Graded Hydrolysis Studies on *Pneumococcus* Type IX Capsular Polysaccharide

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Mild acid hydrolysis of the capsular polysaccharide of *Pneumococcus* type IX (S IX) liberated a number of oligosaccharides which were separated into basic, acidic, and neutral fractions using ion-exchange column chromatography. Each fraction was further separated into its constituent oligosaccharides using high-voltage electrophoresis. The individual oligosaccharides were subjected to methylation studies; when uronic acid was present the methylated oligomer was reduced and the methyl sugars in it were characterised. Based on these studies structures were assigned to the oligomers. The five oligosaccharides characterised support the structure assigned to the repeating unit of S IX.

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 his co-workers leading to a possible tentative structure. Some oligosaccharides were isolated in these investigations but were not unequivocally characterised. Using methylation studies on S IX and some of its degraded products the structure of the repeating unit has been revised by Bhattacharya and Rao.[11] They also characterised some oligosaccharides obtained by mild acid hydrolysis of the fully methylated polysaccharide. S IX was subjected to mild acid hydrolysis and the oligosaccharides were isolated in a homogeneous state and were characterised using methylation studies. We now report the results of these studies.

S IX was hydrolysed by heating with dilute sulphuric acid for 4.5 h. The basic and acidic sugars were adsorbed on Dowex-50 WX–8 (H^+) and Dowex-1 X–4 (HCO_2^-) resin columns respectively. The neutral fraction was found to contain glucose, glucosamine, and immobile material.

The sugar mixture isolated from the cation-exchange-resin column, was separated on high voltage electrophoresis into three fractions designated as fractions B_1, B_2, and B_3 and were characterised as follows.

Fraction B_1 was found to be a mixture of glucosamine and mannosamine, identified by paper chromatography and g.l.c. of the acetylated derivatives. Fraction B_2 on hydrolysis gave glucose, glucosamine, and a trace amount of mannosamine. However this fraction could not be further characterised due to its poor yield. Fraction B_3 on hydrolysis gave glucose, glucosamine, and mannosamine in a molar ratio of 1.0 : 0.8 : 0.8. The material was fully methylated by Hakomori's method [12] and on hydrolysis it gave 2,4,6-tri-*O*-methyl-D-glucose (1.0 mol), 2-amino-2-deoxy-3,4,6-tri-*O*-methyl-D-glucose (0.9 mol), and 2-amino-2-deoxy-4,6-di-*O*-methyl-D-mannose (0.8 mol). The trimethyl glucosamine unit occupied the non-reducing end. Fraction B_3, after reduction with $NaBH_4$ and hydrolysis, showed on paper chromatography spots corresponding to glucosamine and glucose as reducing sugars. The disappearance of the mannosamine unit after reduction indicated that it occupied the reducing end of the trisaccharide. Based on the above results, the structure assigned to this oligosaccharide is GlcNAc $\xrightarrow{1\quad 3}$ Glc $\xrightarrow{1\quad 3}$ ManNAc.

The acid sugar mixture isolated from the anion-exchange resin column was found to contain glucuronic acid and four oligosaccharides (designated as A_1, A_2, A_3, A_4, and A_5) which were resolved by high voltage electrophoresis. The individual fractions were found to be electrophoretically homogeneous and were characterised.

Fraction A_1 was chromotographically indistinguishable from D-glucuronic acid.

Fraction A_2 on hydrolysis gave glucuronic acid (46.5%) and glucose (48.5%). The disaccharide, when fully methylated by Hakomori's method [12] and then hydrolysed, gave 2,4,6-tri-*O*-methyl-D-glucose. The methylated material on reduction with lithium aluminium hydride, followed by hydrolysis, gave 2,4,6-tri-*O*-methyl-D-glucose and 2,3,4-tri-*O*-methyl-D-glucose in a molar ratio of 1.0 : 0.9. As the 2,3,4-tri-*O*-methyl-D-glucose was obtained only after reduction, the glucuronic acid unit must occupy the non-reducing end. The oligosaccharide is an aldobiouronic acid having the structure GlcA $\xrightarrow{1\quad 3}$ Glc; this fragment was isolated and characterised by earlier workers.[2]

Fraction A_3 on hydrolysis gave glucose, glucuronic acid, glucosamine, and mannosamine in a molar ratio of 1.0 : 1.9 : 0.7 : 0.8. The glucuronic acid and glucose were estimated to be 35.5% and 18.8% respectively. The material was methylated and then hydrolysed to yield a mixture containing 2,4,6-tri-*O*-methyl-D-glucose (1.0 mol), 2-amino-2-deoxy-3,4,6-tri-*O*-methyl-D-mannose (0.8 mol), and 2-amino-2-deoxy-4,6-di-*O*-methyl-D-glucose (0.9 mol). On reduction and hydrolysis, the methylated oligosaccharide gave 2,4,6-tri-*O*-methyl-D-glucose (1.0 mol), 2-amino-2-deoxy-3,4,6-tri-*O*-methyl-D-glucose (0.8 mol), 2-amino-2-deoxy-4,6-di-*O*-methyl-D-glucose (0.9 mol), and 2.3-di-*O*-methyl-D-glucose (1.8 mol). The 2,3-di-*O*-methyl-D-glucose, which was not present in the hydrolysate of the unreduced material, originated obviously from the glucuronic acid moiety

and was present in a 2 molar proportion. Heidelberger *et al.*[2] isolated two aldobiouronic acids, *viz.* GlcA $\xrightarrow{1\ \ 3}$ Glc and GlcA $\xrightarrow{1\ \ 3}$ GlcNAc, and Bhattacharya and Rao[11] further established their sequence and that the uronic acid moieties in these were 1,4-linked. Based on these conclusions and the present results the structure of the oligosaccharide in fraction A_3 is ManNAc $\xrightarrow{1\ \ 4}$ GlcA $\xrightarrow{1\ \ 3}$ Glc $\xrightarrow{1\ \ 4}$ GlcA $\xrightarrow{1\ \ 3}$ GlcNAc.

Fraction A_4 on hydrolysis gave glucose, glucuronic acid, glucosamine, and mannosamine in the ratio 2.0 : 1.8 : 1.5 : 0.8. The glucose and glucuronic acid were estimated to be 26.0 and 23.5% respectively. The fully methylated material on hydrolysis gave 2,4,6-tri-*O*-methyl-D-glucose (2.0 mol), 2-amino-2-deoxy-4,6-di-*O*-methyl-D-glucose (1.8 mol), and 2-amino-2-deoxy-4,6-di-*O*-methyl-D-mannose (0.9 mol). A portion of the methylated A_4 was reduced with $LiAlH_4$ and then hydrolysed. The hydrolysate was found to contain 2,4,6-tri-*O*-methyl-D-glucose (2.0 mol), 2-amino-2-deoxy-4,6-di-*O*-methyl-D-glucose (1.7 mol), 2-amino-2-deoxy-4,6-di-*O*-methyl-D-mannose (0.8 mol), 2,3-di-*O*-methyl-D-glucose (0.9 mol), and 2,3,4-tri-*O*-methyl-D-glucose (0.9 mol). The 2,3,4-tri-*O*-methyl-D-glucose and 2-amino-2-deoxy-4,6-di-*O*-methyl-D-glucose were poorly resolved in g.l.c. as their R_t values were very close. The presence of 2,3,4-tri-*O*-methyl-D-glucose in the hydrolysate of the reduced material indicated that one of the glucuronic acid units occupied the non-reducing end. It has been established[11] from deamination studies of S IX that the two glucosamine residues are present in a sequence; the sequences of two aldobiouronic acids are also known. Based on the above results and from the structure of B_3, the structure assigned to this fragment is GlcA $\xrightarrow{1\ \ 3}$ Glc $\xrightarrow{1\ \ 4}$ GlcA $\xrightarrow{1\ \ 3}$ GlcNAc $\xrightarrow{1\ \ 3}$ GlcNAc $\xrightarrow{1\ \ 3}$ Glc $\xrightarrow{1\ \ 3}$ ManNAc.

Fraction A_5 on hydrolysis, gave glucose, glucuronic acid, glucosamine, and mannosamine in the ratio 2.0 : 1.9 : 1.6 : 0.8; glucose and glucuronic acid were estimated to be 27.5 and 26.3% respectively. The fully methylated A_5, on hydrolysis gave 2,4,6-tri-*O*-methyl-D-glucose (2.0 mol), 2-amino-2-deoxy-3,4,6-tri-*O*-methyl-D-mannose (0.8 mol), and 2-amino-2-deoxy-4,6-di-*O*-methyl-D-glucose (1.8 mol). A portion of the methylated A_5 was reduced with $LiAlH_4$ and hydrolysed. The hydrolysate was found to contain 2-amino-2-deoxy-3,4,6-tri-*O*-methyl-D-mannose (0.8 mol), 2-amino-2-deoxy-4,6-di-*O*-methyl-D-glucose (1.8 mol), 2,4,6-tri-*O*-methyl-D-glucose (2.0 mol), and 2,3-di-*O*-methyl-D-glucose (1.8 mol). The 2-amino-2-deoxy-3,4,6-tri-*O*-methyl-D-mannose unit occupied the non-reducing end of the oligosaccharide. The two molar proportions of 2,3-di-*O*-methyl-D-glucose in the hydrolysate of the methylated reduced product were obtained from glucuronic acid residues which were 1,4-linked. Based on the above results and from the structures of A_3 and A_4 the structure assigned to this fragment is ManNAc $\xrightarrow{1\ \ 4}$ GlcA $\xrightarrow{1\ \ 3}$ Glc $\xrightarrow{1\ \ 4}$ GlcA $\xrightarrow{1\ \ 3}$ GlcNAc $\xrightarrow{1\ \ 3}$ GlcNAc $\xrightarrow{1\ \ 3}$ Glc.

The fragments isolated from cation- and anion-exchange resin columns and the neutral fraction represent different portions of S IX. The fragments containing five or more sugar units, *viz.* A_3, A_4, and A_5, on further hydrolysis yielded large quantities of monosaccharides with small amounts of oligosaccharides. The structures of the oligosaccharides isolated in the present studies fully support the revised structure assigned to S IX by Bhattacharya *et al.*[11]

EXPERIMENTAL

Whatman No. 1 filter papers were used for qualitative paper chromatography and large quantities of sugar mixtures were separated on Whatman No. 3MM papers. The solvent systems (v/v) used for paper partition chromatography were (A) n-butanol–acetic acid–water (4 : 1 : 5), (B) ethyl acetate–pyridine–water (8 : 2 : 1), and (C) ethyl acetate–pyridine–acetic acid–water (5 : 5 : 1 : 3). The spray reagents used were (a) alkaline silver nitrate, (b) 0.25% ninhydrin in acetone, and (c) saturated aniline oxalate solution in water. A Hewlett–Packard model 5713A gas chromatograph fitted with a f.i.d. and glass columns (1.83 m × 6 mm) packed with (1) 3% ECNSS-M on Gas Chrom Q (100—120 mesh), (2) 3% OV-225 on Gas Chrom Q (100—120 mesh), and (3) 3% Poly-A 103 on Gas Chrom Q (100—120 mesh) was used. A Hewlett–Packard 3370 B integrator was used for quantitative evaluation of the peaks. A Shandon L-24 high-voltage electrophoresis instrument was used for separation of sugar mixtures and for testing homogeneity; the conditions used were: buffer, pyridine–acetic acid–water (4 : 10 : 1 000 v/v, pH 4.5); *t* 70 min. The distances travelled by the components were reported as (+)-ve or (−)-ve cm depending upon the movements towards the anode or cathode respectively. Optical rotations were recorded with a Perkin-Elmer model 241 MC spectropolarimeter. Spectrophotometric readings were recorded with Carl Zeiss VSU 2-P and Yanaco SP-1 spectrophotometers. Uronic acid and hexose were estimated by carbazole[13] and L-cysteine–sulphuric acid[14] methods respectively. The results were reported as weight % of the material. Unless otherwise stated all evaporations were carried out *in vacuo* at 40 °C.

Partial Hydrolysis of the Polysaccharide.—Guided by the results of pilot experiments for the maximum yield of the oligosaccharides, S IX (120 mg) was heated with 0.125M-sulphuric acid (20 ml) for 4.5 h on a boiling water-bath. The solution was neutralised ($BaCO_3$) and centrifuged. The supernatant was concentrated and then passed successively through columns of Dowex-50 WX-8 (H^+) and Dowex-1 X-4 (HCO_2^-) resins. Each column was washed with water until the eluate was negative to Molisch test. The neutral eluate and the washings were combined, concentrated to a small volume and then freeze-dried; yield 20 mg. Paper chromatographic examination using solvents A and B gave spots corresponding to glucose and glucosamine, together with immobile material.

Examination of the Sugars from Dowex-50 WX-8 Column.—The Dowex-50 WX-8 column was eluted with 0.1M-HCl (150 ml) and the acid was removed by repeated co-distillation with methanol. The material was dissolved in a small

volume of water and then passed through a Sephadex G-25 column. The salt-free material, thus obtained, gave three poorly resolved spots on paper chromatography using solvents A, B, and C. The mixture was separated on Whatman No. 3 MM filter paper using high-voltage electrophoresis (potential gradient 50 V cm^{-1}). The three well separated zones (designated as B_1, B_2, and B_3 in order of decreasing mobilities) which moved towards the cathode, were cut and eluted with water. All the fractions were found to be electrophoretically homogeneous.

Fraction B_1. Yield, 6 mg; electrophoretic mobility, −28.0 cm. Paper chromatography of this fraction using solvents A and B and spraying reagent (b) showed two spots corresponding to glucosamine and mannosamine. The same sugars were identified when the mixture was converted into alditol acetate and analysed by g.l.c.

Fraction B_2. Yield, 1 mg; electrophoretic mobility, −21.0 cm. This fraction was hydrolysed with 3M-HCl for 6 h and the hydrolysate on paper chromatography (solvents A and B) and g.l.c. (as acetates) showed the presence of glucose, glucosamine, and a trace amount of mannosamine. Further experiments were not performed owing to lack of material.

Fraction B_3. Yield, 13 mg; $[\alpha]_D^{30}$ +96° (*c*, 0.3 in H_2O); electrophoretic mobility, −5.4 cm. A portion (1.5 mg) of this fraction was hydrolysed with 3M-HCl for 6 h on a boiling water-bath. The acid was removed by repeated co-distillation with methanol. Paper chromatography (solvents A and B) of the hydrolysate showed three spots corresponding to glucose, glucosamine, and mannosamine. The molar proportion was estimated (g.l.c.) to be 1.0 : 0.8 : 0.8. The oligosaccharide (1 mg) was reduced with $NaBH_4$ and neutralised with acetic acid, and the boric acid was removed by co-distillation with methanol. The reduced fraction B_3 was hydrolysed with 3M-HCl for 6 h on a boiling water-bath. Paper chromatography [solvents A and B and spray reagent (c)] of the hydrolysate gave two spots corresponding to glucose and glucosamine.

Another portion (3.5 mg) was dissolved in dimethyl sulphoxide (2 ml) and was methylated by Hakomori's method using methyl sulphinyl carbanion (2 ml) and methyl iodide (1 ml). The i.r. spectrum of the methylated product showed a weak OH absorption band. Further methylation using the same procedure yielded a fully methylated derivative which was then hydrolysed with 4M-HCl for 5 h and neutralised. The methyl sugars were converted into their alditol acetates and analysed by g.l.c. The results are shown in the Table, column A.

Examination of the Sugars from Dowex-1 X-4 *Column.*—The column was eluted with 10% (v/v) formic acid (150 ml). The acid was removed by repeated co-distillation with water and the material was freeze dried. Paper chromatography did not give satisfactory resolution. The oligosaccharides were separated by high-voltage electrophoresis on Whatman No. 3MM paper (potential gradient 50 V cm^{-1}). Five well separated zones designated as A_1, A_2, A_3, A_4, and A_5 were obtained; A_5 moved towards the negative electrode while the others moved towards the positive electrode. The zones containing the individual sugars were cut, eluted with water, and the solutions were concentrated to syrups. Each fraction was found to be electrophoretically homogeneous.

Fraction A_1. Yield, 3 mg; $[\alpha]_D^{30}$ +34° (*c*, 0.2 in H_2O); electrophoretic mobility, +17.0 cm. On paper chromatography (solvents A and C) it gave a single spot corresponding to glucuronic acid.

Fraction A_2. Yield, 9 mg; $[\alpha]_D^{30}$ +98° (*c*, 0.33 in H_2O); electrophoretic mobility, +11.0 cm. A portion (1.5 mg) was hydrolysed with 3M-HCl for 6 h on a boiling water-bath and after the usual treatments, the hydrolysate on paper chromatography showed spots corresponding to glucose and glucuronic acid; these were estimated to be 48.5% and 46.5% respectively.

Methyl sugars from the hydrolysates of methylated and methylated carboxy-reduced oligosaccharides

Methyl sugars [a]	*t* [b]		Molar proportion [c]								
	ECNSS-M	OV-225	A	B	C	D	E	F	G	H	I
2-Amino-2-deoxy-3,4,6-tri-*O*-methyl-D-glucose	1.00	1.00	0.9								
2-Amino-2-deoxy-3,4,6-tri-*O*-methyl-D-mannose	1.00	1.00				0.8	0.8			0.8	0.8
2,4,6-Tri-*O*-methyl-D-glucose	1.95	1.82	1.0	Present	1.0	1.0	1.0	2.0	2.0	2.0	2.0
2,3,4-Tri-*O*-methyl-D-glucose	2.5	2.22			0.9				0.9		
2-Amino-2-deoxy-4,6-di-*O*-methyl-D-glucose	2.32	2.32				0.9	0.9	1.8	1.7	1.8	1.8
2-Amino-2-deoxy-4,6-di-*O*-methyl-D-mannose	2.82	3.40	0.8					0.9	0.8		
2,3-Di-*O*-methyl-D-glucose	5.38	4.50					1.8		0.9		1.8

[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] Oligosaccharides: A, methylated B_3; B, methylated A_2; C, methylated carboxy-reduced A_2; D, methylated A_3; E, methylated carboxy-reduced A_3; F, methylated A_4; G, methylated carboxy-reduced A_4; H, methylated A_5; I, methylated carboxy-reduced A_5.

The material (3 mg) was methylated by Hakomori's method. A portion of the methylated product was hydrolysed with 4M-HCl for 5 h on a boiling water-bath. Acid was removed by repeated co-distillation with methanol and the methyl sugars were analysed by g.l.c. in the usual way. The results are shown in the Table, column B. The remaining methylated A_2 in a mixture of ether–dichloromethane (4 : 1) was refluxed for 8 h with $LiAlH_4$. The excess of reagent was decomposed by addition of moist ethyl acetate followed by a few drops of water and the solution was neutralised with 1M-phosphoric acid. The solution was centrifuged and the supernatant was washed with water and then evaporated to dryness. This carboxy-reduced material was hydrolysed and the methyl sugars were analysed by g.l.c. Results are given in the Table, column C.

Fraction A_3. Yield, 6 mg; $[\alpha]_D^{30}$ +23° (*c*, 0.25 in H_2O); electrophoretic mobility, +8.5 cm. A portion (1.5 mg) was hydrolysed with 3M-HCl for 6 h at 100 °C, neutralised, and subjected to paper chromatography (solvents A and B) which showed spots corresponding to glucose, glucosamine, glucuronic acid, and mannosamine. Glucose and glucuronic acid were estimated to be 18.8 and 35.5% respectively. The ratio of glucose, glucosamine, and mannosamine was estimated by g.l.c. to be 1.0 : 0.7 : 0.8. A part of this fraction (2 mg) was completely methylated by Hakomori's method and a portion of it was hydrolysed

with 4M-HCl for 5 h, neutralised, and analysed by g.l.c. in the usual way. The results are given in the Table, column D. The remaining portion of the methylated product was reduced with lithium aluminium hydride, hydrolysed, and analysed by g.l.c. The results are given in the Table, column E.

Fraction A_4. Yield, 15 mg; $[\alpha]_D^{30}$ +48.3° (*c*, 0.29 in H_2O); electrophoretic mobility, +4.5 cm. This fraction (1.5 mg) on hydrolysis and paper chromatographic examination gave spots corresponding to glucose, glucuronic acid, glucosamine, and mannosamine. Glucose and glucuronic acid were estimated to be 26.0 and 23.5% respectively. The ratio of glucose, glucosamine, and mannosamine was found to be 2.0 : 1.5 : 0.8. The oligosaccharide (4 mg) was completely methylated by Hakomori's method and a portion was hydrolysed with 4M-HCl for 5 h, neutralised, and the methyl sugars were analysed by g.l.c. in the usual way. The results are shown in the Table, column F. The remaining portion of the methylated product was reduced with lithium aluminium hydride, hydrolysed, and analysed by g.l.c. The results are given in the Table, column G.

Fraction A_5. Yield, 9 mg; $[\alpha]_D^{30}$ +43° (*c* 0.20 in H_2O); electrophoretic mobility, −2.5 cm. A portion (1.5 mg) was hydrolysed with 3M-HCl for 6 h, neutralised, and on paper chromatography (solvent A and B) gave spots corresponding to glucose, glucuronic acid, glucosamine, and mannosamine. G.l.c. of the hydrolysate showed the ratio of glucose, glucosamine, and mannosamine to be 2.0 : 1.6 : 0.8. Glucose and glucuronic acid were estimated to be 27.5% and 26.3% respectively. This fraction (3 mg) was fully methylated and a portion was hydrolysed with 4M-HCl for 5 h, neutralised, and the methyl sugars were analysed by g.l.c. in the usual way. Results are given in the Table, column H. The remaining portion of the methylated product was reduced by $LiAlH_4$, hydrolysed, and analysed by g.l.c. The results are given in the Table, column I.

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