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STRUCTURAL DETERMINATION OF THE CAPSULAR POLYSACCHARIDE OF *Streptococcus pneumoniae* TYPE 18C (56)*,†

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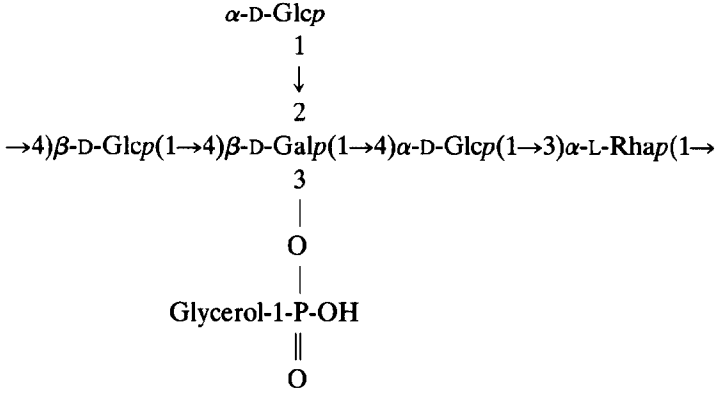
ABSTRACT

The specific capsular polysaccharide of *Streptococcus pneumoniae* type 18C (56) contains D-glucose, D-galactose, L-rhamnose, and glycerol residues, and phosphate and *O*-acetyl groups in the molar ratios of 3:1:1:1:1:1. Accumulated data from methylation analyses of the native and the specifically degraded, native polysaccharide indicated that it is composed of the repeating unit shown; it also contains *O*-acetyl groups, of undetermined location, in the molar ratio to L-rhamnose of 1:1.



INTRODUCTION

The pneumococcal polysaccharides have recently assumed importance owing to their use in a multivalent vaccine against pneumococcal infections[1,2]. Because of the diversity of pneumococcal types, eighty-three serologically defined capsular polysaccharides having been described to date, the choice of capsular polysac-

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†The Danish system of nomenclature is used throughout, followed by the U.S. system in parentheses.

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charide used in the vaccine was based on the most prevalent types found among pneumococcal isolates[2,3]. The current pneumococcal vaccine contains capsular polysaccharide antigens of fourteen different serotypes, including type 18C. The structure of these polysaccharides is an important parameter in their definition, and, to date, knowledge of their structures is almost complete[3].

Estrada-Parra and Heidelberger[4] determined the composition of the 18C polysaccharide as D-glucose, D-galactose, L-rhamnose, glycerol, phosphate, and *O*-acetyl in the molar ratios of 3:1:1:1:1:1. They also provided some linkage and sequence data, including evidence for the presence of side-chain α-glycerophosphate residues. Using fast-atom-bombardment–mass spectrometry and supporting chemical data, Phillips *et al.*[5] proposed the sequence of the repeating unit of the 18C polysaccharide. In this sequence, glycerol was found to be glycosidically linked to some of the terminal L-rhamnopyranosyl residues. Except for the location of its *O*-acetyl groups, we now report the complete structural elucidation of the type 18C polysaccharide, which confirms some of the structural features contained in both of the structures previously proposed[4,5].

RESULTS AND DISCUSSION

Hydrolyses of the type 18C pneumococcal polysaccharide indicated that it contained D-glucose, D-galactose, L-rhamnose, glycerol, and phosphate in the molar ratios of 3:1:1:1:1:1. Also, *O*-acetyl groups were detected by the appearance of a characteristic methyl (-$OCOCH_3$) singlet at δ 1.98 in the ^{1}H-n.m.r. spectrum of the 18C polysaccharide, and were shown to be in the molar ratio to L-rhamnose of 1:1 by the identical intensities of the aforementioned methyl signal at δ 1.98 and a doublet centered at δ 1.21, attributed to the 5-*C*-methyl groups of the rhamnose residues. The same constituents, in the identical molar ratios, had been identified in the type 18C polysaccharide by Estrada-Parra and Heidelberger[4], and, except for the proportion of glycerol, by Phillips *et al.*[5].

Treatment of the 18C polysaccharide with 48% aqueous hydrofluoric acid released all of the glycerol and phosphate, and at the same time resulted in extensive depolymerization of the polysaccharide. A range of fractions of different molecular sizes was isolated, the first two fractions (**1** and **2**) still being polymeric (mol. wt. 5,000–9,000), and fractions **3** and **4** being oligosaccharides. All of the fractions contained D-glucose, D-galactose, and L-rhamnose in the same molar ratios (3:1:1) as in the original polysaccharide. The fact that no glycerol or phosphate could be detected in the polymeric fractions is consistent with the presence, in the 18C polysaccharide, of terminally located glycerophosphate residues, as suggested by Estrada-Parra and Heidelberger[4], and not as linear, inter-residue components, as proposed by Phillips *et al.*[5].

Methylation of the aforementioned fractions was performed following reduction of their terminal (reducing) glycose residues, and the analyses, shown in Table I, indicated that depolymerization of the 18C polysaccharide occurs exclusively by

TABLE I

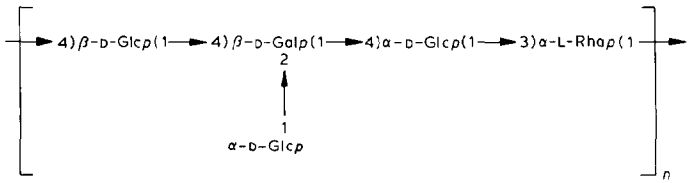
METHYLATION ANALYSES OF THE DEPOLYMERIZED FRAGMENTS (**1**, **2**, **3**, AND **4**) OF THE 18C POLYSACCHARIDE AND OF THE FRAGMENT FROM THE CHLORINE OXIDATION OF PARTIALLY METHYLATED[a] **4**

Methylated glycose derivative[b]	*Molar ratios for*					
					Partially methylated	*Chlorine oxidation of partially methylated*
	1	**2**	**3**	**4**	**4**	**4**
1,2,4,5-Rha	—	0.1	0.4	0.9	1.0	0.9
2,4-Rha	1.0	1.1	1.4	—	—	—
2,3,4,6-Glc	1.0	1.1	1.4	1.9	1.0	1.1
2,3,4,*6-Glc[c]	—	—	—	—	0.9	—
2,3,6-Glc	2.0	1.9	1.5	1.0	1.0	1.0
3,4,*6-Gal[c]	—	—	—	—	—	0.8
3,6-Gal	1.0	1.0	1.0	1.0	1.0	0.2

[a]Partially methylated **4** obtained by depolymerization of methylated **1** with aqueous HF. [b]1,2,4,5-Rha = 1,2,4,5-tetra-*O*-methyl-L-rhamnose, *etc.* [c]Asterisk denotes a deuterium-labeled methyl group (CD_3).

breakage of rhamnopyranosidic bonds. Methylation analysis of **4** yielded 1,2,4,5-tetra-*O*-methylrhamnitol, suggesting that **4** constitutes the fundamental repeating-unit of the 18C polysaccharide (terminating in a 3-*O*-substituted rhamnose residue). The methylation analysis also indicated that **4** contained two terminal (non-reducing) glucopyranosyl groups, a 4-linked glucopyranosyl residue, and a 2,4-linked galactopyranosyl residue.

In the methylation analysis of the larger oligosaccharide **3**, 1,2,4,5-tetra- and 2,4-di-*O*-methylrhamnose were detected in the ratio of 1:1. This, together with the



1 n = ~9–11
2 n = ~6–8
3 n = 2
4 n = 1

detection of a 50% increase in the proportion of 2,3,6-tri-*O*-methylglucose over that found in methylated **4**, and a decrease of similar magnitude in that of 2,3,4,6-tetra-*O*-methylglucose, indicated that **3** comprises two of the fundamental repeating-units of the 18C polysaccharide. Also, the increase in the proportion of 2,3,6-tri-*O*-methyl glucose is consistent with linkage of the fundamental repeating-unit **4** through Rha*p*-(1→4)-Glc*p* in the 18C polysaccharide.

This was confirmed by methylation analysis of the polymeric fractions **1** and **2**, in which 2,3,6-tri- and 2,3,4,6-tetra-*O*-methylglucose were detected in the ratio of 2:1. Furthermore, treatment of fully methylated **1** with aqueous hydrofluoric acid, and deuteriomethylation of part of the newly formed, partially methylated **4** with deuteriomethyl iodide, introduced a labeled methyl group at O-4 of half of the 2,3,4,6-tetra-*O*-methylglucose.

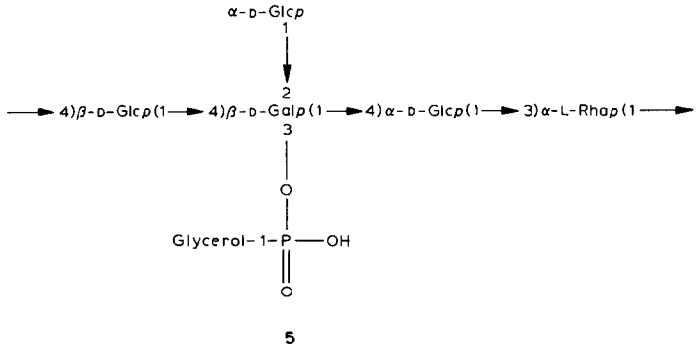
Further data regarding the sequence could be obtained by the chlorine oxidation of the 4-hydroxyl group of the terminal glucopyranosyl group of the remainder of the partially methylated **4**. In this procedure[6], treatment of the oxidized product with base causes elimination to occur in this terminal glucopyranosyl group, which can then be readily removed by mild acid. This procedure liberates the hydroxyl group on the glycose residue penultimate to the oxidized residue, and this group can now be labeled by methylation with trideuteriomethyl iodide. The results of the application of this procedure to the partially methylated **4** are shown in Table I. Although complete removal of the terminal D-glucopyranosyl group was not accomplished, as indicated by the presence of a small proportion of 3,6-di-*O*-methylgalactose, the appearance of substantial quantities of a new 3,4,6-tri-*O*-methylgalactose derivative, deuterium-labeled at O-4, indicated that the terminal glucopyranosyl group of **4** is linked to O-4 of the galactopyranosyl residue, thus demonstrating the presence of the sequence Glc*p*-(1→3)-Rha*p*-(1→4)-Gal in the 18C polysaccharide. This sequence had previously been identified[4,5] in the 18C polysaccharide.

From the methylation data accumulated, and because **4** contains only one galactopyranosyl residue, the sequence of glycose substituents in **4**, the fundamental repeating-unit of the 18C polysaccharide can now be postulated. This sequence of glycose substituents in the repeating unit of the 18C polysaccharide is identical to that proposed by Phillips *et al.*[5], except that they identified some rhamnopyranosyl residues glycosidically linked to glycerol. Some of our assignments of the anomeric configurations of the individual glycose constituents in the 18C polysaccharide also differ from those reported by Phillips *et al.*[5], as will be discussed.

Fully acetylated, polymeric **1**, obtained by the depolymerization of the 18C polysaccharide with aqueous hydrogen fluoride, was oxidized with chromium trioxide, a reagent that selectively oxidizes sugars in the β-D or -L configuration[7]. Following hydrolysis of part of the products of oxidation, rhamnose, glucose, and galactose in the ratios of 6:10:1 were detected by g.l.c. analysis. Although some galactose survived the oxidation, these results are consistent with the conclusion that the galactopyranosyl residue and one of the three glucopyranosyl residues in the repeating unit of the 18C polysaccharide have the β-D configuration. This result was confirmed by the ^{1}H-n.m.r. spectrum of **1**, which exhibits four anomeric-proton doublets which are listed, together with coupling constants in parentheses, at δ 5.29 (3.8), 5.10 (3.2), 4.84 (small, not resolved), and 4.64 (7.8 Hz). Because the last signal is composed of two overlapping doublets, as shown by comparative inte-

gration of all of the anomeric signals, this result is consistent with the 18C polysaccharide's having a five-sugar repeating-unit in which two of the sugars have the β, and three have the α, configuration.

To locate the glucopyranosyl residues that survived the oxidation, the rest of the products of oxidation was reduced with sodium borohydride, and the resultant, reduced disaccharide fragments were detected by g.l.c.–m.s. analysis, one being a hexitol-(1→3)-rhamnitol unit, and the other a hexitol-(1→2)-hexitol unit. Methylation and hydrolysis of the mixture of reduced disaccharides gave 1,2,4,5-tetra-*O*-methylrhamnitol, 1,3,4,5,6-penta-*O*-methylgalactitol, and 2,3,4,6-tetra-*O*-methylglucose in the molar ratios of ~1:1:2. Thus, one of the α-D-glucopyranosyl residues in the repeating unit of the 18C polysaccharide is linked to O-3 of the rhamnopyranosyl residue, and the other to O-2 of the galactopyranosyl residue. Consequently, except for the location of the glycerophosphate and *O*-acetyl groups, it had now been ascertained that **5** is the repeating unit of the 18C polysaccharide.



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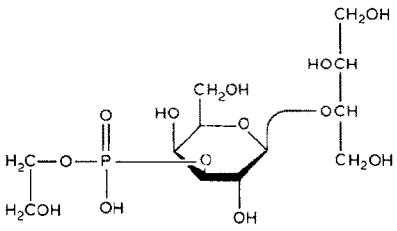
The position of linkage of the glycerophosphate group to the repeating unit of the 18C polysaccharide was determined by the method of Fiege and Radziejeweska-Lebrecht[8]. The native 18C polysaccharide was fully methylated, and the phosphate groups were removed by treatment with aqueous hydrofluoric acid, to generate a partially methylated product that was methylated with trideuteriomethyl iodide in order to label the hydroxyl group originally involved in the phosphate linkage. In g.l.c.–m.s. studies on the hydrolysis product, 3,6-di-*O*-methylgalactose fully labeled with deuterium (CD_3) at O-3 was identified; this is consistent with attachment of the glycerophosphate group to O-3 of the β-D-galactopyranosyl residue in the repeating unit of the 18C polysaccharide **5**. Of the other methylated components identified, namely 2,4-di-*O*-methylrhamnose, 2,3,6-tri-*O*-methylglucose, and 2,3,4,6-tetra-*O*-methylglucose, the last was 40% labeled with deuterium (CD_3) at O-4. This result is consistent with the previously demonstrated susceptibility of the rhamnopyranosyl residues in the 18C polysaccharide to hydrolysis by aqueous hydrofluoric acid.

The structural features previously proposed for the repeating unit of the 18C polysaccharide **5** were confirmed by treatment of the 18C polysaccharide with sodium metaperiodate by the Smith degradation-procedure[9]. This procedure also revealed the position of linkage of the phosphoric diester bond to the terminal glycerol residue. The oxidized 18C polysaccharide was reduced, and the intermediate polyalcohol derivative formed was hydrolyzed under mildly acidic conditions, to yield two saccharides (**6** and **7**), the former being the larger of the two, as determined from the elution profile of their separation by gel-filtration. The isolation of **6** and **7** is consistent with susceptibility of all three of the D-glucopyranosyl residues in **5** to oxidation by periodate. The structures of **6** and **7** were confirmed by the following procedures.



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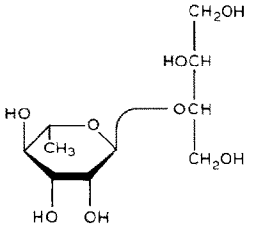

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Treatment of **6** with aqueous hydrogen fluoride yielded phosphate, ethylene glycol, glycerol, and erythritol in the molar ratios of 1.00:0.95:0.04:0.12. Further hydrolysis of some of this product under stronger conditions yielded galactose and erythritol in the molar ratio of 1.00:0.95. The small proportion of glycerol detected in the first hydrolysis, as compared to the release of equimolar quantities of phosphate and glycerol from the 18C polysaccharide under similar conditions of hydrolysis, is indicative of the presence of terminal α-glycerophosphate in the 18C polysaccharide, as suggested by Estrada-Parra and Heidelberger[4]. This was confirmed by the detection of ethylene glycol, the expected Smith degradation product of glycerol phosphorylated at one primary hydroxyl group, on hydrolysis of **6** with aqueous hydrofluoric acid. Dephosphorylated **6** was methylated, and the product was analyzed by g.l.c.–m.s. One major component was detected, the fragmentation pattern of which was identical to that given by fully methylated 2-*O*-D-galactopyranosylerythritol[10].

On hydrolysis, saccharide **7** yielded L-rhamnose and erythritol in approximately equimolar proportions. Methylation of **7**, and analysis of the derivative by g.l.c.–m.s., indicated one component, the fragmentation pattern of which was identical to that given by fully methylated 2-*O*-L-rhamnopyranosylerythritol[11].

On the basis of the periodate oxidation of the 18C polysaccharide, no definitive assignment of the location of *O*-acetyl groups could be made. Phillips *et al.*[5] proposed, on the basis of their periodate-oxidation studies, that the *O*-acetyl group of their preparation of the 18C polysaccharide is situated at O-3 of the (terminal)

D-glucopyranosyl group. Our results do not agree with their assignment, as all three of the glucopyranosyl units in our preparation of the 18C polysaccharide were oxidized. The reason for this discrepancy is not known; however, the possibility that it could be due to differences[12] in the strain or the growth medium must be allowed for.

EXPERIMENTAL

Materials. — Pneumococcal capsular polysaccharide type 18C (56) was the generous gift of Dr. J. Carlo of Merck Sharp and Dohme, Rahway, NJ. The oligosaccharide was purified on a column (25 × 2 cm) of DEAE-Sepharose 6B ion-exchange resin, using a sodium chloride gradient (0→0.6M) in 0.01M sodium phosphate buffer, pH 7.0. The eluate was monitored for hexose[13] and phosphorus[14], and the pure, type 18C polysaccharide was eluted at a gradient concentration of 0.25M. The type 18C polysaccharide was *O*-deacetylated as described by Bhattacharjee *et al.*[15]. α- and β-DL-Glycerophosphate were obtained from Sigma Chemical Co., St. Louis, MO.

Analytical methods. — Column chromatography was performed with water as the eluant, unless stated otherwise, and solutions were evaporated under diminished pressure below 40°. Gas–liquid chromatography (g.l.c.) was performed with a Hewlett–Packard 5830A instrument equipped with a flame-ionization detector and a Model 18850A electronic integrator. The glass columns (180 × 0.15 cm) used contained the following liquid phases on Gas Chrom Q: (*i*) 30% (w/w) of SP-2340 at 220°, (*ii*) 3% (w/w) of OV-225 at 190°, and (*iii*) 3% (w/w) of OV-1 (175 → 250°, at 4°/min). Glass-capillary chromatography was performed in an SP-1000 W.C.O.T. column (25 m × 0.25 nm). Combined g.l.c.–m.s. was conducted with a Finnegan 3100D or a Hewlett–Packard 5985 system, using the aforementioned columns.

^{13}C-N.m.r. spectra were recorded for solutions in 10-mm tubes at 37°, in the pulsed, Fourier-transform mode with complete proton-decoupling. Chemical shifts are reported in p.p.m. downfield from external tetramethylsilane, and the ^{2}H resonance of deuterium oxide was used as a field-frequency lock-signal. Polysaccharides and oligosaccharides were examined as solutions in deuterium oxide (25–50 mg/mL).

^{1}H-N.m.r. spectra were recorded at 80° with a Varian 400-MHz spectrometer (Regional High Resolution NMR Laboratory, University of Montreal, Quebec) in the pulsed, Fourier-transform mode. The polysaccharides and oligosaccharides (3–5 mg) were twice lyophilized from 99.7% D_2O, and examined in the same solvent. The apparent, first-order coupling-constants (Hz) were measured directly, and the chemical shifts (δ) are expressed relative to external sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS). The HOD signal was removed by saturation.

Glycose analysis. — Analyses for glycose constituents were conducted as previously described[16]. The polysaccharide (5 mg), mixed with inositol (1 mg) as the

internal standard, was treated at 4° with 48% hydrofluoric acid (1 mL), a reagent that has been shown to promote the facile cleavage of phosphoric esters[17], and the release of free phosphate[14] was monitored at various time-intervals. Complete dephosphorylation had occurred after 4 d, and the hydrogen fluoride was removed under diminished pressure over sodium hydroxide in a desiccator. Ethylene glycol, glycerol, and erythritol in the hydrolyzates were detected, and quantified, as their peracetates by g.l.c.–m.s. analysis, using a glass-capillary column, and glycerol, erythritol, and inositol as external standards.

For the analysis of the other glycose constituents, the aforementioned dephosphorylation of the type 18C polysaccharide with 48% hydrogen fluoride was repeated. The residue was hydrolyzed with 0.25M sulfuric acid for 16 h at 100°, and the glycoses were reduced with sodium borohydride, the alditols peracetylated, and the acetates detected[18] by g.l.c.–m.s. using column (*i*). The D configuration was assigned to glucose and galactose, and the L configuration to rhamnose, by g.l.c. analysis of their (−)-2-butyl glycosides, as described by Gerwig *et al.*[19,20].

Oligosaccharide repeating units. — The type 18C polysaccharide (300 mg) was treated with 48% hydrogen fluoride as previously described for the removal of its glycerol phosphate moiety. The residue was fractionated on a column (2.4 × 90 cm) of Sephadex G-25, to give two oligosaccharides of different sizes. The smaller one is referred to as oligosaccharide **4** (35 mg), and the larger one, as oligosaccharide **3** (51 mg). A material of higher molecular weight was also detected that gave a broad peak tailing from the void volume of the column; this material was divided into a fraction of larger molecular size (**1**; 94 mg), in the vicinity of the void volume of the column, and a fraction of smaller molecular size (**2**; 63 mg).

Methylation analysis. — The native, and modified, type 18C polysaccharide and derived oligosaccharides were methylated with methyl iodide in the presence of methylsulfinyl anion according to the method of Hakomori[21], and the methylation products were purified, hydrolyzed, and acetylated as described by Jennings *et al.*[16]. The specifically methylated, alditol acetate derivatives were then analyzed[22] by g.l.c.–m.s., using column (*ii*). Methylated oligosaccharides were analyzed directly by g.l.c.–m.s., using column (*iii*).

Sequential-degradation procedure. — The procedures were conducted essentially as described by Jansson *et al.*[6]. The fraction of the hydrofluoric acid-treated, type 18C polysaccharide having the largest molecular size (10 mg) was fully methylated, and the permethylated product was treated with 48% hydrofluoric acid (1 mL) for 2 d at 4°. The acid was removed as already described, and the residue purified by gel-filtration on a column[9] of Sephadex LH-20. A portion of the residue (2 mg) was methylated with trideuteriomethyl iodide, the product hydrolyzed, the sugars reduced with sodium borohydride, the alditols acetylated, and the acetates analyzed by g.l.c.–m.s. in column (*ii*).

The rest (7 mg) of the preparation was oxidized[6] with M chlorine in dichloromethane–dimethyl sulfoxide–triethylamine for 6 h at −45°. The products were purified by passage of the mixture through a column of Sephadex LH-20, and

the preparation was treated with M sodium ethoxide in ethanol for 1.5 h at room temperature, made neutral, evaporated to dryness, and hydrolyzed with 50% acetic acid for 3 h at 100°. The hydrolyzate was evaporated to dryness, and the methylated oligosaccharides were extracted with chloroform, and the extract purified by passage through a column of Sephadex LH-20. Evaporation of the solution yielded a residue (5 mg) which was methylated with trideuteriomethyl iodide, and the permethylated product was hydrolyzed, the sugars acetylated, and the acetates analyzed by g.l.c.–m.s. as previously described, using column (*ii*).

Location of phosphate groups. — The location of phosphate groups in the type 18C polysaccharide was achieved essentially as described by Fiege and Radziejeweska-Lebrecht[8]. The methylated, type 18C polysaccharide was treated with 48% hydrofluoric acid, to remove phosphate groups, and the resultant, partially methylated polysaccharide was methylated with trideuteriomethyl iodide. The permethylated polysaccharide was hydrolyzed, and the component methylated sugars were identified by g.l.c.–m.s., using column (*ii*) as previously described. Because the type 18C polysaccharide contains other linkages sensitive to breakage by hydrofluoric acid, a control experiment was conducted in which the dephosphorylated polysaccharide (a fraction of high molecular weight from treatment of the 18C polysaccharide with hydrofluoric acid) was also subjected to the procedure.

Chromium trioxide oxidation. — The oxidation of the 18C polysaccharide was conducted according to the method of Hoffman and Lindberg[7]. The high-molecular weight fraction of the hydrofluoric acid-treated 18C polysaccharide (20 mg) was fully acetylated, and the peracetylated polysaccharide was dissolved in acetic acid (glacial) together with *myo*-inositol acetate as an internal standard. An aliquot of the solution was withdrawn for control sugar-analysis, and the rest was treated with powdered chromium trioxide (400 mg) in an ultrasonic bath for 1 h at 45°. Ice–water (10 mL) was slowly added to the mixture, and the resultant solution was extracted with chloroform. Aliquots of both the extract and the control solution were evaporated to dryness, and the residues subjected to glycose analyses as previously described.

The oxidized, peracetylated polysaccharide was dissolved in 1:1 (v/v) ethanol–1,4-dioxane (10 mL), and, following the addition of sodium borohydride, the solution was stirred overnight. After addition of water, Dowex-50 (H^+) ion-exchange resin was added to the solution until pH 3.0 was reached, the suspension was filtered, the filtrate was evaporated to dryness, and methanol was four times added to and evaporated from the residue. The residue was dissolved in water, and fractionated on a column of Bio-Gel P2. Fractions associated with the disaccharide region of the elution profile were evaporated to dryness, and the methylated oligosaccharides were identified by g.l.c.–m.s. using column (*iii*), as previously described.

Periodate oxidation. — A Smith degradation was performed as described by Goldstein *et al.*[9]. Native 18C polysaccharide (60 mg) was oxidized with 0.2M sodium metaperiodate (25 mL) in 0.1M sodium aceate buffer, pH 3.9 (75 mL) for

120 h at 4°. The excess of periodate was decomposed by the addition of ethylene glycol (4 mL), and the solution was dialyzed against water. Sodium borohydride (250 mg) was added, and the mixture was kept for 16 h. The excess of sodium borohydride was decomposed by addition of Dowex-50 (H^+) ion-exchange resin, the resin filtered off, and the boric acid removed by 3 additions and evaporations of methanol under vacuum. The residue was hydrolyzed with 0.1M HCl for 16 h at 25°, and the neutral hydrolyzate was fractionated on a column (1.5 × 90 cm) of Bio-Gel P2. Two oligosaccharide fractions (**1** and **2**) were obtained in the order of size.

The fraction (**1**) of larger molecular size was hydrolyzed with 48% aqueous HF for 3 d at 4°, and the hydrolyzate was analyzed for the presence of phosphate, ethylene glycol, glycerol, and erythritol. Some of the hydrolyzate was further hydrolyzed with 0.25M sulfuric acid for 16 h at 100°, and the glycose components were identified by g.l.c.–m.s. as their alditol acetate derivatives, as already described. The rest of the hydrofluoric acid hydrolyzate was methylated, and the products identified by g.l.c.–m.s. using column (*iii*).

The fraction (**2**) of smaller molecular size from the Bio-Gel P2 column was methylated, and identified by g.l.c.–m.s. using column (*iii*).

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REFERENCES

1 P. SMITH, D. OBERHOLZER, S. HAYDEN-SMITH, H. J. KOORNOF, AND M. R. HILLEMAN, *J. Am. Med. Assoc.*, 238 (1977) 2613–2616.
2 R. AUSTRIAN, *Rev Infect. Dis.*, 3 (1981) s1–s17.
3 H. J. JENNINGS, *Adv. Carbohydr. Chem. Biochem.*, 41 (1983) 155–208.
4 S. ESTRADA-PARRA AND M. HEIDELBERGER, *Biochemistry*, 2 (1963) 1288–1294.
5 L. R. PHILLIPS, O. NISHIMURA, AND B. A. FRASER, *Carbohydr. Res.*, 121 (1983) 243–255.
6 P.-E. JANSSON, L. KENNE, B. LINDBERG, H. LJUNGGREN, J. LONNGREN, U. RUDÈN, AND S. SVENSSON, *J. Am. Chem. Soc* , 99 (1977) 3812–3815.
7 J. HOFFMAN AND B. LINDBERG, *Methods Carbohydr. Chem.*, 8 (1980) 117–122.
8 V. FIEGE AND J. RADZIEJEWESKA-LEBRECHT, *Proc. Int. Symp. Glycoconjugates*, (1979) 12–13.
9 I. J. GOLDSTEIN, G. W. HAY, B. A. LEWIS, AND F. SMITH, *Methods Carbohydr. Chem.*, 5 (1965) 361–370.
10 B. LINDBERG, B. LINDQVIST, J. LONNGREN, AND W. NIMMICH, *Carbohydr. Res.*, 58 (1977) 443–451.
11 M. CURVALL, B. LINDBERG, J. LONNGREN, AND W. NIMMICH, *Carbohydr. Res.*, 42 (1975) 73–82.
12 H. J. JENNINGS, A. K. BHATTACHARJEE, D. R. BUNDLE, C. P. KENNY, A. MARTIN, AND I. C. P. SMITH, *J. Infect. Dis., Suppl.*, 136 (1977) s78–s83.
13 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
14 P. S. CHEN, J. Y. TORIBA, AND H. WARNER, *Anal. Chem.*, 28 (1956) 1756–1758.
15 A. K. BHATTACHARJEE, H. J. JENNINGS, C. P. KENNY, A. MARTIN, AND I. C. P. SMITH, *J. Biol. Chem.*, 250 (1975) 1926–1932.
16 H. J. JENNINGS, A. K. BHATTACHARJEE, L. KENNE, C. P. KENNY, AND G. CALVER, *Can. J. Biochem.*, 58 (1980) 128–136.
17 D. LIPKEN, B. E. PHILLIPS, AND J. W. ABRELL, *J. Org. Chem.*, 34 (1969) 1539–1547.

18 J. S. SAWARDEKER, J. H. SLONEKER, AND A. R. JEANES, *Anal. Chem.*, 37 (1965) 1602–1604.
19 G. J. GERWIG, J. P. KAMERLING, AND J. F. G. VLIEGENTHART, *Carbohydr. Res.*, 62 (1978) 349–357.
20 G. J. GERWIG, J. P. KAMERLING, AND J. F. G. VLIEGENTHART, *Carbohydr. Res.*, 77 (1979) 1–7.
21 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
22 B. LINDBERG, *Methods Enzymol.*, 28 (1972) 178–195.