A Reinvestigation on the Structure of the Capsular Polysaccharide from *Pneumococcus* Type IX

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The capsular polysaccharide of *Pneumococcus* Type IX (S IX) was subjected to methylation. The methylated derivative was reduced with $LiAlH_4$ to convert the uronic acid units into neutral sugars, and the native polysaccharide was also reduced with diborane. The uronic acid residues were subjected to base degradation. The uronic acid-reduced product was deacetylated and deaminated. All these products were converted into their methylated derivatives and the methyl sugars in the hydrolysates were characterised and estimated. The polysaccharide was subjected to periodate oxidation followed by Smith degradation. At every stage of the reaction the material was subjected to methylation studies. The fully methylated S IX was hydrolysed using mildly acidic conditions to yield five methyl oligosaccharides. The mixture was re-methylated and then separated into its component. One mixture and three homogeneous fractions were obtained. The methyl sugars in the homogeneous fragments were identified. From these studies a revised structure is assigned to the repeating unit of S IX. Chromium trioxide oxidation studies indicated that all the glycosidic linkages are of the α-type.

STUDIES on the structural [1-3] and immunochemical [4-10] aspects of *Pneumococcus* Type IX capsular polysaccharide (S IX) have been reported by Heidelberger and others, leading to a possible tentative structure. Oligomers were isolated in these investigations, but were not shown to be homogeneous and were not unequivocally characterised. With these points in view further work has been undertaken on S IX.

RESULTS AND DISCUSSION

In this paper results of methylation studies on S IX, and its various modified and/or degraded products, are reported. In all cases, methylation was effected by Hakomori's procedure [11] followed by Purdie's [12] method. The methyl sugars in the hydrolysates of the products were converted into their alditol acetates, and then characterised either by g.l.c.[13] or g.l.c.–m.s.[14] As the alditol acetates of some methyl sugars could not be separated by g.l.c., the methyl sugar mixture was first separated by high-pressure liquid chromatography into mixtures containing one or two components, which were converted into alditol acetates and then identified by either g.l.c. or g.l.c.–m.s.

Fully methylated S IX on hydrolysis gave 2,3,6-tri-*O*-methyl-D-glucose, 2,4,6-tri-*O*-methyl-D-glucose, 4,6-di-*O*-methyl-*N*-methylglucosamine,† and 4,6-di-*O*-methyl-*N*-methylmannosamine; a trace of 4,6-di-*O*-methyl-*N*-methylgalactosamine was also detected on the g.l.c. chromatogram but it could not be fully characterised by g.l.c.–m.s. due to the small quantity.

In Table 1 column A the molar proportions of different methyl sugars are given. Retention times of 2,3,6-tri-*O*-methyl-D-glucose and 4,6-di-*O*-methyl-*N*-methyl-

TABLE 1

Methylation studies of S IX

Methyl sugars [a]	R_t [b] ECNSS-M	R_t [b] OV-225	Molar proportions [c] A	B	C	D	E	F
2,3,4,6-Tetra-*O*-methyl-D-glucose [f]	1.00	1.00				0.8	0.8	
3,4,6-Tri-*O*-methyl-*N*-methylglucosamine [g]	1.00	1.00				0.6		
2,4,6-Tri-*O*-methyl-D-glucose [h]	1.95	1.75	1.6	1.8	1.7	1.0	2.9	1.9
2,3,6-Tri-*O*-methyl-D-glucose [i]	2.50	2.32 [d]	0.8	0.8	2.6	0.8	2.8	2.8
4,6-Di-*O*-methyl-*N*-methylglucosamine [j]	2.32	2.32 [d]	1.7	1.9	1.8	1.3		1.9
4,6-Di-*O*-methyl-*N*-methylmannosamine [j]	2.82	3.40	1.8	1.8	1.9	1.6		1.8
2,3-Di-*O*-methyl-D-glucose [k]	5.39	4.50		1.9				
2,5-Anhydro-1,4,6-tri-*O*-methyl-D-mannitol [l]		0.23					0.7 [e]	
2,5-Anhydro-1,3,4,6-tetra-*O*-methyl-D-mannitol		0.11					0.6 [e]	

[a] The methyl sugars identified are the corresponding alditol acetates. [b] Retention times are relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. [c] Polysaccharide: A, methylated S IX; B, methylated–carboxy-reduced S IX; C, carboxy-reduced–methylated S IX; D, carboxy degradation of methylated S IX; E, deaminated and methylated S IX; F, chromium trioxide oxidised–methylated S IX. [d] The retention times of 2,3,6-tri-*O*-methyl-D-glucose and 4,6-di-*O*-methyl-*N*-methylglucosamine are very similar. [e] Part of the alditol acetate is probably lost during concentration. [f] 1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol; *m/e* 43 (100%), 45 (47), 71 (14), 85 (6), 87 (20), 101 (69), 113 (9), 117 (32), 129 (34), 131 (6), 145 (25), 161 (24), and 205 (5). [g] 1,5-Di-*O*-acetyl-3,4,6-tri-*O*-methyl-D-hexosamine; *m/e* 43 (100%), 45 (39), 74 (27), 87 (9), 116 (34), 129 (6), 142 (7), 145 (7), 158 (5), 161 (1), 202 (1), and 205 (2). [h] 1,3,5-Tri-*O*-acetyl-2,4-6-tri-*O*-methyl-D-glucitol; *m/e* 43 (100%), 45 (32), 73 (21), 81 (11), 101 (18), 117 (37), 129 (28), 161 (8), and 233 (5). [i] 1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol; *m/e* 43 (100%), 45 (22), 71 (9), 73 (8), 87 (14), 99 (17), 101 (16), 113 (18), 117 (39), 129 (7), 131 (6), 161 (3), and 233 (9). [j] 1,3,5,-Tri-*O*-acetyl-4,6-di-*O*-methyl-D-hexosamine; *m/e* 43 (100%), 45 (36), 74 (32), 87 (5), 116 (45), 129 (5), 142 (2), 145 (1), 158 (11), 161 (1), 170 (1), 231 (1), 242 (1), and 274 (1). [k] 1,4,5,6-Tetra-*O*-acetyl-2,3-di-*O*-methyl-D-glucitol; *m/e* 43 (100%), 45 (3), 58 (4), 71 (4), 85 (18), 99 (18), 101 (21), 117 (70), 127 (19), 129 (5), 159 (5), 161 (4), 201 (6), and 256 (4). [l] 3-*O*-Acetyl-2,5-anhydro-1,4,6-tri-*O*-methyl-D-mannitol; *m/e* 43 (100%), 45 (99), 46 (42), 71 (15), 87 (16), 129 (75), 138 (8), 143 (9), 144 (14), 157 (7), 171 (2), and 203 (20).

† Abbreviations: glucosamine = 2-deoxy-2-acetamido-D-glucopyranose: mannosamine = 2-deoxy-2-acetamido-D-mannopyranose. After methylation and hydrolysis: *N*-methylglucosamine = 2-deoxy-2-(*N*-methylacetamido)-D-glucopyranose: *N*-methylmannosamine = 2-deoxy-2-(*N*-methylacetamido)-D-mannopyranose.

glucosamine (2.50 and 2.32 respectively) are very close on an ECNSS-M column, and almost the same on an OV-225 column. In the former column two humps were observed in the chromatogram corresponding of these two methyl sugars. Taking advantage of the shape of the curve they were integrated (using a reporting integrator) to obtain the areas of individual components. From these values, it was estimated that the amounts of 2,3,6-tri-*O*-methyl-D-glucose and 4,6-di-*O*-methyl-*N*-methylglucosamine were 0.8 and 1.7 mol, respectively. The amounts of 2,4,6-tri-*O*-methyl-D-glucose and 4,6-di-*O*-methyl-*N*-methylmannosamine were found to be 1.6 and 1.8 mol, respectively. The presence of 2,3,6- and 2,4,6-tri-*O*-methyl-D-glucose units indicates that S IX contains 1,4- and 1,3-linked D-glucopyranose residues. D-Glucosamine and D-mannosamine residues are 1,3-linked.

Polysaccharide S IX contained glucuronic acid residues, and the glycosidic linkage of these acid sugars could not be hydrolysed completely without affecting the constituent sugar units. So the methylated S IX was reduced with lithium aluminium hydride, hydrolysed, and the methyl sugars identified and estimated. The results are given in Table 1, column B. Comparison of the values in columns A and B showed that the reduced methylated polysaccharide yielded 2,4,6-tri-*O*-methyl-D-glucose (1.8 mol), 2,3,6-tri-*O*-methyl-D-glucose (0.8 mol), 4,6-di-*O*-methyl-*N*-methylglucosamine (1.9 mol), and 4,6-di-*O*-methyl-*N*-methylmannosamine (1.8 mol); whereas the unreduced methylated polysaccharide gave 1.6, 0.8, 1.7, and 1.8 mol, respectively. The other methyl sugar *viz.* 2,3-di-*O*-methyl-D-glucose, which obviously originated from the D-glucuronic acid units in S IX, was obtained in 1.9 mol proportion, indicating that the uronic acid units in the polysaccharide were 1,4-linked. Of the three molecules of D-glucose, two are 1,3- and the third is 1,4-linked.

The carboxy-groups of the uronic acid residues in the native polysaccharide were reduced with diborane. The product was methylated and hydrolysed, and the methyl sugars were analysed in the usual way. The results are given in Table 1, column C. In this hydrolysate 2,3-di-*O*-methyl-D-glucose was not present, whereas the proportion of 2,3,6-tri-*O*-methyl-D-glucose increased from 0.8 mol to 2.6 mol, indicating that the D-glucuronic acid residues in S IX were 1,4-linked. This is in agreement with the earlier conclusions by Heidelberger and his co-workers [2] regarding the linkage of uronic acid units in the polysaccharide. The molar proportions of other methyl sugars remained almost the same.

To identify the position of the uronic acid residues, the polysaccharide was subjected to base degradation studies, in which acid sugar units were selectively cleaved,[15] and the degraded material was re-methylated. The results of re-methylation are summarised in Table 1, column D. 2,3,4,6-Tetra-*O*-methyl-D-glucose and 3,4,6-tri-*O*-methyl-*N*-methylglucosamine have the same retention time. Quantitisation of the results was made by separating the hydrolysate with high pressure liquid chromatography and then t.l.c. using methyl ethyl ketone–water azeotrope. The spots were developed by spraying with 20% sulphuric acid and the intensities, measured with Chromoscan,* were found to be in the ratio 4 : 3. The molar proportions of 2,3,4,6-tetra-*O*-methyl-D-glucose and 3,4,6-tri-*O*-methyl-*N*-methylglucosamine are 0.8 and 0.6, respectively, rather than whole numbers. This could be explained by the fact that the degradation of the uronic acid residues in a polysaccharide is <100% complete, as observed by Lindberg and his co-workers.[15] In the case of 4,6-di-*O*-methyl-*N*-methylmannosamine, 1.6 rather than 2.0 mol were obtained; further β-elimination [15] during degradation with base might be responsible for the lower yield of this sugar. The fact that the uronic acid portion, degraded and then methylated, yielded 2,3,4,6-tetra-*O*-methyl-D-glucose and 3,4,6-tri-*O*-methyl-*N*-methylglucosamine, indicates that the two aldobiouronic acid fragments in the polysaccharide have the structures (⟶4)GlcA(1⟶3)Glc(⟶) and (⟶4)GlcA(1⟶3)GlcNAc(⟶).

Selective cleavage based on *N*-deacetylation followed by deamination was applied to the polysaccharide to establish the position of the amino-sugars. Diborane-reduced S IX was *N*-deacetylated,[16] followed by gel filtration on a Sephadex G-50 column. The material eluted was of high molecular weight, showing that no degradation took place during deacetylation. The material was deaminated, reduced, and then hydrolysed. The hydrolysate contained small quantities (<1%) of glucosamine and mannosamine as compared with the initial amounts, indicating that not less than 95% of the amino-sugar residues were deaminated. The deaminated product on methylation and hydrolysis gave 2,3,4,6-tetra-*O*-methyl- (0.8 mol) and 2,3,6- (2.8 mol) and 2,4,6-tri-*O*-methyl-D-glucose (2.9 mol), along with 2,5-anhydro-1,4,6-tri-*O*-methyl-D-mannitol (0.7 mol). Another peak close to that of solvent was also obtained. Its retention time corresponded to that of 2,5-anhydro-1,3,4,6-tetra-*O*-methyl-D-mannitol (0.6 molar proportion) (Table 1, column E). Deamination, methylation, and hydrolysis of S IX yielded 2,4,6-tri-*O*-methyl-D-glucose instead of mannosamine, non-reducing end units of D-glucose (2,3,4,6-tetra-*O*-methyl-D-glucose), and one each of the tri- and tetra-*O*-methyl derivative of reduced 2,5-anhydromannitol, the latter two being derived from D-glucosamine residues. From these results it is concluded that two *N*-acetyl-D-glucosamine residues are present in a sequence. As the D-glucuronic acid unit is linked glycosidically to a glucosamine residue, the structure of a fragment is (⟶4)-GlcA(1⟶3)GlcNAc(1⟶3)GlcNAc(1⟶3)-Glc(⟶). That the end D-glucose unit in the above fragment is linked at the C-3 position is supported by periodate oxidation studies (see later).

Earlier workers [3] used periodate oxidation to identify some of the linkages in S IX : 90% of the uronic acid residues and *ca.* 40% of the glucose residues were

* Chromoscan with the thin-layer scanner: Joyce Loebl & Co. Ltd., Gateshead.

TABLE 2

Methylation analysis of the products of periodate-oxidised S IX

Methyl sugars [a]	R_t [b] ECNSS–M	R_t [b] OV-225	Molar proportions [c] A	B	C
2,3,4,6-Tetra-*O*-methyl-D-glucose	1.00	1.00			
3,4,6-Tri-*O*-methyl-*N*-methylglucosamine	1.00	1.00		3.0	1.0
3,4,6-Tri-*O*-methyl-*N*-methylmannosamine	1.00	1.00			
2,4,6-Tri-*O*-methyl-D-glucose	1.95	1.75	2.0	1.0	0.9
4,6-Di-*O*-methyl-*N*-methylglucosamine	2.32	2.32	1.9	0.9	
4,6-Di-*O*-methyl-N-methylmannosamine	2.82	3.40	1.9	0.9	0.9

[a] The methyl sugars identified are the corresponding alditol acetates. [b] Retention times are with respect to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. [c] Polysaccharide treatment: A, periodate oxidation–methylation; B, periodate oxidation–reduction–mild hydrolysis–methylation; C, periodate oxidation–reduction–mild hydrolysis–second periodate oxidation–reduction–mild hydrolysis–methylation.

oxidised; all the amino-sugars survived the oxidation. In the present studies the polysaccharide was subjected to oxidation by periodate and Smith degradation, and the products obtained at various stages were subjected to methylation. Polysaccharide S IX was oxidised with IO_4^- and the resulting oxidised polysaccharide was fully methylated. The results are summarised in Table 2, column A. Three methyl sugars, *i.e.* 2,4,6-tri-*O*-methyl-D-glucose, 4,6-di-*O*-methyl-*N*-methylglucosamine, and 4,6-di-*O*-methyl-*N*-methylmannosamine, were obtained almost in the same molar proportions. In addition, the methyl derivatives of lower alcohols and aldehydes were also found, but they came out of the column much earlier than 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, and were very close to the solvent peak. In the repeating unit of S IX, one 1,4-linked D-glucose and two 1,4-linked D-glucuronic acid residues are susceptable to oxidation, leaving the other sugar residues intact.

The polysaccharide S IX was subjected to periodate oxidation, reduction, mild hydrolysis, full methylation, and then hydrolysis; one molar proportion of each of the above three methyl sugars together with 2,3,4,6-tetra-*O*-methyl-D-glucose, 3,4,6-tri-*O*-methyl-*N*-methylglucosamine, and 3,4,6-tri-*O*-methyl-*N*-methylmannosamine were obtained; the total amount of the last three sugars was 3 molar proportions (Table 2, column B). The mixture containing these three methyl sugars could not be separated and the mass-fragmentation analysis showed characteristic fragments for tetra-*O*-methyl-D-hexose and tri-*O*-methyl-D-hexosamine.

From these results the following conclusion can be drawn. One molar proportion each of 2,4,6-tri-*O*-methyl-D-glucose, 4,6-di-*O*-methyl-*N*-methylglucosamine, and 4,6-di-*O*-methyl-*N*-methylmannosamine disappeared, and were replaced by similar quantities of tetra-*O*-methyl-D-glucose and two tri-*O*-methyl-D-hexosamines. This indicates that one unit of uronic acid was linked to C-3 of the D-glucose residue, and the other to C-3 of D-glucosamine; one unit of 1,4-linked glucose was glycosidically linked to C-3 of the D-mannosamine residue in a repeating unit. Heidelberger *et al.*[2,3] identified an oligosaccharide having the structure ManNAc(1 → 3)-Glc(1 → 3)ManNAc, in the graded hydrolysis product, whereas the present results give evidence for the presence of a grouping (→ 4)Glc(1 → 3)ManNAc(1 →) in the molecule. Therefore, the structure of the trisaccharide fragment is (→ 3) ManNAc(1 → 4)Glc(1 → 3)ManNAc(1 →).

The periodate oxidised–Smith degraded S IX was subjected to a second periodate oxidation followed by another Smith degradation. The resulting product was fully methylated and then hydrolysed to yield methyl sugars which were identified as 3,4,6-tri-*O*-methyl-*N*-methylglucosamine (1.0 mol), 2,4,6-tri-*O*-methyl-D-glucose (0.9 mol), and 4,6-di-*O*-methyl-*N*-methylmannosamine (0.9 mol) (Table 2, column C). These results show that the periodate-resistant fragment was a trisaccharide with D-glucosamine at the non-reducing end. Its structure is (→ 3)GlcNAc(1 → 3)Glc(1 → 3)-ManNAc(→). The deamination reaction supports the structure.

Fully methylated S IX was hydrolysed with 90% formic acid and the hydrolysate was further methylated by Purdie's method. The mixture gave spots corresponding to five substances on paper chromatography.

TABLE 3

Methylated oligosaccharides obtained on partial hydrolysis of the methylated S IX

Fraction	Yield (mg)	Paper chromatographic mobility [a]	Methyl sugars [b]	R_t [c] ECNSS–M	R_t [c] OV-225	Molar proportion
F_5	4.0	0.85	2,4,6-Tri-*O*-methyl-D-glucose	1.95	1.80	trace
F_3 and F_4	6.2	0.65	3,4,6-Tri-*O*-methyl-*N*-methylglucosamine	1.00	1.00	0.8
			2,4,6-Tri-*O*-methyl-D-glucose	1.95	1.82	1.0
			4,6-Di-*O*-methyl-*N*-methylglucosamine	2.32	2.32	0.6
F_2	5.1	0.41	3,4,6-Tri-*O*-methyl-*N*-mannosamine	1.00	1.00	0.9
			2,3,6-Tri-*O*-methyl-D-glucose	2.50	2.30	1.0
			4,6-Di-*O*-methyl-*N*-methylmannosamine	2.82	3.40	0.9
F_1	3.5	0.20	4,6-Di-*O*-methyl-*N*-methylglucosamine	2.30	2.30	1.7
			2,4,6-Tri-*O*-methyl-D-glucose	1.95	1.82	1.0

[a] Relative to 2,3,4,6-tetra-*O*-methyl-D-glucose. [b] Methyl sugars identified are the corresponding alditol acetates. [c] Retention times are with respect to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

The slowest moving material was designated as F_1 and the fastest as F_5. The mixture was separated on thick filter papers. Oligosaccharides F_1, F_2, and F_5 were obtained in a homogeneous state, whereas F_3 and F_4 were isolated as a mixture which could not be further separated. I.r. studies gave signals for the CO_2Me group in F_1, F_5, and the F_3–F_4 mixture, indicating the presence of the uronic acid unit as a component. The results are given in Table 3.

Fraction F_5 gave on hydrolysis 2,4,6-tri-*O*-methyl-D-glucose. This oligosaccharide is obviously the methyl derivative of aldobiouronic acid containing glucose and glucuronic acid units, whose structure is GlcA(1 → 3)-Glc.

Fraction F_2 did not contain uronic acid and on hydrolysis gave 3,4,6-tri- and 4,6-di-*O*-methyl-*N*-methylmannosamine and 2,3,6-tri-*O*-methyl-D-glucose in almost equal molar proportions. This methyl trisaccharide obviously originated from the portion of the repeating unit having the structure (→ 3)ManNAc(1 → 4)-Glc(1 → 3)ManNAc.

Fraction F_1 contained uronic acid and on hydrolysis gave 2,4,6-tri-*O*-methyl-D-glucose (1.0 mol) and 4,6-di-*O*-methyl-*N*-methylglucosamine (1.7 mole). Due to the presence of the amino-sugar and uronic acid units the oligosaccharide did not undergo complete hydrolysis, and as a result the yield of di-*O*-methyl-*N*-methylglucosamine was less than theoretical. As no tetra-*O*-methyl-D-hexose or tri-*O*-methyl-D-hexosamine was detected in the hydrolystate, the uronic acid moiety occupied the non-reducing end. From this, and from the structure assigned to the aldobiouronic acid containing glucuronic acid and glucosamine, the structure assigned to this fragment is GlcA(1 → 3)GlcNAc(1 → 3)-GlcNAc(1 → 3)Glc. Periodate oxidation indicated that the 1,3-linked glucose residue was linked to the C-3 position of the mannosamine residue in the repeating unit.

The mixture of fractions F_3 and F_4 contained uronic acid, and gave on hydrolysis 3,4,6-tri-*O*-methyl-*N*-methylglucosamine (0.8 mol), 4,6-di-*O*-methyl-*N*-methylglucosamine (1.0 mol), and 2,4,6-tri-*O*-methyl-D-glucose (0.6 mol). Probably there are two disaccharides in this mixture having the structures GlcA(1 → 3)-GlcNAc and GlcNAc(1 → 3)Glc, which formed from the tetrasaccharide in fraction F_1 during the hydrolysis. From the results of these analyses the sequence of the two aldobiouronic acid units is as follows: (→ 4)-GlcA(1 → 3)Glc(1 → 4)GlcA(1 → 3)GlcNAc(→).

From all these considerations the structure assigned to the repeating unit of the capsular polysaccharide of *Pneumococcus* Type IX is as follows: (→ 4)GlcA-(1 → 3)Glc(1 → 4)GlcA(1 → 3)GlcNAc(1 → 3)-GlcNAc(1 → 3)Glc(1 → 3)ManNAc(1 → 4)Glc-(1 → 3)ManNAc(1 →).

A part of the carboxy-reduced acetylated polysaccharide was treated with chromium trioxide–acetic acid,[17] a procedure known to oxidised β-pyranosidic residues leaving α-pyranosidic residues intact. On methylation followed by hydrolysis it was observed that no methyl sugar was lost to any extent (Table 1, column F). This indicates that there is no β-linked sugar residue in the macromolecules. In agreement with earlier findings,[2,3] all the glycosidic linkages in S IX are of the α-type.

EXPERIMENTAL

General Methods.—Concentration of solutions was carried out under reduced pressure at bath temperature not exceeding 40 °C. Specific rotations were determined at 30 °C with a Perkin-Elmer polarimeter model 241 MC and are equilibrium values. I.r. spectra were taken in chloroform solutions or in potassium bromide pellets with a Perkin-Elmer Infracord spectrometer. Paper chromatography was carried out by the descending method using Whatmann No. 1 filter papers using as solvent the methyl ethyl ketone–water azeotrope. Spray reagents used were saturated aqueous aniline oxalate and 0.1% ninhydrin in butan-ol. G.l.c. was performed with a Hewlett-Packard models 5730A gas chromatograph equipped with flame-ionization detector. The columns used were: (A) 3% ECNSS-M on Gas Chrom Q (100—120 mesh) (180 mm × 4 mm); and (B) 3% OV-225 on Gas Chrom Q (100—120 mesh) (180 mm × 2 mm) at 170—200 °C; the carrier gas was nitrogen and the pressure and flow rates were maintained as required. For quantitative evaluation of the areas under different peaks in the chromatogram, a Hewlett-Packard model 3370B integrator was used. A Varian MAT 311 gas chromatograph–mass spectrometer system SS-100 was used for identification of the alditol acetates of methyl sugars. Mass spectrometer conditions were: ionisation potential, 70 eV; acceleration voltage, 3 kV: emission current, 1 000 μA: ion-source temperature, 160 °C. Computer conditions were: exponential scan speed, 4 sec/decade; sampling rate, 6 kHz. The column used was 3% OV-225 at 195 °C with helium as a carrier gas. In some cases the sugar mixtures were separated by high-pressure liquid chromatography using a Water-Associates' model 440 liquid chromatography, fitted with u.v. and refractive index detectors; the conditions used were: column, μ-Bondapack-Carbohydrate; solvent, methyl cyanide–water (85 : 15); flow rate, 1.5 ml min^{-1}. N.m.r. spectra of materials in D_2O solutions at 85 °C were recorded using a Varian XL-100 instrument.

Methylation Analysis.—The polysaccharide (60 mg, dried over P_2O_5), in a 100-ml serum bottle sealed with a rubber cap, was dissolved in dry dimethyl sulphoxide (20 ml). The bottle was flushed with nitrogen and 2M methylsulphinylsodium in methyl sulphoxide (12 ml) was added using a syringe. The gelatinous solution was agitated in an ultrasonic bath for 30 min and then kept at room temperature overnight. Methyl iodide (10 ml) was added dropwise with external cooling and the resulting turbid solution was agitated for 30 min in the ultrasonic bath. The clear solution was diluted with water (25 ml), dialysed against distilled water, and the non-dialysable material (65 mg) was recovered by freeze-drying. This product showed slight hydroxy absorption in the i.r. spectrum and was further subjected to two successive methylations by Purdie's method.[12] The product had no hydroxy absorption in the i.r.; $[\alpha]_D^{30}$ +20.6 (*c*, 2.0 in $CHCl_3$); yield 60 mg.

The methylated S IX (1.5 mg) was heated in 4N·HCl (1 ml) on a boiling water-bath for 5 h. The acid was removed

by storage over KOH in vacuo; the last traces of acid were removed as silver chloride. The methyl sugars so obtained were analysed as their alditol acetates by g.l.c. and g.l.c.-m.s. Results are shown in Table 1, column A.

Carboxyl Reduction of the Methylated S IX.—The fully methylated S IX (10 mg) in dry tetrahydrofuran (5 ml), was added cautiously to a suspension of lithium aluminium hydride (25 mg) in tetrahydrofuran (5 ml). The reaction mixture was refluxed for 20 h, and moist ethyl acetate was added to decompose the excess of hydride. The insoluble salts were centrifuged off and washed with chloroform. The combined washings and centrifugate were evaporated to dryness (8 mg). This product showed hydroxy absorption in the i.r. spectrum and the band previously present at 1 735 cm^{-1} (carboxy ester) disappeared. Hydrolysis and analysis of the constituent methyl sugars were performed as described for the methylated polysaccharide. The results are shown in Table 1, column B.

Carboxy Reduction of S IX *and Methylation.*—Dried S IX (20 mg) was well dispersed in formamide–pyridine (2 : 1) (30 ml), by stirring the suspension for 3—4 h. After addition of acetic anhydride (10 ml) the mixture was kept at room temperature for 18 h. Water (10 ml) was added dropwise to the cooled solution and the mixture was dialysed against distilled water. Concentration and purification were effected by passing it through a column (60 × 5 cm) of Sephadex LH-20 which was irrigated with acetone. The eluant was monitored by polarimeter and the acetylated polysaccharide (18 mg) eluted out in the void volume.

The acetylated product was suspended in dry tetrahydrofuran (18 ml) in a serum vial fitted with a rubber septum. Excess of diborane in the same solvent was added and the mixture was stirred for 16 h at room temperature. Methanol was cautiously added to decompose the excess of diborane and the methyl borate was removed in the usual way. The yield of acetylated reduced product was 12 mg. I.r. spectroscopy showed no carboxy absorption band.

The substance was methylated by the methods of Hakomori and Purdie, hydrolysed, and the methyl sugars identified and estimated as usual. The results are summarised in Table 1, column C.

Carboxy Degradation of the Methylated Polysaccharide.—Fully methylated, dried S IX (10 mg) and a trace of toluene-*p*-sulphonic acid were dissolved in dimethyl sulphoxide–2,2-dimethoxypropane (19 : 1 v/v) (2 ml) contained in a serum vial sealed with a rubber septum. The vial was flushed with nitrogen and then agitated ultrasonically for 30 min at 20—25 °C to ensure complete solution. After addition of 2M methylsulphinylsodium (1 ml) in methyl sulphoxide the solution, which turned dark yellow, was agitated ultrasonically at 20—25 °C for an additional 30 min, and then kept overnight at room temperature. The vial was opened and 50% aqueous acetic acid (10 ml) was added with external cooling. The reaction mixture was poured into water (50 ml) and the aqueous solution was extracted with chloroform (3 × 25 ml). The combined organic phase was washed with water, dried over anhydrous sodium sulphate, and concentrated to dryness. The product was suspended in 10% aqueous acetic acid (10 ml) and heated on a boiling water-bath for 1 h; the modified polysaccharide was recovered by freeze-drying. It was re-methylated by Hakomori's procedure, and then recovered by partition between water and chloroform and hydrolysed. The resulting methyl sugars were analysed in the usual way. The results are summarised in Table 1, column D.

Deamination of S IX.—Before deamination was effected the carboxy groups of the acid sugars were reduced, followed by *N*-deacetylation. The *O*-acetylated S IX (90 mg) was treated with diborane to convert the uronic acid units into the corresponding neutral sugar residues. The resulting material did not show the band corresponding to the carbonyl group in i.r. spectrum (yield 75 mg). The acetylated and carboxy-reduced polysaccharide (60 mg) and sodium thiophenolate (6 g) were dissolved in water (6 ml). 2M Methylsulphinylsodium in methyl sulphoxide (40 ml) was added and the mixture was heated to 100 °C in a sealed tube for 17 h. Sodium hydroxide precipitated when the reagents were added and a precipitate of diphenyl disulphide was formed during the reaction. The reaction mixture was diluted with water, filtered, dialysed against water, and freeze-dried. The deacetylated product was added to a column of Sephadex G-50 (80 × 1.6 cm) which was eluted with water. The material, isolated as a single peak from the column, was then lyophilised (yield 45 mg). After *N*-deacetylation the signals at δ 2.05—2.08 in the n.m.r. spectrum disappeared and the signals at δ 4.76—4.80 shifted upfield by *ca.* 0.15 p.p.m.

N-Deacetylated, carboxy-reduced S IX (30 mg) was dissolved in water (1 ml) and cooled to 0 °C; ice-cold sodium nitrite solution (5%, 1.5 ml) was added with stirring, followed by acetic acid (33%, 1.5 ml) dropwise at such a rate that the temperature did not exceed 2 °C. The solution was stirred for 2 h at 0 °C; ninhydrin did not give a positive test with the reaction mixture. The solution was brought to 20—25 °C, nitrogen was bubbled through for 30 min to remove the remaining nitrous acid, and the solution was freeze-dried. The resultant tacky solid was dispersed in methanol (25 ml), which was then removed under reduced pressure. The material was passed through a column of Dowex 50 (H^+) resin, and then reduced with $NaBH_4$. After removal of excess of $NaBH_4$, followed by usual treatments, the product was isolated. A hydrolysate of a portion (2 mg) of this material was found to contain glucosamine (0.8%) and mannosamine (0.9%). The remaining material was methylated by Hakomori's method, and fully methylated material was hydrolysed. The constituent methyl sugars were analysed in the usual way. The results are summarised in Table 1, column E.

Periodate Oxidation of S IX.—S IX (30 mg) in water (5 ml) was allowed to react with sodium metaperiodate (0.2M, 30 ml) for 96 h at 5 °C in the dark. The excess of periodate in the reaction mixture was destroyed with ethylene glycol (3 ml). The solution was dialysed against distilled water and lyophilised (yield 25.5 mg).

A portion (*ca.* 5 mg) of the periodate-oxidised S IX was methylated twice by Hakomori's procedure.[11] After hydrolysis and the usual treatment, the methyl sugars were converted into their alditol acetates and identified in the usual way. The results are summarised in Table 2, column A.

Smith Degradation of S IX.—A portion (*ca.* 10 mg) of the periodate-oxidised S IX was dissolved in water (5 ml) and reduced with $NaBH_4$. The reaction mixture was acidified with acetic acid to pH 4, dialysed for 2 days to remove salts, and then freeze-dried. The periodate-oxidised–reduced polysaccharide was then treated with 2N H_2SO_4 (1 ml) at room temperature for 3 days. After the usual treatment the solution was lyophilised. The lyophilised material was methylated by Hakomori's method, hydrolysed, and the resulting methyl sugars were identified in the usual way. The results are given in Table 2, column B.

Second Periodate Oxidation.—The solution containing periodate-oxidised–reduced–partially hydrolysed S IX (10 mg) was treated with sodium metaperiodate solution (0.2M, 10 ml). After setting aside at 5 °C in the dark for 24 h iodate and periodate ions were precipitated out as their insoluble barium salts with barium hydroxide solution (using Bromothymol Blue as indicator). The precipitate was removed by centrifugation and the supernatant was concentrated to a small volume (*ca.* 5 ml). Sodium borohydride (20 mg) was added to the solution and the solution was left overnight. Excess of $NaBH_4$ was removed in the usual way and the product was treated with 2N H_2SO_4 (2 ml) at room temperature for 48 h. The solution was then neutralised with $BaCO_3$, centrifuged, and the supernatant concentrated to dryness. The resulting material was fully methylated by Hakomori's method and then hydrolysed. The methyl sugars obtained were analysed as their alditol acetates; the results are summarised in Table 2, column C.

Fragmentation Studies on Methylated S IX.—The fully methylated polysaccharide (20 mg) was heated with 90% formic acid (9 ml) at 70 °C for 45 min. The solution was concentrated to dryness and the solid obtained was suspended in water and freeze-dried. The hydrolysed product was further methylated by Purdie's method. The mixture gave spots corresponding to five methylated oligosaccharides, which were designated as F_1, F_2, F_3, F_4, and F_5 respectively. The R_{TMG} values of these spots are given in Table 3. The oligosaccharides F_3 and F_4 (R_{TMG} 0.65) did not separate well. The mixture was resolved on Whatman No. 3 MM filter papers using the methyl ethyl ketone–water azeotrope as irrigating solvent. The strips containing different methylated oligomers were cut and eluted with chloroform. Fractions F_1, F_2, and F_5 were found to be homogeneous, whereas F_3 and F_4 were collected as one fraction. I.r. spectra of F_1, F_5, and the mixture containing F_3 and F_4 showed the presence of the CO_2Me group indicating the presence of uronic acid units in them. All the fractions were hydrolysed and the methyl sugars obtained were analysed in the usual way. The results are given in Table 3.

Chromium Trioxide Oxidation.[17]—The acetylated S IX (20 mg) was treated with diborane as described earlier to convert the uronic acid units into the corresponding neutral sugar residues. Powdered chromium trioxide (30 mg) was added to the solution of acetylated, carboxy-reduced polysaccharide (10 mg) in glacial acetic acid (0.3 ml) and the suspension was agitated in an ultrasonic bath at 50 °C for 1 h. The dark reaction mixture was added to a column of Sephadex LH-20 (80 × 0.8 cm) which was irrigated with acetone. The oxidised material was converted into its fully methylated derivative by Hakomori's method and was isolated from the reaction mixture by parition between water and chloroform. It was hydrolysed and the resulting methyl sugars were analysed in the usual way. The methyl sugars identified are shown in Table 1, column F.

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