Structure of the Complex Group-Specific Polysaccharide of Group B *Streptococcus*†

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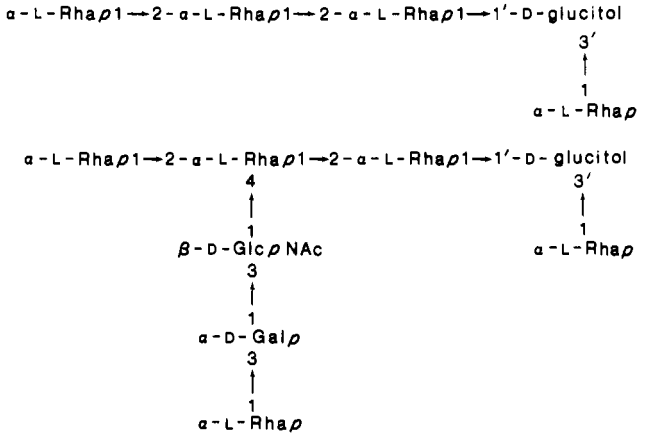
Received May 27, 1986; Revised Manuscript Received September 16, 1986

ABSTRACT: The group-specific antigen was isolated from a type Ia group B streptococcal strain and is a complex polysaccharide composed of α-L-rhamnopyranosyl, α-D-galactopyranosyl, 2-acetamido-2-deoxy-β-D-glucopyranosyl, D-glucitol, and phosphate residues. The complexity of the group B polysaccharide antigen is evident from the fact that when depolymerized by basic hydrolysis it yielded three structurally related, but nevertheless significantly different, oligosaccharides. These oligosaccharides were obtained in different molar quantities as their monophosphate esters. This evidence strongly suggests that they are linked by phosphodiester bonds in the original group B antigen. If these oligosaccharides are in fact randomly situated throughout the linear polysaccharide, then this type of heterogeneous repeating unit is unusual for a polysaccharide of bacterial origin. However, this structural arrangement of the oligosaccharides has yet to be unambiguously established because the alternate explanation of there being three different polysaccharides in the group B antigen cannot be discounted in the evidence presented here. The oligosaccharides were enzymatically dephosphorylated, and the structures of two of the three oligosaccharides are



Despite their structural differences, the two oligosaccharides are related by the smaller being an integral part of the larger. In the structural analysis of the group B antigen, methylation analysis, periodate oxidation, nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry, fast atom bombardment mass spectrometry, and various specific chemical and enzymatic degradations were the principal methods used. Of particular interest was the use of an α-rhamnosidase to selectively degrade the larger oligosaccharide. This facilitated the assignment of signals in its ^{1}H and ^{13}C NMR spectra.

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 has been shown to contain 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 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 information and have in addition 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. While the structural studies presented in this paper indicate that some of the aforementioned structural detail proposed for the group B antigen is correct, it is far from complete and presents a greatly oversimplified picture of the highly complex group B antigen.

† This work was supported in part by NIH Grant A120626-01. This is National Research Council of Canada Publication No. 26707.

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Experimental Procedures

Growth of the Organism and Purification of the Group Antigen. Prototype strain 090, type Ia, group B streptococcus

0006-2960/87/0426-0476$01.50/0

was originally obtained from the late Dr. Rebecca Lancefield. The lyophilized strain was rehydrated with Todd-Hewitt broth (Difco) and incubated overnight at 37 °C on a blood agar plate. Strain 090 was inoculated from the blood agar plate into two nephalometry flasks containing 50 mL each of Columbia broth (Difco), and after 3 h growth into mid-late log phase, the organisms are transferred to an Erlenmeyer flask containing 50 mL of Columbia broth supplemented with 8% glucose. After incubation for 5–6 h at 37 °C, the contents of the starter flask were transferred to 16 L of the same broth in a Biolafite fermentor (France). The organisms were grown for 24 h at 37 °C, and the pH of the medium was maintained at 7.2 by neutralization of the acidic metabolic products with 10 M NaOH using the titrator. A small aliquot of organisms was removed and checked for purity by subculture on aerobic and anaerobic plates.

The organisms were removed from the culture supernatant by centrifugation in a Beckman Model J-6B centrifuge at 5000*g* for 15 min. The supernatant was filtered through a series of membranes of smaller pore sizes on a Millipore filter apparatus (Millipore Corp., Bedford, MA). After being passed through a 0.4-μm filter, the culture supernatant was concentrated to 200 cm^3 on a Pellicon cassette system (Millipore) using a 10 000 molecular weight pore-size cassette. The retentate was washed 4 times with 100 cm^3 of 10 mM tris(hydroxymethyl)aminomethane (Tris)[1] buffer, pH 7.2. The washed supernatant was fractionated with 30% (v/v) ethyl alcohol (4 °C) and the precipitate discarded. The supernatant was adjusted to 80% ethyl alcohol (4 °C) and after sitting for 18 h at 4 °C, centrifuged. the precipitate was solubilized in 10–20 cm^3 of 10 mM Tris buffer, pH 7.2, with 0.001 M $MgCl_2$ and 0.001 M $CaCl_2$ added. The solution was treated with DNase, RNase, and Pronase as previously described (Kasper et al., 1978) and dialyzed exhaustively against H_2O. The solution was chromatographed on DEAE-Sephacel (Pharmacia) equilibrated in 10 mM Tris buffer, pH 7.2 (250 cm^3 bed volume). Group B activity was eluted separately from type Ia serological activity by using a 2-L gradient from the starting buffer to 0.2 N NaCl in 10 mM Tris. The fractions were tested for serologic activity by capillary precipitation using the specific antisera defined by McCarty and Lancefield (1955), and the group B activity eluted as a single peak at 5.2 ms/cm osmolarity. All fractions containing only group B activity were pooled, concentrated on an Amicon ultrafiltration membrane (PM-10), and exhaustively dialyzed against distilled water. The final material was lyophilized and found to be free of contaminants absorbing at 260 or 280 nm.

Serological Methods. Group-specific and type Ia specific rabbit antiserum was made to strain 090R and strain 090, respectively, according to the method of McCarty and Lancefield (1955).

Analytical Methods. Solutions were concentrated under reduced pressure below 40 °C. Phosphorus was determined by the method of Chen et al. (1956). Analysis for glycose constituents was carried out essentially by the method of Dmitriev et al. (1975) as previously described (Jennings et al., 1980). Following hydrolysis (0.5 M trifluoroacetic acid at 100 °C for 16 h) of the polysaccharide, the individual glycoses were reduced and peracetylated (Sawardeker et al., 1965) and identified by GLC–MS using column i. The configuration (D or L) of the monosaccharide components was achieved by the method of Gerwig et al. (1979) by GLC analysis of their (−)-butylglycosides on column ii. The D configuration of glucitol was ascertained by its failure to be enzymatically converted to D-glucose (Root et al., 1985). A dephosphorylated oligosaccharide (III) component (10 mg) from the group B polysaccharide (see Preparation of Oligosaccharides I, II, and III) was hydrolyzed with 0.5 M trifluoroacetic acid at 100 °C for 16 h. The hydrolysate was then added to 0.1 M phosphate buffer at pH 7.0 and treated with 60 units of D-galactose oxidase (EC 1.1.3.9) (Millipore Corp., Feeehold, NJ) in the presence of 90 units of horseradish peroxidase (EC 1.11.1.7) (Sigma, St. Louis, MO). After 48 h, the solution was monitored for the presence of D-glucose using D-glucose oxidase (EC 1.1.3.4) (Sigma, St. Louis, MO) according to the procedure of Roth et al. (1965).

Methylation Analysis. The polysaccharide and oligosaccharides were methylated with methyl or trideuteriomethyl iodide in the presence of methylsulfinyl anion according to the method of Hakomori (1964). The products were then purified on a small Sephadex LH-20 column (20 cm × 0.5 cm) using chloroform as eluant. The fractions were monitored by spotting the eluant on silica gel TLC plates, spraying the plates with 10% H_2SO_4 in ethanol, and charring the plates in an oven for 5 min at 130 °C. Fractions shown to contain permethylated polysaccharide or oligosaccharides were pooled and evaporated to dryness, and the residue was hydrolyzed with 0.5 M trifluoroacetic acid for 16 h at 100 °C. Following evaporation of the acid, the partially methylated monomers were reduced with $NaBH_4$ or $NaBD_4$ and acetylated, and the products were analyzed by GLC–MS (Lindberg, 1972) using column ii. Permethylated oligosaccharides were analyzed directly by GLC–MS in both EI and CI modes using columns ii and iii. The location of the phosphate groups in the polysaccharide and oligosaccharides was achieved essentially as described by Feige and Radziejewska-Lebrecht (1979). Following methylation (Hakomori, 1964), the phosphate groups were removed from the methylated derivatives by using 48% hydrogen fluoride (3 days at 4 °C), and then the dephosphorylated, methylated oligosaccharides were remethylated by using trideuteriomethyl iodide. The individual methylated components were then identified by GLC–MS analysis by methods described previously.

Instrumental Methods. Optical rotations were determined on a Perkin-Elmer 243 instrument using 1-dm semimicro cells at 23 ± 1 °C.

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 m) containing 3% OV17 operated at 180–230 °C at 2 °C/min and held for 10 min at 230 °C. Combined gas–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 Dexsil 300 glass column (iii) operated at 150–330 °C at 10 °C/min. Chemical ionization (CI) spectra were obtained by using methane or isobutane as the carrier and reagent gas at a source temperature or 150 °C and an ionization voltage of 230 eV.

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; GLC, gas–liquid chromatography; GLC–MS, gas–liquid chromatography–mass spectrometry; EI, electron impact; CI, chemical ionization; FAB-MS, fast atom bombardment mass spectrometry; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

Fast atom bombardment (FAB) spectrometry was carried out in the Department of Chemistry, Ottawa University, by using a VG7070E (Manchester, England) mass spectrometer at an accelerating voltage of 6 kV in the positive and negative modes. Xenon was used as the bombarding gas, and the atom gun was operated at 9 kV and 1.2 mA. FAB mass spectra were obtained by loading 40–100 μg of substance onto a drop of glycerol on the stainless-steel target. The mass units were calibrated by using glycerol.

^{13}C and ^{1}H NMR spectra were recorded on a Bruker AM500 spectrometer. Acetone was used as an internal chemical shift reference for ^{1}H NMR (2.225 ppm) and for ^{13}C NMR (31.07 ppm). ^{13}C NMR spectra were recorded at 310 K in 5-mm tubes at concentrations of 20–30 mg in 0.4 mL of D_2O at 125.0 MHz in the pulsed Fourier-transform mode, and coupling constants ($^{1}J_{C1,H1}$) were determined by the gated ^{1}H-decoupling technique to retain nuclear Overhauser enhancements. ^{1}H NMR spectra were recorded at 343 K at 500.0 MHz in 5-mm tubes. Samples (1–5 mg) were exchanged 3 times with 99.7% D_2O and finally dissolved in 0.4 mL of 99.99% D_2O.

The ^{13}C–H shift correlation with proton decoupling in the F1 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 F2 domain and 15 Hz per point in the F1 domain.

^{31}P NMR spectra were obtained in the Fourier-transform mode on a Bruker CXP 3000 instrument. Spectra were recorded at 121.5 MHz at 303 K in 10-mm tubes containing 45–75 mg of oligosaccharide or polysaccharide in 1.5 mL of D_2O. Paramagnetic contaminants were removed from the samples by chromatography on Chelex-100 (Bio-Rad Laboratories). The spectrometer was field–frequency locked on the deuterium resonance of D_2O, and chemical shifts are reported in parts per million from the external reference signal of 85% H_3PO_4, contained in a sealed capillary tube, taken as δ 0.00. Polysaccharide samples were recorded at different pHs (3.6, 7.0, and 8.8) with the decoupler on.

Preparation of Oligosaccharides I–III. The streptococcal group B antigen was subjected to alkaline hydrolysis in order to break phosphodiester bonds. To optimize the hydrolysis, the native group B polysaccharide (2.5 mg) was treated with 0.5 M NaOH (0.5 mL) at 20 and 50 °C over a period of 72 h. Aliquots were removed at intervals, and the amount of phosphomonoester generated was measured in the following way. The aliquots were neutralized and treated with alkaline phosphatase (EC 3.1.3.1) (Millipore Corp., Freehold, NJ), and the inorganic phosphate was determined by the method of Chen et al. (1956). The optimal conditions are described below.

The group B specific polysaccharide (100 mg) was treated with 0.5 M NaOH (2.0 mL) for 48 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 0.02 M pyridinium acetate (pH 5.4) and fractionated by passage through a Bio-Gel P-4 (Bio-Rad Laboratories) column using the above buffer as eluant. The eluate was monitored for the appearance of the oligosaccharides by using a Waters R403 differential refractometer, and fractions were also monitored for hexose (Dubois et al., 1956) and phosphate (Chen et al., 1956). The phosphorylated oligosaccharides, numbered in order of their elution from the column, were obtained in the approximate ratio of 3:4:1, respectively. The phosphorylated oligosaccharides were then individually treated with alkaline phosphatase to yield the dephosphorylated oligosaccharides (I, II, and III).

HF Treatment of the Streptococcal Group B Polysaccharide. The group B polysaccharide (100 mg) was treated at 4 °C with 48% HF (1.5 mL), a reagent which has been shown to promote the facile cleavage of phosphate esters (Lipkin et al., 1969). Samples (10 μL) were withdrawn, neutralized, and analyzed for the release of inorganic phosphate by using the method of Chen et al. (1956). When the amount of free phosphate had maximized (48 h), the reaction mixture was diluted with water, neutralized with Dowex 1 × 8 (OH^-) ion-exchange resin, and lyophilized. The residue was then applied to a Bio-Gel P-2 column (Bio-Rad Laboratories) in aqueous solution and eluted with water. The column eluate was monitored by using a Waters R403 differential refractometer, and among other oligosaccharides, three pure oligosaccharides (III, IV, and V) were isolated and identified by methylation and ^{1}H NMR analyses.

Removal of Terminal Rhamnose Residues from II. Oligosaccharide II (15 mg) was dissolved in 200 mM sodium citrate buffer at pH 5.0 and treated with 10 units of Naringinase from *Penicillium* species (EC 3.2.1.40, 400 units/g) (Sigma, St. Louis, MO). After 24 h at 40 °C, the degradation products were separated on a Bio-Gel P-4 column (Bio-Rad Laboratories) in a 0.02 M pyridinium acetate buffer at pH 5.4. The column eluate was monitored with a Waters R403 differential refractometer, and in addition to undegraded II, two partially separated smaller oligosaccharides (VI and VII) were identified. Oligosaccharides VI and VII were purified by rechromatography of the fraction of the eluate containing them on the above column.

Deamination of II. Oligosaccharide II (15 mg) was dissolved in 2 mL of 2 M NaOH to which sodium borohydride (2 mg) was added. The solution was sealed in a glass tube which was heated at 100 °C for 10 h. After being cooled, the solution was neutralized with 2 M HCl, desalted on a Sephadex G-15 column (Pharmacia) using water as eluant, and lyophilized. The N-deacetylated II was then deaminated essentially by the method of Dmitriev et al. (1975) as described by Jennings et al. (1983). The products of the deamination were fractionated on a Bio-Gel P-4 column (Bio-Rad Laboratories) with 0.02 M pyridinium acetate buffer at pH 5.4 using a Waters R403 differential refractometer to monitor the eluate. Two major fractions were identified as III and VIII by methylation and NMR spectroscopic analysis.

Periodate Oxidation of II. Oligosaccharide II was subjected to a Smith degradation as described by Goldstein et al. (1965). Oligosaccharide II (25 mg) was treated with 0.05 M $NaIO_4$ (10 mL) and the mixture kept at room temperature in the dark for 72 h. Excess periodate was precipitated from the solution by using Ba_2CO_3, and the product of oxidation was reduced by the addition of sodium borohydride (15 mg). Excess sodium borohydride 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 1% acetic acid for 1 h at 100 °C and lyophilized, and the products of hydrolysis were reduced with sodium borodeuteride in aqueous solution. The products were fractionated on a Bio-Gel P-2 column (Bio-Rad Laboratories) using water as the eluate, and the resultant oligosaccharides (IX, X, and XI) were detected in the eluate using a Waters R403 differential refractive index monitor.

RESULTS

Composition of the Group B Antigen. Analytical results

Table I: Component Analyses of Group B Antigen and Its Phosphorylated Oligosaccharide Constituents

component	molar ratio			
	group B antigen	IP	IIP	IIIP
rhamnose[a]	5.6	6.5	4.9	3.8
galactose[a]	1.0[c]	2.0[c,d]	1.0[c]	
glucitol[a]	0.5	0.7	0.7	1.0[c]
N-acetylglucosamine[a]	0.8	1.7	0.8	
phosphorus[b]	1.0	1.0	1.0	1.0

[a] Estimated following dephosphorylation, hydrolysis, and deamination. Glucosamine was estimated as its 2,5-anhydromannitol derivative. [b] Estimated by the method of Chen et al. (1956). [c] Selected as unity reference. [d] Ratio consistent with NMR and FAB-MS data (Jennings et al., unpublished experiments).

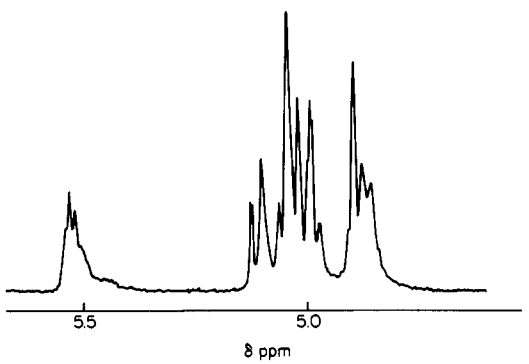

FIGURE 1: Partial ^1H NMR spectrum (anomeric proton region) of the group B polysaccharide antigen in D_2O at 330 K.

indicate that the group B antigen contains L-rhamnose, D-galactose, 2-acetamido-2-deoxy-D-glucose, D-glucitol, and phosphate in the molar ratio of 5.6:1.0:0.8:0.44:1.0, respectively (Table I). The result confirms the previous identification of these components in the group B antigen as described by Pritchard et al. (1981, 1984). It is interesting to note that the D configuration of glucitol was ascertained by its inability to be oxidized by D-galactose oxidase. This enzymatic oxidation is known to occur with the L enantiomer of glucitol (Root et al., 1985). Variability in the component analysis of different preparations of the group B antigen, in which, for example, the molar ratio of L-rhamnose to phosphate varied from 4.9:1.0 to 6.1:1.0, gave some preliminary indication of its heterogeneity. This was confirmed by the complexity of the anomeric proton signals observed in the anomeric region (δ 4.8–5.5) of the ^1H NMR spectrum of the group B antigen (Figure 1) and was especially apparent in the complex signal at δ 5.5, which was later assigned to the anomeric proton of the α-D-galactopyranosyl residues. The fact that this signal was not a simple doublet ($^3J_{H1,H2}$) is indicative of the galactopyranosyl residues being situated in different environments within the group B polysaccharide. The ^1H NMR spectrum of the group B polysaccharide also served to confirm the accuracy of the component analysis, when integration of the CH_3 signals of the 2-acetamido-2-deoxy-D-glucopyranosyl (singlet at δ 2.067) and L-rhamnopyranosyl (multiplet centered at δ 1.29) residues gave an intensity ratio of 1.0:5.8, consistent with the analytical result reported previously for this particular preparation of the group B polysaccharide (Table I).

The heterogeneous nature of the group B antigen was also confirmed by the identification of different oligosaccharides obtained as products of its alkaline hydrolysis. Complete depolymerization of the group B polysaccharide by alkaline hydrolysis yielded three different phosphorylated oligosaccharides as the major components which were separated by column chromatography. The elution profile of the separation is shown in Figure 2, and the oligosaccharides were

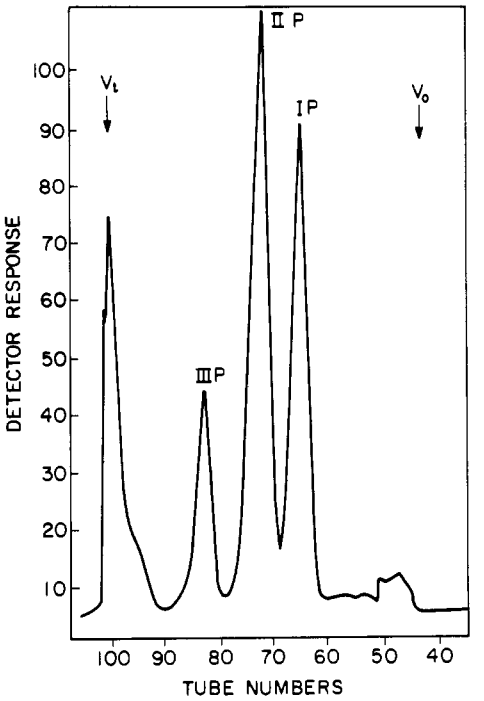

FIGURE 2: Chromatographic separation of phosphorylated oligosaccharides (IP, IIP, and IIIP) using a Bio-Gel P4 column and 0.02 M pyridinium acetate buffer at pH 5.4.

designated IP, IIP, and IIIP in order of their elution from the molecular exclusion column, the largest one being eluted first. The oligosaccharides were obtained on comparative yields of approximately 3:4:1, respectively. The fact that the group B polysaccharide was completely depolymerized under basic conditions indicated that it was composed of a linear arrangement of oligosaccharides linked through phosphodiester bonds, and this was confirmed by the observation of only one phosphorus signal at δ 0.97 in its ^{31}P NMR spectrum. The chemical shift and pH insensitivity of this signal are characteristic of the presence of a phosphodiester bond. In addition, the fact that each of the oligosaccharides was stable to further treatment with base implied that the group B polysaccharide did not have a repeating unit but was in fact composed of a series of structurally related but different oligosaccharides. However, the alternate possibility that the group B antigen could be composed of three different polysaccharides cannot be discounted on the above evidence.

As a prerequisite to determine the structure of the group B antigen, it was necessary to first determine the structures of its individual oligosaccharide components. Also, in order to simplify the structural determination of IP, IIP, and IIIP, they were dephosphorylated to yield I, II, and III, respectively.

Structure of III. Glycose analysis indicated that III contained L-rhamnose and D-glucitol in the molar ratio of 3.8:1 (Table I) and permethylated III yielded the following components on hydrolysis: 2,3,4-tri-*O*-methylrhamnose, 3,4-di-*O*-methylrhamnose, and 2,4,5,6-tetra-*O*-methylglucitol in the approximate molar ratio of 2:2:1, respectively (Table II). The glucitol residue was not labeled with deuterium at C1, indicating that III contained two terminal rhamnopyranosyl residues, two interchain rhamnopyranosyl residues linked at O2, and a glucitol residue linked at O1 and O3.

The sequence of the above components in III was obtained by FAB-MS analysis and by direct GC–MS (EI and CI) analysis of its permethylated derivative. From the EI spectrum, the fragmentation pattern (Figure 3) is consistent with the structure shown in Figure 4a. The following deductions

Table II: Methylation Analysis of Oligosaccharides Obtained by Selective Degradations of Group B Antigen and II[a]

methylated sugar	detector response (%)										
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI
2,3,4-Me_3Rha[b]	27.7	42.2	39.1	25.6	50.0	32.7	23.3	29.6	–	–	–
3,4-Me_2Rha[b]	10.6	15.6	43.5	51.3	–	18.2	21.3	–	–	–	–
2,3-Me_2Rha[b]	10.6	–	–	–	–	–	19.1	–	38.5	38.5	–
2,4-Me_2Rha[b]	11.7	–	–	–	–	–	–	–	–	–	–
2,4,5,6-Me_4-glucitol[c]	8.5	12.5	17.4	–	–	12.7	17.0	–	–	–	–
2,3,4,5,6-Me_5-glucitol[c]	–	–	–	23.1	–	–	–	–	–	–	–
3-MeRha[b]	10.6	14.1	–	–	–	18.2	–	–	–	–	–
2,4,6-Me_3Gal[b]	17.9	15.6	–	–	50.0	–	–	37.1	–	–	–
4,6-Me_2GlcNMeAc[b]	+	+	+	–	–	+	+	–	+	+	+
1,3,5,6-Me_4GlcNMeAc[b]	–	–	–	–	+	–	–	–	–	–	–
2,3,4,6-Me_4Gal[b]	–	–	–	–	–	18.2	21.3	–	42.3	52.4	66.7
1-Me-3-deoxyglycerol[c]	–	–	–	–	–	–	–	–	+	–	–
1,3-Me_2-4-deoxyerythritol[c]	–	–	–	–	–	–	–	–	–	–	33.3
2,3,4-Me_3-threitol[c]	–	–	–	–	–	–	–	–	19.2	–	–
1,3-Me_2-glycerol[b]	–	–	–	–	–	–	–	–	–	+	–
3-Me-glycerol[c]	–	–	–	–	–	–	–	–	+	–	–
1,4,6-Me_3-2,5-anhydromannitol[b]	–	–	–	–	–	–	–	33.3	–	–	–

[a] (+) Slight nonquantitative response; (–) not detected. [b] Identified as alditol acetates deuteriated at C1, using column ii. [c] Identified as alditol acetates not deuteriated at C1, using column ii.

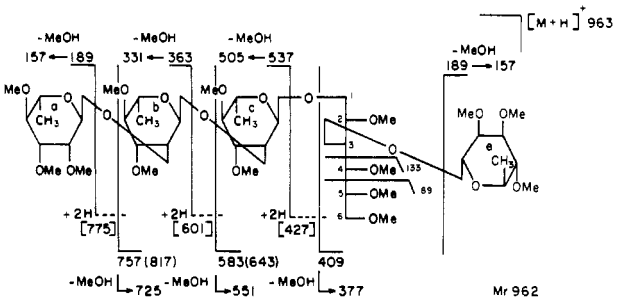

FIGURE 3: Fragmentation pattern of the EI mass spectrum of oligosaccharide III depicting some important primary ions and some diagnostic CI (isobutane) fragments in brackets. Corresponding J_1 fragment ions are shown in parentheses.

in support of the proposed structure may be drawn from the presence or absence of specific fragment ions in the EI and CI mass spectrum of permethylated III. In the CI spectrum, an abundant molecular ion was observed at m/z 963 $(MH)^+$, and in both the CI and EI spectrum fragments, m/z 189 (a A_1), 363 (ab A_1), and 537 (abc A_1) indicated that the sequence Rha-Rha-Rha was present in III. These primary ions were also accompanied by secondary ions m/e 157, 331, and 505 derived from the primary ions by the loss of methanol. Fragments m/e 409 (ed A_1), 583 (edc A_1), and 757 (edcb A_1) in the EI mass spectrum were also consistent with the presence of the overlapping sequence Rha-glucitol-Rha-Rha in III. Fragments m/z 775, 601, and 427 in the CI spectrum of permethylated III are also indicative of this latter sequence. The above sequence was also supported by characteristic fragment ions found in the FAB (positive) mass spectrum of III. In the pattern of fragmentation, the molecular ion was represented by the pseudomolecular ions at m/z 767 $(M + H)^+$ and 789 $(M + Na)^+$, and the fragment ions detected at m/z 293, 439, 474, and 621 were also consistent with the structure shown in Figure 4a.

The presence of fragment ions in the EI spectrum of permethylated III at m/z 89, 133, and 409 and the absence of an ion at m/z 177 demonstrated the presence of a glucitol residue substituted at O3, and this was confirmed by the methylation analysis which indicated that glucitol was substituted at positions O1 and O3 in III. The fact that a terminal rhamnopyranosyl residue was linked to O3 of glucitol was deduced from further analysis of II. In this analysis, the substituents linked to O1 and O3 of glucitol were unambig-

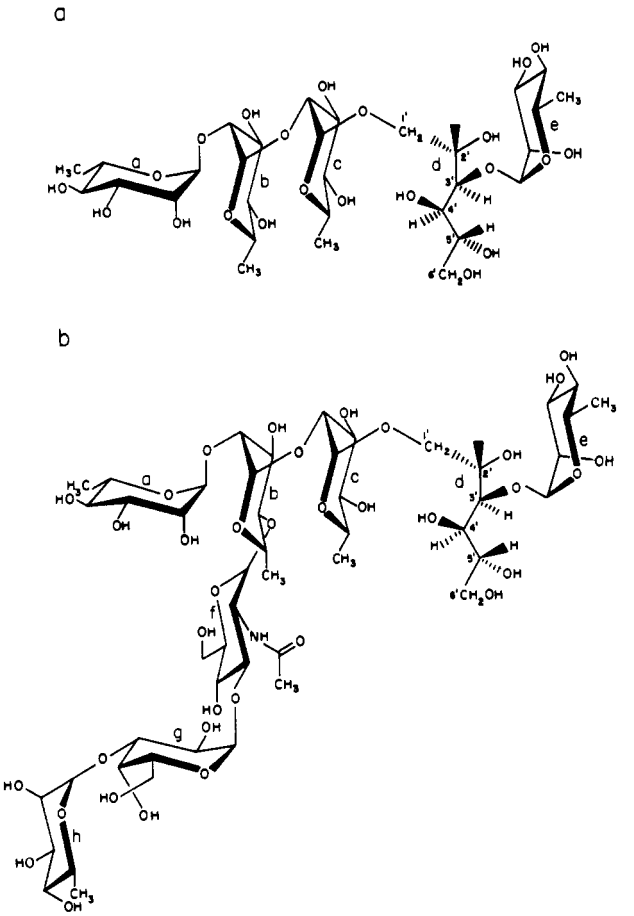

FIGURE 4: Structure of oligosaccharides (a) III and (b) II. The conformation of the oligosaccharides has not yet been confirmed.

uously assigned, and further, it was demonstrated by degradation (deamination) of II that III was an integral part of the structure of II. The configurations of the anomeric linkages in III were assigned by ^1H and ^{13}C NMR spectroscopic analysis and were consistent with those depicted in Figure 4a; both the ^1H and ^{13}C NMR spectra exhibited four signals in their anomeric regions (Tables III and IV, respectively). Assignment of the anomeric signals in the ^1H NMR and ^{13}C NMR spectra of III was made initially by reference to assignments made from model compounds by Josephson and Bundle (1980) and Bock (1983), respectively, and were con-

Table III: Proton Chemical Shifts[a] and Coupling Constants ($^3J_{1,2}$)[b] of Anomeric Protons of Oligosaccharides Obtained from Group B Antigen

proton	II	III	IV	V	VI	VII	VIII	IX	X	XI
H1a	4.971(d)[d] (1.6)	4.971(d) (1.7)	4.974(d) (1.6)		4.971(d)[c]			4.960(d) (1.8)		
H1b	5.117(d) (1.6)	5.102(d) (1.8)	5.102(d) (1.6)		5.117(d)[c]	4.971(d)[c]		4.785(t)[e] (13.0)	4.963(d) (1.8)	
H1c	4.869(d) (1.7)	4.871(d) (1.6)	4.876(d) (1.7)		4.869(d)[c]	4.896(d)[c]		4.785(t)[e] (13.0)		
H1e	5.038(d) (1.7)	5.030(d) (1.8)			5.022(d)[c]	5.020(d)[c]				
H1f	4.835(d) (8.1)			5.167(d) (2.6)	4.837(d) (7.5)	4.844(d) (7.5)		4.773(d) (7.8)	4.837(d) (7.6)	4.710(d) (8.0)
				4.766(d) (8.0)						
H1g	5.441(d) (3.9)			5.420(t)[f] (3.0)	5.406(d) (3.5)	5.407(d) (3.5)	5.213(d) (2.8)	5.408(d) (3.0)	5.405(d) (3.0)	5.394(d) (2.9)
H1h	5.022(d) (1.7)			5.034(d) (1.5)			5.035(d) (1.7)			

[a] Measured at 343 K in D_2O with acetone (1%) as internal reference (2.225 ppm). [b] In parentheses (Hz). [c] Unresolved doublet. [d] Doublet. [e] Oxidized and reduced ($NaBH_4$) open-chain rhamnopyranosyl residue of oligosaccharide IX (Figure 7). [f] Doublet split into a triplet, because of terminal reducing GlcNAc residue.

Table IV: Chemical Shifts[a] and $^1J_{^{13}C,H}$ Coupling Constants[b] of Signals in ^{13}C NMR Spectra of Oligosaccharides Obtained from Group B Antigen

carbon	II	III	VI	VII
C1a	102.95 (172.0)	102.92 (169.5)	103.11	
C1b	101.17 (172.0)	101.59 (170.6)	101.24	102.75
C1c	99.56 (171.0)	99.57 (167.3)	99.61	99.56
C1e	102.42 (172.0)	102.41 (167.7)	102.51	102.49
C1f	101.99 (164.0)		102.21	102.20
C1g	99.56 (172.0)		99.83	99.83
C1h	103.02 (170.5)			
C6d	63.70	63.63	63.70	63.67

[a] In ppm from internal acetone. Assignments confirmed by using two-dimensional NMR (C,H) shift-correlated experiments. [b] In parentheses (hertz) and obtained by gated decoupling.

sistent with assignments made by using two-dimensional $^{13}C,^1H$ shift-correlated NMR spectroscopy (Bax & Freeman, 1981; Michon et al., 1985a,b). These assignments were also consistent with those made on the equivalent oligosaccharide moiety of the structurally related II using sequential degradation techniques (see later).

Although the $^3J_{H1,H2}$ coupling constants of the four rhamnopyranosyl residues in III could be measured (Table III), they are small and cannot be used to differentiate rhamnopyranoside anomers. However, the large couplings on the H4 triplet ($^3J_{H3,H4}$, $^3J_{H4,H5}$ = 11.7 Hz) indicate a transdiaxial disposition of H3, H4, and H5 which is consistent with the rhamnopyranosyl residues adopting the $_1C^4$ (L) configuration. The most convincing evidence in favor of all the rhamnopyranosyl residues in III having the α-L configuration was obtained from the proton-coupled ^{13}C NMR spectrum of III. $^1J_{^{13}C,H}$ coupling constants are sensitive to change in anomeric configuration (Perlin et al., 1970), and those of the L-rhamnopyranosyl residues in III (Table IV) are characteristic of all the L-rhamnopyranosyl residues being in the α-L configuration.

Structure of IIIP. Freaction IIIP was obtained directly from the chromatographic separation of the products of the alkaline hydrolysis of the group B polysaccharide. In addition to the components listed for III in Table I, IIIP also contained 1 mol equiv of phosphate as shown by analysis (Table I). The only difference in the methylation analysis of IIIP as compared to that of III (Table II) was the failure to detect any methylated glucitol derivatives in the former, thus indicating that the phosphate esters of IIIP were located on its glucitol residue. To detect the methylated glucitol residues, methylated IIIP was treated with aqueous hydrofluoric acid to remove phosphate substituents, and the dephosphorylated product was remethylated with deuterium-labeled methyl iodide in order to locate the position of phosphate substitution.

The following labeled methylated glucitol derivatives were obtained following hydrolysis of labeled permethylated III. The detection of a 2,4,5,6-tetra-*O*-methylglucitol derivative, labeled at either O5 (70%) or O6 (30%), indicated that IIIP was in fact a mixture of two distinct phosphate monoesters in the ratio of 7:3, respectively, the phosphate groups being located at O5 or O6 of its glucitol residue. A further 2,3,4,5,6-penta-*O*-methylglucitol derivative was also detected by GLC–MS analysis in the intensity ratio of 3:7, respectively, with the aforementioned tetra-*O*-methyl derivative. The penta-*O*-methyl derivative was also labeled at either O5 or O6 in the same ratio as the tetra-*O*-methyl derivative but in addition with fully labeled at O3. This indicated that the dephosphorylation of permethylated IIIP not only produced the anticipated III but also yielded a substantial amount (30%) of a product of further hydrolysis of IIIP in which terminal

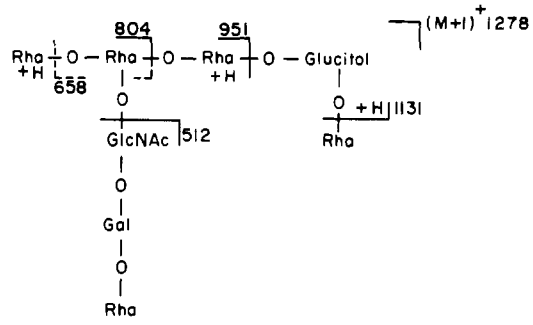

FIGURE 5: Pertinent primary ions from the positive FAB mass spectrum of oligosaccharide II.

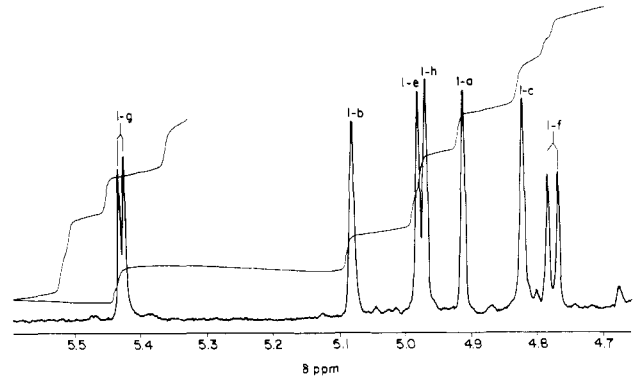

FIGURE 6: Partial ^1H NMR spectrum (anomeric proton region) of oligosaccharide II in D_2O at 330 K.

rhamnopyranosyl residues were also selectively removed from its glucitol residue.

The location of the phosphate groups was also confirmed by NMR spectroscopic evidence. In the ^{13}C NMR spectrum of IIIP, only two signals in the region characteristic of primary hydroxymethyl carbons were detected at 61.82 and 67.24 ppm in the approximate intensity ratio of 0.3:0.7, respectively. This ratio is fairly consistent with that found for the two different phosphate esters in IIIP in its methylation analysis, and in fact, these signals can be respectively assigned to C6 of a mixture of O5 and O6 phosphate-substituted glucitol residues in IIIP on the basis of displacements anticipated on C6 of the glucitol residue of III (signal previously assigned at 63.63 ppm) due to the above substitution pattern (Jennings & Smith, 1978). A phosphate substituent at O5 of III is consistent with the observed upfield displacement of its C6 signal by 2.01 ppm, and the presence of a phosphate substituent at O6 of III is consistent with the observed downfield displacement of its C6 signal by 3.61 ppm.

The presence of two different phosphate esters in IIIP was also confirmed by data obtained from its ^{31}P NMR spectrum. The spectrum exhibited two major singlets in the region of the spectrum characteristic of monophosphate esters at δ 3.891 and 5.595. These signals had an intensity ratio of 2.2:1.0 which is approximately the same ratio found for the respective O5- and O6-substituted phosphate esters in IIIP by both methylation analysis and ^{13}C NMR spectroscopy. In addition in the proton-coupled ^{31}P NMR spectrum of IIIP, the signal at δ 3.891 was split into a doublet, and the signal at δ 5.595 was split into a triplet. This is consistent with the phosphorus atom responsible for the former being coupled to a single proton, as in the case of a phosphate ester on O5 of the glucitol residue of IIIP, and for the phosphorus atom responsible for the latter signal being coupled to two protons, as in the case of a phosphate ester on the primary O6 position of the glucitol residue of IIIP.

Structure of II. Glycose analysis of II indicated that it contained L-rhamnose, D-galactose, 2-acetamido-2-deoxy-D-glucose, and D-glucitol in the molar ratio of 4.9:1.0:0.8:0.7 (Table I), and hydrolysis of permethylated II yielded the methylated glycose derivatives listed in Table II. In addition to those obtained from the hydrolysis of permethylated III, another 2,3,4-tri-*O*-methylrhamnose and additional 3-*O*-methylrhamnose, 2,4,6-tri-*O*-methylgalactose, and 4,6-di-*O*-methylglucosamine derivatives were also detected. The presence of these derivatives is consistent with the structure of II being formed by the addition of a trisaccharide (e.g., Rha*p*1→3Gal*p*1→3Glc*p*NAc) branch to one of the interchain rhamnopyranosyl residues of III. The structure proposed for II is shown in Figure 4b and was deduced on the following additional evidence. The sequence of the above components in II was deduced from the pattern of fragment ions produced when II was subjected to FAB-MS analysis (Figure 5). These fragment ions were consistent with II being similar to III except for the addition of a trisaccharide (Rha*p*1→3Gal*p*1→3GlcNAc) branch situated on the penultimate rhamnopyranosyl residue (b) of III. Pseudomolecular ions were detected at m/z 1278 $(M + H)^+$ and 1300 $(M + Na)^+$, and other fragment ions were detected at m/z 1131, 951, 804, 658, and 512. The fragment ion at m/z 658 was the result of double cleavage of II with the hydrogen transfer as depicted in Figure 5, and the ion at m/z 512 was very intense, corresponding to glycosidic cleavage at a hexosaminyl residue. The above sequence was also corroborated by degradation procedures carried out on II. That II contained seven glycopyranosyl residues was confirmed by examination of signals in the anomeric region of its ^1H NMR spectrum (Figure 6) and ^{13}C NMR spectrum. The chemical shifts and coupling constants associated with the ^1H NMR and ^{13}C NMR signals are listed in Tables III and IV, respectively. Seven proton doublets were observed in the anomeric region of the ^1H NMR spectrum of II, and seven carbon signals (two of them coincident) were observed in the equivalent region of its ^{13}C NMR spectrum. Assignments of the anomeric signals in the ^1H NMR and ^{13}C NMR spectra of II were made by using the same methods as described previously for III. In addition, they were confirmed by degradation techniques in which rhamnopyranosyl residues were selectively removed from II by using both chemical and enzymatic techniques. Thus, the ^1H and ^{13}C signals of missing residues could then be identified by comparing the ^1H NMR and ^{13}C NMR spectra of II with the equivalent spectra of the oligosaccharide degradation products (Tables III and IV).

When II was treated with aqueous hydrogen fluoride, it was split into two oligosaccharides (IV and V), the structures of which are shown in Figure 7 and the chemical shifts and coupling constants are listed in Table III. Of interest is the fact that IV and V together constitute the total structure of II (Figure 4b) with the exception of residue e. Thus, the selective cleavage of this terminal rhamnopyranosyl residue (e) enabled an unambiguous assignment of its anomeric proton signal in the ^1H NMR spectrum of II to be made (Table III).

A rhamnosidase contained in a commercial preparation of naringinase was used to carry out the enzymatic degradation of II. Romero et al. (1985) have recently reported a method for assaying its rhamnosidase activity using *p*-nitrophenyl α-L-rhamnopyranoside as the substrate. When II was treated with naringinase, the aqueous HF-sensitive terminal rhamnopyranosyl residue (e) (Figure 4b) was not cleaved, but instead cleavage occurred at the other two terminal rhamnopyranosyl residues (a and h in Figure 4b). In addition, because residue h was cleaved faster than residue a, it was possible to

FIGURE 7: Structures of the oligosaccharides (IV–XI) obtained by chemical and enzymatic degradations of oligosaccharide II. (IV and V) Aqueous HF; (VI and VII) rhamnosidase; (VIII) deamination; (IX–XI) periodate oxidation.

isolate VI and VII, corresponding to II minus residue h and II minus both residue h and residue a, respectively (Figure 7), and thus to assign the anomeric protons of these residues in the ^1H NMR and ^{13}C NMR spectra of II. Unfortunately, when III (Figure 4a) was treated with naringinase, both residues a and b were cleaved simultaneously, leading to difficulties in making assignments directly from the ^1H NMR of III. These latter results and more in depth studies on the specificity of the rhamnosidase in naringinase will be published at a later date (H. J. Jennings et al., unpublished experiments). Other chemical degradations were carried out on II in which different oligosaccharide fractions were produced. These oligosaccharides were consistent with the proposed structure for II (Figure 4b) and also assisted in the assignment of signals in the ^1H NMR of II (Table III).

In preparation for deamination, II was first subjected to basic hydrolysis to remove the *N*-acetyl group from its 2-acetamido-2-deoxy-glucopyranosyl residue. The N-deacetylation was only accomplished with difficulty, approximately 80% N-deacetylation requiring treatment of II with 2 M NaOH at 100 °C for 10 h. Oligosaccharide II was extremely resistant to hydrazinolysis even in the presence of hydrazine sulfate, and this resistance to hydrazinolysis of 3-O-substituted 2-acetamido-2-deoxyhexopyranosides has been previously reported (Fujinaga & Matsushima, 1966; Dmitriev et al., 1973; Inoue & Katajima, 1985). N-Deacetylated II was deaminated with nitrous acid (Dmitriev et al., 1975), and the major products, oligosaccharides III and VIII, were isolated from the reaction mixture by gel filtration. The larger oligosaccharide (III) was identified by procedures described previously following its direct isolation from the group B antigen. Oligosaccharide VIII, shown in Figure 7, probably originated from the trisaccharide branch of II, the 2-acetamido-2-deoxyglucopyranosyl residue being converted to a 2,5-anhydromannose residue by deamination (Williams, 1975). To confirm this hypothesis, VIII was reduced with $NaBD_4$, and permethylated and methylated VIII was subjected to GC–MS analysis. The fragmentation pattern is shown in Figure 8 and is consistent with the proposed structure of VIII (Figure 7).

Smith degradation of II yielded three major oligosaccharide fractions (IX, X, and XI) which were isolated by column chromatography and numbered respectively in order of their elution (molecular size) from the column. The structures of IX, X, and XI are shown in Figure 7 and are consistent with the proposed structure of II (Figure 4b) from which they were derived. Oligosaccharide IX originated from residues g, f, b, c, and d of II (Figure 4b), and the smaller oligosaccharides

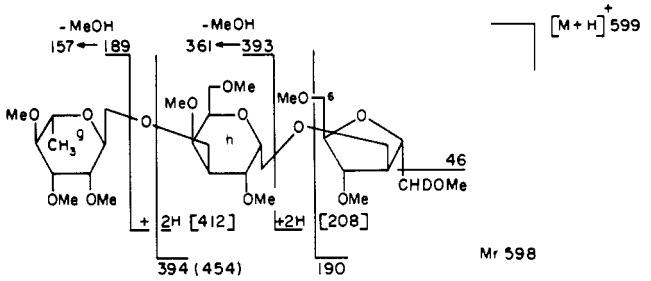

FIGURE 8: Fragmentation pattern of the EI mass spectrum of oligosaccharide VIII showing some important primary ions and some diagnostic CI (isobutane) fragments in brackets. A J_1 fragment ion is shown in parentheses.

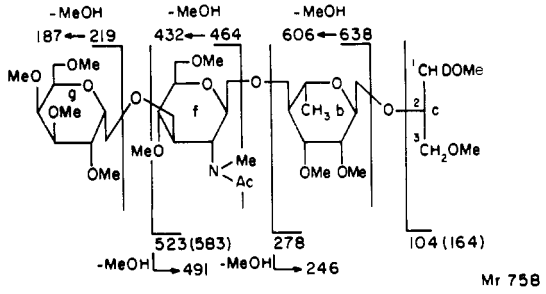

FIGURE 9: Fragmentation pattern of the EI mass spectrum of oligosaccharide X showing some important primary ions. J_1 fragment ions are shown in parentheses.

(X and XI) were the products of further degradation of IX. The anticipated Smith degradation product was X (Figure 7), the structure of which is consistent with its methylation analysis (Table II) and the fact that it exhibited only three anomeric doublets in its ^{1}H NMR spectrum (Table III) corresponding to residues g, f, and b of II (Figure 4b). The fragmentation pattern of the mass spectrum of permethylated X is shown in Figure 9.

Oligosaccharide IX gave four anomeric doublets in its ^{1}H NMR spectrum (Table III) of which three (b, f, and g) corresponded to the same residues in II. The fourth doublet, not present in the ^{1}H NMR spectrum of II, had an unusually large $^3J_{H1,H2}$ coupling constant (13.0 Hz) and could only logically correspond to a chemically transformed residue c of II. This hypothesis was confirmed by the methylation analysis of IX (Table II) in which 1-*O*-methyl-3-deoxyglycerol and 3-*O*-methylglycerol were detected in addition to the other anticipated methylated derivatives. The presence of the above components indicated that the O2-linked L-rhamnopyranosyl residue c of II was oxidized to an open-chain form by sodium metaperiodate but following reduction did not fragment in the hydrolysis step of the Smith degradation procedure. The resistance of oxidized O2-linked rhamnopyranosyl residues to acid hydrolysis in the Smith degradation of *Klebsiella* polysaccharides has been previously reported (Dutton & Mackie, 1977). The presence of XI in the Smith degradation products of II (Figure 7) can only be rationalized if one assumes that the remnant of residue a following the oxidation of II (dialdehyde moiety) is partially cleaved from II during the oxidation, thus exposing the normally resistant residue b to oxidation. Because of the weight of evidence confirming the structure of II (Figure 4b), the alternate explanation of the presence of structural variability in II in not valid.

Structure of I. Glycose analysis of the largest oligosaccharide (I) indicated that it contained L-rhamnose, D-galactose, 2-acetamido-2-deoxy-D-glucose, and D-glucitol in the molar ratio of 6.5:2.0:1.7:0.7, indicating that it contained possibly two more L-rhamnose residues and one more D-galactose and 2-acetamido-2-deoxy-D-glucose residues than II. The methylation analysis (Table II) indicates the further structural complexity of I and demonstrates the presence of additional 3O- and 4O-linked rhamnopyranosyl residues. The structural elucidation of I is still in progress and will be reported at a later date; however, early analysis of its FAB-MS spectrum indicates the presence of fragment ions which are consistent with oligosaccharide II being an integral part of the structure of I.

Discussion

Although morphological and biochemical tests are used to identify group B streptococci, the latter can be more accurately defined immunologically on the basis of the presence of common immunodeterminants. These determinants are structural features of the complex group B polysaccharide antigen which is present in all strains (Lancefield, 1933, 1934, 1938). Hence, 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.

Attempts to define the structure of the group B polysaccharide have been thwarted by its extremely complex nature. However, some preliminary structural data have recently been reported by Pritchard and co-workers (Pritchard et al., 1981, 1984). These authors have demonstrated that the group B antigen has a teichoic acid like structure, the repeating units probably being linked through glucitol diphosphate esters. They also proposed some rudimentary structural features of the repeating unit of the group B antigen which, except for the omission of one terminal and one interchain αL-rhamnopyranosyl residue, responds closely to the sequence of glycose residues that we proposed for oligosaccharide II. Although the above structural work has provided insights into some of the features of the group B polysaccharide, its highly complex and heterogeneous nature was not established.

Our initial analytical and NMR spectroscopic studies had been hampered by the heterogeneity of the group B antigen, and in order to tackle the problem of its structure, it was first necessary to split it into some of its smaller component parts. This was achieved most effectively by the basic hydrolysis of the group B antigen in which its diphosphate ester bonds were broken. The hydrolysis yielded three different oligosaccharide monophosphate esters (IP, IIP, and IIIP), and the fact that all these oligosaccharides were stable to further basic treatment indicated that they were all discrete oligosaccharide moieties of the group B antigen. Thus, the heterogeneity of the group B antigen was established.

The structures of two of the complex dephosphorylated oligosaccharides (II and III) (Figure 4) were determined by using a number of degradative and spectroscopic techniques including NMR, GC–MS, and FAB-MS. These structural studies were facilitated by the isolation of a number of fragment oligosaccharides obtained by the chemical and enzymatic degradation of II (Figure 7). With these oligosaccharides, the complex sequence of glycoses in II was elucidated, and unambiguous anomeric proton and carbon signal assignments were made possible in the ^{1}H NMR and ^{13}C NMR spectra of II. Of particular note was the use of a rhamnosidase, contained in naringinase, to remove specific terminal α-L-rhamnopyranosyl residues from II. This enzyme should find great utility in probing the structures of complex rhamnans or other molecules containing terminal rhamnopyranosyl residues. Interestingly, although these oligosaccharides (II and III) were structurally different, they were not unrelated, because the smaller of the two (III) was found to be a part of the structure of the larger (II). The additional structural feature of the larger oligosaccharide (II) was the presence of

a trisaccharide (α-L-Rha*p*1→3α-D-Gal*p*1→3β-D-Glc*p*NAc) branch. Although the structure of the largest oligosaccharide (I) has not yet been fully determined, the identification of fragment ions common to II in its FAB-MS spectrum provided strong evidence that I is also similarly related to II and III (Jennings et al., unpublished experiments).

The identification of three different oligosaccharide moieties in the group B antigen poses the question as to whether it does in fact have a repeating unit. It could be constructed of a linear arrangement of randomly mixed oligosaccharides (I, II, and III) linked together by diphosphate ester bonds, although it is also possible that the group B antigen was originally homogeneous, being based on a single repeating unit of oligosaccharide I, and that some of its trisaccharide branches were subsequently removed by glycosidases secreted into the growth medium by the organism. Certainly, if this latter hypothesis is not true, then in order to be compatible with a structural arrangement involving repeating units, the group B antigen would have to consist of three different polysaccharides. However, these polysaccharides could of course still be covalently linked to each other in some way, thus reconciling the concept of the repeating unit with the complexity of the antigen. Sufficient evidence is not available at this time to definitively eliminate any of the above possibilities.

The isolation of the oligosaccharides as their monophosphate esters (IP, IIP, and IIIP) from the basic hydrolysis of the group B antigen is compatible with the oligosaccharides being linked together through phosphodiester bonds as previously proposed (Pritchard et al., 1984). The failure to detect a methylated glucitol derivative in the methylation analysis of the group B antigen was interpreted as indicating that the phosphodiester bond was attached to one end of the glucitol residue. This was confirmed by methylation and ^{13}C NMR analyses of IIIP, from which it was deduced that IIIP consisted of a mixture of two monophosphate esters, the ester groups being located on either O5 or O6 of its glucitol residue. The oligosaccharide having the monophosphate ester at O5 of its glucitol residue was predominant (60–70%) in the mixture. The above evidence could be indicative of heterogeneity in the diphosphate ester linkages of the group B antigen, but it is more likely that heterogeneity was the result of a phosphate group migration, which is known to occur during the basic hydrolysis of this type of structure (Egan et al., 1982). The fact that only one major phosphorus signal was detected in the ^{31}P NMR of the group B antigen is strongly supportive of this latter hypothesis. It is interesting to note that no signal characteristic of an intermediate 5,6-cyclic phosphate ester was detected in the ^{31}P NMR spectrum of the products (IP, IIP, and IIIP) of the base-treated group B antigen. Such a signal would have confirmed the migration mechanism, as these intermediates have been previously identified by using this technique (Egan et al., 1982). However, failure to detect this type of intermediate does not necessarily negate the possibility of migration, as it could possibly be due to complex conformational and mechanistic factors.

All the above structural work was carried out on the group B polysaccharide isolated from one particular strain of type Ia group B *Streptococcus*. However, preliminary work on the group B antigen isolated from strains of different group B streptococcal serotypes (types II and III) provided physical evidence that the group B polysaccharide is a common antigen of group B streptococci as originally proposed by Lancefield (1933, 1934, 1938) on the basis of serological studies. Basic hydrolyses of both the latter polysaccharide preparations yielded, with minor variations in relative quantities, the same three major components as defined by the elution profile of the chromatographic separation of the products of basic hydrolysis of the type Ia preparation (Figure 2). In addition, on the basis of their identical ^{13}C and ^{1}H NMR spectra, the structural identity of each of the three components (oligosaccharides I, II, and III) from all the above sources can be proposed.

The structural complexity of the group B antigen implies that it probably also has complex serological properties. However, by elucidating its structure, it might eventually be possible to identify some of the more simple individual determinants which constitute this highly complex polysaccharide.

ACKNOWLEDGMENTS

We thank Fred Cooper for the EI-MS and CI-MS, Dr. Kasekoff for the FAB-MS, and Dr. J. R. Brisson for recording the two-dimensional NMR correlated spectra.

Registry No. PO_4^{3-}, 7723-14-0; L-rhamnose, 3615-41-6; D-galactose, 59-23-4; D-*N*-acetylglucosamine, 7512-17-6; D-glucitol, 50-70-4.

REFERENCES

Bax A., & Freeman, R. (1981) *J. Magn. Reson. 44*, 542.
Bock, K. (1983) *Adv. Carbohydr. Chem. Biochem. 41*, 27.
Carey, R. B., Eisenstein, K. T., Shockman, G. D., Greber, T. F., & Swenson, R. M. (1980) *Infect. Immun. 28*, 195.
Chen, P. S., Toriba, J. Y., & Warner, H. (1956) *Anal. Chem. 28*, 1756.
Dmitriev, B. A., Knirel, Yu. A., & Kochetkov, N. K. (1973) *Carbohydr. Res. 29*, 451.
Dmitriev, B. A., Backinowsky, L. V., Lvov, V. L., Kochetkov, N. K., & Hoffman, I. L. (1975) *Eur. J. Biochem. 50*, 539.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956) *Anal. Chem. 28*, 350.
Dutton, G. G. S., & Mackie, K. L. (1977) *Carbohydr. Res. 55*, 49.
Egan, W., Schneerson, R., Werner, K. E., & Zon, G. (1982) *J. Am. Chem. Soc. 104*, 2898.
Fiege, V., & Radziejewska-Lebrecht, J. (1979) *Proc. Int. Symp. Glycoconjugates*, 12.
Fujinaga, M., & Matsushima, Y. (1966) *Bull. Chem. Soc. Jpn. 39*, 185.
Gerwig, G. J., Kamerling, J. P., & Vliegenthart, J. F. G. (1979) *Carbohydr. Res. 77*, 1.
Goldstein, I. J., Hay, G. W., Lewis, B. A., & Smith, F. (1965) *Methods Carbohydr. Chem. 5*, 361.
Hakomori, S. (1964) *J. Biochem. (Tokyo) 55*, 205.
Heidelberger, M., Davie, J. M., & Krause, R. M. (1967) *J. Immunol. 99*, 794.
Inoue, Y., & Kitajima, K. (1985) *Carbohydr. Res. 135*, 342.
Jennings, H. J., & Smith, I. C. P. (1978) *Methods Enzymol. 50*, 39.
Jennings, H. J., Bhattacharjee, A. K., Kenne, L., Kenny, C. P., & Calver, G. (1980) *Can. J. Biochem. 58*, 128.
Jennings, H. J., Johnson, K. G., & Kenne, L. (1983) *Carbohydr. Res. 121*, 233.
Josephson, S., & Bundle, D. R. (1980) *J. Chem. Soc., Perkin Trans. 1*, 297.
Kane, J. A., & Karakawa, W. W. (1977) *J. Immunol. 118*, 2155.
Kasper, D. L., Goroff, D. K., & Baker, C. J. (1978) *J. Immunol. 121*, 1096.
Lancefield, R. C. (1933) *J. Exp. Med. 57*, 571.
Lancefield, R. C. (1934) *J. Exp. Med. 59*, 441.
Lancefield, R. C. (1938) *J. Exp. Med. 67*, 25.
Lindberg, B. (1972) *Methods Enzymol. 28B*, 178.

Lipkin, D., Phillips, B. E., & Abrell, J. W. (1969) *J. Org. Chem. 34*, 1539.
McCarty, M., & Lancefield, R. C. (1955) *J. Exp. Med. 102*, 11.
Michon, F., Brisson, J. R., Roy, R., Ashton, F. E., & Jennings, H. J. (1985a) *Biochemistry 24*, 5592.
Michon, F., Brisson, J. R., Roy, R., Ashton, F. E., & Jennings, H. J. (1985b) *Can. J. Chem. 63*, 2781.
Perlin, A. S., Casu, B., & Koch, H. J. (1970) *Can. J. Chem. 48*, 2596.
Pritchard, D. G., Coligan, J. E., Speed, S. E., & Gray, B. M. (1981) *J. Clin. Microbiol. 13*, 89.
Pritchard, D. G., Gray, B. M., & Dillon, H. C., Jr. (1984) *Arch. Biochem. Biophys. 235*, 385.
Romero, C., Manjon, A., Bastida, J., & Iborra, J. L. (1985) *Anal. Biochem. 149*, 566.
Root, R. L., Durrwachter, J. R., & Wong, C.-H. (1985) *J. Am. Chem. Soc. 107*, 2997.
Roth, H., Segal, S., & Bertoli, D. (1965) *Anal. Biochem. 10*, 32.
Sawardeker, J. S., Sloneker, J. H., & Jeanes, A. R. (1965) *Anal. Chem. 37*, 1602.
Williams, J. M. (1975) *Adv. Carbohydr. Chem. Biochem. 31*, 9.

Characterization of α_2-Macroglobulin–Plasmin Complexes: Complete Subunit Cleavage Alters Receptor Recognition in Vivo and in Vitro†

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Received July 23, 1986; Revised Manuscript Received October 3, 1986

ABSTRACT: When human α_2-macroglobulin (α_2M) binds proteinases, it undergoes subunit cleavage. Binding of small proteinases such as trypsin results in proteolysis of each of the four subunits of the inhibitor. By contrast, previous studies suggest that reaction of plasmin with α_2M results in cleavage of only two or three of the inhibitor subunits. In this paper, we demonstrate that the extent of subunit cleavage of α_2M is a function of plasmin concentration. When α_2M was incubated with a 2.5-fold excess of plasmin, half of the subunits were cleaved; however, at a 20-fold enzyme to inhibitor ratio, greater than 90% of the subunits were cleaved with no additional plasmin binding. This increased cleavage was catalyzed by free rather than bound plasmin. It is concluded that this "nonproductive" subunit cleavage is dependent upon the molar ratio of proteinase to inhibitor. The consequence of complete subunit cleavage on receptor recognition of α_2M–plasmin (α_2M–Pm) complexes was studied. Preparations of α_2M–Pm with only two cleaved subunits bound to the murine macrophage receptor with a K_d of 0.4 nM and 60 fmol of bound complex/mg of cell protein. When preparations of α_2M-Pm with four cleaved subunits were studied, the K_d was unaltered but ligand binding increased to 140 fmol/mg of cell protein. The receptor binding behavior of the latter preparation is equivalent to that observed when α_2M is treated with small proteinases such as trypsin. This study suggests that receptor recognition site exposure is not complete in the α_2M–Pm complex with half of the subunits cleaved. Proteolytic cleavage of the remaining subunits of the inhibitor results in a further conformational change exposing the remaining receptor recognition sites.

Human α_2-macroglobulin (α_2M) is a glycoprotein composed of four identical ($M_r \sim 180\,000$) subunits that inhibits endopeptidases of all four major classes (Barrett & Starkey, 1973; Hall & Roberts, 1978; Swenson & Howard, 1979; Sottrup-Jensen et al., 1983). The mechanism of inhibition is unique in that specific, limited proteolysis of α_2M at a "bait region" located near the middle of each subunit (Barrett & Starkey, 1973; Harpel, 1973; Mortensen et al., 1981) results in a conformational change in the inhibitor that physically entraps the proteinase (Barrett & Starkey, 1973; Barrett et al., 1979). The activity of the bound proteinase toward small substrates is retained (Ganrot, 1966, 1967), but activity toward macromolecular substrates is greatly diminished (Harpel & Mosesson, 1973; Bieth et al., 1981; Gonias & Pizzo, 1983a).

Each mole of α_2M is capable of inhibiting 2 mol of trypsin or chymotrypsin (Pochon et al., 1978; Barrett et al., 1979; Swenson & Howard, 1979) but only 1 mol of plasmin (Ganrot, 1967; Pochon et al., 1978; Gonias et al., 1982a) or a synthetic chymotrypsin dimer (Pochon et al., 1981). This difference in binding can be explained by a model of α_2M structure that contains two adjacent and equivalent binding sites (Pochon et al., 1981; Pochon & Bieth, 1982; Feldman et al., 1985). A large proteinase such as plasmin can bind to one site and sterically inhibit the binding of a second proteinase (Pochon et al., 1981; Gonias & Pizzo, 1983a; Feldman et al., 1985).

Limited reduction and alkylation of α_2M results in the generation of two functional "half-molecules", each containing one proteinase binding site (Gonias & Pizzo, 1983a,b). When a proteinase enters a binding site, it cleaves both subunits (Pochon et al., 1981), two thiol groups are generated (Sottrup-Jensen et al., 1980, 1981), and a change in the conformation of the inhibitor occurs exposing two receptor recognition sites (Imber & Pizzo, 1981; Kaplan et al., 1981; Van Leuven et al., 1979). Methylamine can also cause the appearance of these thiol groups and an equivalent conformational change in human α_2M (Gonias et al., 1982; Barrett et al., 1979). This change in conformation can be demonstrated by a "slow" to "fast" shift in electrophoretic mobility in

† This work was supported by National Heart, Lung, and Blood Institute Grants HL-24066 and HL-31932 and by National Cancer Institute Grant CA-29589.

0006-2960/87/0426-0486$01.50/0