^{13}C NMR spectra of XI and XIII. The ^{31}P NMR spectra of XIII, like that of XI, exhibited only one complex signal at δ 0.6. In addition, the chemical shifts and ^{31}P - ^{13}C coupling constants associated with the ethylene glycol carbons and C6 of the galactopyranosyl residues of both XI and XIII were very similar (Table III). As in the case of XI, the position of the phosphate linkage in XIII was also established from its ^{13}C NMR (DEPT) spectrum.

DISCUSSION

Group B streptococci can be defined immunologically on the basis of the presence of common determinants. These determinants are structural entities of the group B polysaccharide antigen which is present in all strains (Lancefield, 1933, 1934, 1938), and therefore, it is important to be able to identify these determinants in order to understand more precisely the structural basis of the antigenic classification of group B streptococci.

Pritchard and co-workers (Pritchard et al., 1981, 1984) were able to demonstrate that the group B antigen has a teichoic acid like structure, the repeating unit being linked through glucitol phosphate esters. They also proposed some rudimentary structural features of the repeating unit. However, although the above work provided insights into some of the features of the group B antigen, they were unaware of its unique and highly complex and heterogeneous nature. This was first established by Michon et al. (1987) when complete alkaline hydrolysis of the group B antigen yielded three different alkali-stable oligosaccharides (I, II, and III) obtained in unequal amounts (3:4:1, respectively). The structures of II and III were also elucidated by a number of degradative and spectroscopic techniques.

Methylation analysis of I, obtained from both the complete (Michon et al., 1987) and partial alkaline hydrolysis (Table I), indicated that it contained structural features not found in either II or III. The detection of 2,3-di-*O*- and 2,4-di-*O*-methylrhamnose was indicative of the presence of unique O3- and O4-linked rhamnose residues in I. However, it was subsequently discovered that I, obtained by both alkaline hydrolyses, was in fact a mixture of two oligosaccharides (I and IV), the O3-linked rhamnose residues belonging exclusively to IV. The evidence for this was that, although I and IV were inseparable by both ion-exchange and molecular exclusion chromatography (Figure 1a), I and a large fragment of IV could be obtained in a pure state by use of different isolation procedures. For example, in contrast to the native group B antigen, complete alkaline hydrolysis of fraction 1, obtained from the partial alkaline hydrolysis of the native group B antigen (Figure 1a), yielded the same components (I, II, and III) except that in this case I was obtained free of IV. Also, because of the inate stability of IV to periodate oxidation, a large part of it was recovered from the Smith degradation of the group B antigen.

The structures of the two complex dephosphorylated oligosaccharides (I and IV) (structure c and d of Figure 2, respectively) were determined with a number of degradative and spectroscopic techniques including NMR, GC-MS, and FAB-MS. These structural studies were also facilitated by

the isolation of a number of key fragment oligosaccharides (Figure 7) obtained by both chemical and enzymatic (exorhamnosidase) degradation of I and IV. In the case of I, this evidence showed that it was closely related to both II and III, in that III was found to be a part of II and II was found to be a part of I (Figure 2c). The additional structural feature added to III to give the larger oligosaccharides (II and I) was respectively one or two identical terminal α -L-Rhap1 \rightarrow 3 α -D-Galp1 \rightarrow 3 β -D-GlcNAc.

Although IV also contained the above terminal trisaccharide moiety, its overall structure (Figure 2d) was quite unique. The trisaccharide was linked to O3 of the terminal α -L-rhamnopyranosyl residue of an O3-linked L-rhamno-pentose, which in turn was linked to O4 of a reducing terminal 2-acet-amido-2-deoxyglucopyranosyl residue. Of particular note was the fact that the L-rhamnopyranosyl residue glycosidically linked to the latter reducing residue is the only β -L-linked rhamnopyranosyl residue in IV and also in the whole group B antigen.

The isolation of I, II, and III as their monophosphate esters (IP, IIP, and IIP) from the basic hydrolysis of group B antigen (Michon et al., 1987) is compatible with its oligosaccharide units being linked together through phosphodiester bonds as previously proposed (Pritchard et al., 1984). That IIIP was obtained as a mixture of two monophosphate esters, the ester being located at either O5 or O6 or its D-glucitol residue (Michon et al., 1987), was interpreted in two ways. Either heterogeneity exists in the group B antigen phosphodiester linkages or phosphate migration (Egan et al., 1982) occurs on alkaline cleavage of homogeneous phosphodiester bonds in the group B antigen. Consistent with the latter interpretation, the ^{31}P NMR spectrum of the group B polysaccharide exhibited only one major phosphorus signal (Michon et al., 1987); this is also confirmed by structural studies on some of the oligosaccharide constituents of the group B antigen.

Oligosaccharide V (Figure 8), obtained by the partial alkaline hydrolysis of the group B antigen, contains both III and II linked together by an intact phosphodiester bond. That this bond is representative of all the phosphodiester bonds in the group B antigen was indicated by the fact that its ^{31}P NMR spectrum exhibited an identical phosphorus signal having the same chemical shift as that exhibited in the ^{31}P NMR spectrum of the group B antigen (Michon et al., 1987). ^{13}C NMR studies on V revealed that the phosphodiester bond was linked from O6 of the D-glucitol residue of III to O6 of the α -D-galactopyranosyl residue of II, and this was confirmed by treatment of V with naringinase. The specificity of this enzyme in relation to oligosaccharides III and II has been previously established (Michon et al., 1987). However, while the exorhamnosidase was still able to remove the terminal rhamnose moiety from III even when it was a constituent of V, it was not able to remove the normally sensitive terminal α -L-rhamnopyranosyl residue from II (Michon et al., 1987). Presumably the phosphodiester linkage at O6 of the α -D-galactopyranosyl residue of V renders the adjacent terminal α -L-rhamnopyranosyl residue inert to cleavage by this enzyme. Further evidence that all the phosphodiester bonds in the group B antigen are linked to α -D-galactopyranosyl residues was obtained by studying the fragments from a Smith degradation of the group B antigen. Two fragments (XI and XIII) (Figure 7) which originated from the IV and I moieties, respectively, of the group B antigen had all their α -D-galactopyranosyl residues substituted with ethylene glycol phosphate.

The final task in the structural elucidation of the group B antigen is to determine how all of its different component

oligosaccharides (I, II, III, and IV) are assembled. Evidence has been provided that all the oligosaccharides are linked through phosphodiester bonds and that despite their structural differences all the linkages between the oligosaccharides appear to be homogeneous. It has also been established that all the phosphodiester linkages are attached from O6 of a D-glucitol residue to O6 of an α -D-galactopyranosyl residue. This provides an important clue as to how the group B antigen is assembled, because as III and IV contain only one each of the linkage residues (D-glucitol and α -D-galactopyranosyl, respectively) they must be situated in terminal positions in the group B antigen. In addition, the presence of one of each of the above linkage residues in II implies that it is an interchain residue, and because I has two α -D-galactopyranosyl residues and one D-glucitol residue, it constitutes a potential branch point in the group B antigen.

Confirmatory evidence for the above hypothesis was obtained by treating the group B antigen with an exorhamnosidase (naringinase) (Michon et al., 1987). This enzyme removed only the terminal 1 \rightarrow 2-linked α -L-rhamnose unit from component III and left components I, II, and IV intact. Thus III must be the nonreducing terminal residue of the group B polysaccharide, and because of the sequence data obtained from the structural elucidation of V, it must always be linked through a phosphodiester bond to II. It is significant that the enzyme did not remove any terminal α -L-rhamnopyranosyl residues from components I, II, and IV of the group B antigen, because in the form of their free oligosaccharides II (Michon et al., 1987), I, and IV are susceptible to this enzyme. Studies on V indicate that this stability is due to the presence of a phosphate linkage on O6 of an α -D-galactopyranosyl residue adjacent to a normally susceptible terminal α -L-rhamnopyranosyl residue. Thus all of the α -D-galactopyranosyl residues of I, II, and IV must be substituted in this way. This is consistent with both I and II being interchain residues and furthermore with I being a branch point in the group B antigen. Additional evidence for the latter was obtained when XIII, a fragment originating from I, was obtained from the Smith degradation of the group B antigen with both its α -D-galactopyranosyl residues substituted with ethylene glycol phosphate. The above evidence is also consistent with IV being the reducing terminal oligosaccharide moiety of the group B antigen. This was confirmed when it was discovered that IV could be deuterium labeled at its terminal 2-acet-amido-2-deoxyglucopyranosyl residue although there is evidence to suggest that IV probably exists as a glycosidic phosphate derivative in the original group B antigen. This can be deduced from the following proposed sequence of events. The glycosidic phosphate which stabilizes the terminal residue of IV during the periodate oxidation step of the Smith degradation is subsequently cleaved during the final acid hydrolysis step. This then leaves the terminal 2-acetamido-2-deoxyglucopyranose residue vulnerable to reduction with sodium borodeuteride. It is interesting to speculate that such a terminal glycosidic phosphate group may be an important component in the linkage of the group B antigen to the group B streptococcal cell wall, and there is evidence to suggest that the group B antigen is in fact covalently linked to the peptidoglycan of the above organisms (Yeung & Mattingly, 1986).

The simplest structure for the group B antigen that satisfies all the above criteria is shown in Figure 9. A tetraantennary structure was preferred in order to accommodate the molecular size (approximate average M_w 20 000) of the group B antigen, which was deduced on the basis of the molar ratios of 3:8:4:1 found for the component oligosaccharides I, II, III,

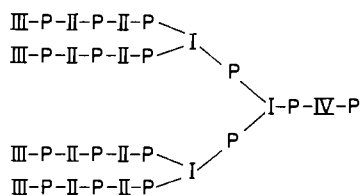


FIGURE 9: Tentative overall structure for the group B antigen.

and IV, respectively. That I and IV were in an approximate ratio of 3:1 was deduced from the ^1H NMR spectrum of the contents of the fraction containing I and IV obtained by the complete alkaline hydrolysis of the group B antigen (Michon et al., 1987). Integration of the H_2 (d) signal of residue IV (Table IV) compared with that of H_1 (b) of I (Table II) gave an approximate ratio of 1:3, respectively. For simplicity, we have presented a symmetrical structure, but there is no evidence to rule out other possible structures. Internal sequence data are difficult to obtain, and our only success to date has been to identify the sequence II-P-II which was obtained by controlled HF treatment of the group B antigen (Michon and Jennings, unpublished results). This is consistent with the proposed structure, but the group B antigen could still consist of a number of other possible symmetrical or unsymmetrical structures or a mixture of any combination of the above structures. Alternative structures could be produced by simply interchanging II in the four branches of the group B antigen or locating it between the I-P-I or I-P-IV sequences. Obviously the total elucidation of the structure of the group B antigen is an extremely difficult task and may be virtually impossible should it prove to be very heterogeneous. However, the structure depicted in Figure 9 is probably highly representative of the group B antigen.

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