Immunochemical Studies on the Capsular Polysaccharide of Pneumococcal Type IX

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1. In addition to the previously identified components, D-glucose, N-acetyl-D-glucosamine and D-glucuronic acid, the immunologically specific capsular polysaccharide of pneumococcal type IX (polysaccharide S IX) is now found to contain mannosamine and galactosamine, probably as their N-acetyl derivatives. 2. Partial hydrolysis yields a complex mixture of oligosaccharides, several of which have been separated. 3. One of these is α -D-glucuronopyranosyl-(1→3)-D-glucose. It is an inhibitor of the precipitation of anti-pneumococcal type IX serum by polysaccharide S IX. 4. Two others, D-glucuronopyranosyl-(1→3)-D-glucosamine and D-glucuronopyranosyl-(1→3)-D-galactosamine have been identified and a trisaccharide and pentasaccharide partially characterized.

Chemical studies on the immunologically specific capsular polysaccharide polysaccharide S IX‡ were initiated to facilitate interpretation of cross-reactions shown by certain polyglucoses and other polysaccharides with antibodies to polysaccharide S IX (Heidelberger, 1963). Polysaccharide S IX was initially found to consist of D-glucose, D-glucuronic acid and N-acetyl-D-glucosamine (Heidelberger *et al.*, 1954; Rao & Heidelberger, 1966). The polysaccharide is now shown to contain, in addition, mannosamine and a small amount of galactosamine, both of which presumably occur as the N-acetyl derivatives. Oligosaccharides produced by partial hydrolysis are described and the inhibitory effects of salts of acidic disaccharides on precipitation of polysaccharide S IX and isolichenin in anti-(pneumococcal type IX) sera are presented and discussed in the light of their contributions to the knowledge of the structure of polysaccharide S IX.

Materials and Methods

These were essentially as given in an earlier paper (Rao & Heidelberger, 1966), with the following additions: solvents used for chromatography were (a) ethyl acetate–pyridine–acetic acid–water (5:5:1:3, by vol.); (b) butan-1-ol–acetic acid–water (3:1:1, by vol.); (c) ethyl acetate–acetic acid–formic acid–water (18:3:1:4, by vol.). Staining reagents were: (A) alkaline silver nitrate; (B) benzidine–periodate (Gordon *et al.*, 1956); (C) 0.5% ninhydrin in acetone. Evaporations *in vacuo* were carried out at 30–35°C unless otherwise stated.

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‡ Abbreviation: polysaccharide S IX, specific capsular polysaccharide of pneumococcal type IX.

Hexose (glucose) was determined by a modification of the cysteine–H₂SO₄ assay (Dische & Danilchenko, 1967) or by the phenol method (Dubois *et al.*, 1956). Total 2-amino-2-deoxyhexose was determined by the Gatt & Berman (1966) modification of the Elson–Morgan (1933) method. Neutral or acidic and basic components of polysaccharide S IX hydrolysates, freed of excess of HCl by azeotropic distillation with ethanol–benzene (4:1, v/v), were separated by elution from Bio-Rad AG50W (X8; H⁺ form) resin with water and 2M-HCl, respectively. Portions of the eluates were dried, converted into O-trimethylsilyl derivatives with Trisil (Pierce Chemical Co., Rockford, Ill., U.S.A.) at 37°C for 30 min. Excess of reagents was evaporated before reconstitution in cold *n*-hexane before injection into a Packard modular gas chromatograph equipped with a dual flame ionization detector. The coiled glass column (6ft×0.4cm) was packed with 3% S.E. 30 on Supelcoorte (Supelco Inc., Bellefonte, Pa., U.S.A.) and temperature-programmed from 150°–200°C at 7°C/min.

D-Galactosamine and D-glucose were determined by Galactostat and Glucostat reagents respectively (Worthington Biochemical Corp., Freehold, N.J., U.S.A.), and free amino groups by the ninhydrin method of Yemm & Cocking (1955).

Isolichenin was a gift from Professor D. J. Manners, nigerose from Professor K. Matsuda. The acetylated methyl esters of α -D-glucuronopyranosyl-(1→4)-D-glucose (maltobiouronic acid) and the isomeric α -D-glucopyranosyl-(1→4)-D-glucuronic acid were supplied by Dr. Y. Hirasaka and Dr. I. Matsunaga of the Chugai Pharmaceutical Co. Ltd., Tokyo, Japan. Before use in the experiments on inhibition recorded in Tables 2 and 3 the substances were deacetylated, saponified, and neutralized, essentially as described by Hirasaka & Matsunaga (1965) for the second of these disaccharides.

Experimental and Results

Partially purified polysaccharide S IX (100mg, corresponding to 143A) (Rao & Heidelberg, 1966) was dissolved in 5ml of buffer (30mM-KH₂PO₄-4mM-Na₂HPO₄, pH 6) and added at 4°C to a DEAE-Sephadex A-50 column (4.1cm×45cm) which had been equilibrated with the buffer. The polysaccharide remained on top of the column even after passage of 200ml of buffer. An NaCl gradient (0-1M) was applied and fractions were monitored for hexose (Dubois *et al.*, 1956) and for nucleic acid (*E*₂₆₀). The hexose-containing fractions were pooled, dialysed and freeze-dried. Yield: 83.5mg. This preparation of polysaccharide S IX failed to precipitate types I and VII anti-(pneumococcal) sera containing much antibody to pneumococcal C-substance. The *E*₂₆₀ indicated 0.9% nucleic acid.

A similar preparation of polysaccharide S IX was subjected to immunoelectrophoresis in agarose at concentrations of 1mg, 5mg and 10mg/ml in barbital buffer at pH 8.6. It gave a single arc with anti-(pneumococcal IX) serum and faint ones with anti-(pneumococcal I) and anti-(pneumococcal VII) sera only at 10mg/ml.

Analytical composition of polysaccharide S IX

Analyses of polysaccharide S IX are shown in Table 1. Optimum hydrolytic conditions for the hexosamines were 4M-HCl for 5-6h at 100°C. Hexosamines were determined after removal of glucuronic acid and glucose on an AG50W (X8; H⁺ form) column by elution with water. The subsequent acid eluate (2M-HCl) was freed of acid and samples were analysed both colorimetrically and by g.l.c. Standards of glucosamine hydrochloride and mannosamine hydrochloride were similarly treated. Galactosamine was determined from the height of its peak in g.l.c. relative to that found for mannosamine in the hydrolysate of polysaccharide S IX.

Interference of glucuronic acid in the determination of glucose was corrected by use of standards of glucose-glucuronic acid (3:2, w/w). Similar standards were used to correct for interference of glucose in the assay for glucuronic acid.

Polysaccharide S IX-cyanine dye complex

By the method of Edstrom (1969) a complex of the cyanine dye {1-ethyl-2-[3-(1-ethylnaphtho[1,2*d*]-thiazolin-2-ylidene)-2-methylpropenyl]naphtho-[1,2*d*]-thiazolium bromide, Eastman Kodak Co., Rochester, N.Y., U.S.A.} with polysaccharide S IX had an extinction at 640nm (the maximum) of 0.353 relative to a dye-water blank (maximum at 508nm). This, according to Edstrom's (1969) results, should be characteristic of a polymer with about one anionic site per three monosaccharide units (22% anhydroglucuronic acid found, Table 1).

Partial hydrolysis of polysaccharide S IX: with 0.1M- and 0.15M-H₂SO₄

Polysaccharide S IX (4g of a preparation analogous to 143A₁) was hydrolysed with 200ml of 0.1M-H₂SO₄ at 100°C for 6h, then for 3h with 0.15M-H₂SO₄ and neutralized with BaCO₃ that had been extracted with boiling water. The solution was evaporated *in vacuo* to about 25ml, filtered, passed through a column (3.5cm×15cm) of Amberlite IR-120 (H⁺ form) and eluted with 4 litres of water. The eluate was evaporated *in vacuo* to about 50ml, added to a column (3.5cm×15cm) of Dowex 1 (X4; HCO₃⁻ form) and eluted with water. The eluate (5 litres) showed chromatographic spots corresponding to glucose and *N*-acetylglucosamine, which had previously been characterized (Rao & Heidelberg, 1966).

The Amberlite column, eluted with 0.25M-H₂SO₄, released the expected monosaccharides as

Table 1. Composition of polysaccharide S IX

For experimental details see the text. —, Not done.

	%*	%†	Mole ratio
Anhydroglucose	28	33	3
Anhydro- <i>N</i> -acetylglucosamine	25	23	2
Anhydro- <i>N</i> -acetylmannosamine	—	20	2
(Anhydro- <i>N</i> -acetylgalactosamine)		(0.7)	
Anhydroglucuronic acid	23	22	2
Nitrogen	3.0	—	—
Ash (as Li)	0.8	—	2
Phosphorus	<0.1	—	—
[α] _D ²³	+121°	—	—

* Lot 143, Fr. A₁. From Rao & Heidelberg (1966).

† DEAE-Sephadex-purified preparation.

well as oligosaccharides which have not yet been separated.

For extraction of the acidic fractions, the Dowex column was eluted with 2 litres of aq. 0.5M-NH₃. The eluate was evaporated *in vacuo*, filtered, and, at a volume of 7ml, precipitated with small amounts of methanol. The precipitates were combined, dissolved in water, reprecipitated with methanol, and dried (fraction A1). This contained all of the sugars of polysaccharide S IX and was of sufficiently high molecular weight to precipitate anti-(pneumococcal type IX) horse serum: 0.02 and 0.04mg of fraction A1 precipitated 56 and 59 μ g of antibody N from an antiserum giving 166 μ g with polysaccharide S IX.

The supernatants from the methanol precipitates were evaporated *in vacuo* and chromatographed with solvent *b*. There were six mobile spots, of which the principal ones were designated A2, A3 and A4 in the order of increasing mobility.

Fraction A4, D-glucuronic acid, yielded 5mg of crystals, which were esterified and converted into the methyl glycoside with boiling 1% methanolic HCl under reflux, reduced with KBH₄, and hydrolysed for 20h with M-H₂SO₄ at 100°C, yielding D-glucose as confirmed by chromatography and reactivity with Glucostat.

Fraction A3, (120mg) light brown, was dissolved in 1 ml of water and treated with a few drops of ethanol. Inorganic material was deposited. The filtrate was decolorized on the boiling-water bath with activated charcoal (Darco G 60), filtered and freeze-dried. It gave single spots on chromatography in three solvents: in solvent *b*, R_{Glc} was 0.40 and R_{GlcA} 0.43; in solvent *a*, R_{GlcA} 0.32. $[\alpha]_D^{22}$ was +106° (*c* 2.56 in water). Hydrolysis with 0.5M-H₂SO₄ at 100°C for 2h showed glucose, glucuronic acid and glucuronolactone. The glucose/glucuronic acid ratio was 1:1 (Dische & Danilchenko, 1967; Dische, 1947); the standard used

contained these sugars in the same ratio. Fraction A3 (5.1 mg) was dissolved in 2ml of water and reduced at 4°C with 30.2mg of NaBH₄ added in two portions. Excess of reagent was decomposed with cold 6M-acetic acid. Hydrolysis with M-H₂SO₄ at 100°C for 20h gave spots corresponding to glucuronic acid and sorbitol on chromatography with solvent *a*.

An aqueous solution of fraction A3 that had been passed through Amberlite IR-120 was titrated to pH7 with 0.01M-NaOH and gave an equivalent weight of 355. This solution was cooled to 4°C, treated with 6ml of 0.1M-periodic acid and diluted to 50ml. Reduction of periodate was determined by the arsenite method with 2ml portions and liberation of formic acid was determined by titration of 2ml with 0.01M-NaOH with Methyl Red as indicator. Fraction A3 reduced 3.05mol of periodate/mol and liberated 1.12mol of formic acid/mol. After 4 days the residual solution was precipitated in the cold with Ba(OH)₂, the excess was neutralized with CO₂, and the solution was evaporated *in vacuo*. A small precipitate was filtered off and the filtrate reduced at 4°C with NaBH₄. Excess of reagent was decomposed and removed as described above. The solution was deionized with Amberlite IR-120 (H⁺ form), and once more hydrolysed, neutralized and deionized as above. Paper chromatography of the product in solvents *b* and *c* gave spots corresponding to arabitol and glyceric acid with staining reagent *B*.

Inhibition experiments showing fraction A3 to be a major antigenic determinant of polysaccharide S IX are given in Tables 2 and 3.

The aqueous solution of acid fraction A2 was concentrated, inorganic material was filtered off, and the solution was deionized and freeze-dried. Chromatography in solvents *b* and *c* showed compact spots with R_{Glc} 0.077 (solvent *b*) and R_{GlcA} 0.046 (solvent *c*). After hydrolysis (3M-HCl, 100°C, 7.5h) fraction

Table 2. *Inhibition of homologous precipitation in anti-(pneumococcal type IX) serum by salts of acidic disaccharides*

Dilutions of anti-(pneumococcal type IX) sera H623C and R91 were used (C, absorbed with group-specific pneumococcal C-polysaccharide). The total volume was 0.5 ml in all runs. Nigerose (48 μ M), had a slight inhibitory action in the rabbit system, but was not tested in the horse system.

Sugar added	Antiserum	Time at 0°C (days)	Antibody N pptd. (μ g)	Inhibition (%)
None	Horse	8	24	
Fraction A3 (30 μ mol)	Horse	8	13	46
None	Rabbit	8	23	
Fraction A3 (30 μ mol)	Rabbit	8	12	48
None	Rabbit	7	34	
α -4-O-D-Glucuronopyranosyl-D-glucose (60 μ mol)	Rabbit	7	30.5	10
None	Rabbit	8	32	
α -4-O-D-Glucopyranosyl-D-glucuronic acid (76 μ mol)	Rabbit	8	27	16

Table 3. *Inhibition of precipitation of isolichenin in anti-(pneumococcal type IX) sera by salts of acidic disaccharides*

Dilutions of anti-(pneumococcal type IX) sera H623C and R91 were used. The total volume was 0.5ml in all runs. Nigerose (16 μ M) inhibited the horse and rabbit systems strongly; gentiobiose (46 μ M) (Nutritional Biochemicals Corp.) inhibited the rabbit system more strongly than the horse system.

Sugar added	Antiserum	At 0°C (days)	Antibody N pptd. (μ g)	Inhibition (%)
None	Horse	8	18.5	
Fraction A3 (5 μ mol)	Horse	8	11.5	38
None	Rabbit	8	36	
Fraction A3 (5 μ mol)	Rabbit	8	2	94
Fraction A3 (1.25 μ mol)	Rabbit	18	6	83
None	Rabbit	9	33.5	
α -4-O-D-Glucuronopyranosyl-D-glucose (30.5 μ mol)	Rabbit	9	17.5	48
α -4-O-D-Glucopyranosyl-D-glucuronic acid (38 μ mol)	Rabbit	9	11	67

A2 was found to contain 40% glucose, 28% glucuronic acid, 9.2% glucosamine and 7.1% mannamine, present in the original fraction as their *N*-acetyl derivatives. A strong colour yield (19.5% as *N*-acetylglucosamine according to Aminoff *et al.*, 1952) showed that alkali-labile terminal reducing residues of *N*-acetylhexosamine were present in fraction A2.

Fraction A2 (5mg in 2ml of water) was reduced with 42mg of NaBH₄ as described above, hydrolysed with 5ml of 0.5M-H₂SO₄ at 100°C for 20h, and neutralized with BaCO₃. Chromatography in solvent *a* gave spots corresponding to glucose, sorbitol, glucuronic acid, glucosamine and glucosaminitol. The two polyols are indicative of a mixture of at least two oligosaccharides. This accords with a determination of molecular weight by the method of Hirst *et al.* (1949) which furnished a value, 635, between those calculated for tri- and tetra-saccharides.

Partial hydrolysis of polysaccharide S IX: with m-H₂SO₄

Polysaccharide S IX (1 g) was dissolved in 150ml of m-H₂SO₄, saturated with N₂ and heated at 100°C for 15h. The hydrolysate was neutralized to pH 7.0 with saturated Ba(OH)₂, concentrated by rotary evaporation to about 30ml, filtered and applied to a column (57cm $\times$ 2.6cm, 300ml) of AG50W(X8; H⁺ form). Elution with 350ml of water was followed by 500ml of m-HCl. Fractions of 6ml were collected (Fig. 1).

Samples from selected tubes were analysed by g.l.c. both before and after further hydrolysis (4M-HCl, 100°C, 6h). Peak 1 contained glucose, glucuronic acid, and traces of two unidentified components. Peaks 2, 3 and 4 were oligosaccharides contaminated with glucose from 'tail' of peak 1. Peak 5 was a mixture of glucosamine and mannamine hydrochlorides.

Refractionation of peak 1. Material from tubes 1–30 (Fig. 1) was concentrated and refractionated on a column (300ml) of Dowex 1 (X4; HCO₃⁻ form). Elution was with water (300ml), followed by aq. 0.5M-NH₃ (310ml). Fractions (4.5ml) were collected and analysed for glucose and glucuronic acid. One peak was observed in each eluant and nothing more was eluted with aq. m- or 4M-NH₃. Analyses by colorimetry and g.l.c. of the aqueous eluate showed only glucose (81mg) and glycosidically linked glucuronic acid–glucose (11mg). Analysis by Gluco-stat gave 90mg of glucose, showing once more that the glucose in polysaccharide S IX is the D-isomer. The aq. NH₃ eluate contained only ammonium glucuronate.

Refraction of peak 2. The contents of tubes 71–85 (Fig. 1) were freed of excess of HCl by repeated rotary evaporation with ethanol–benzene (4:1, v/v) and refractionated on a column (300ml) of AG50W (X8; H⁺ form). Elution was with water (500ml), followed by m-H₂SO₄ (250ml). Fractions (4.0ml) were analysed for glucose and hexosamines. The aqueous eluate contained only glucose, the acid eluate a large peak consisting of one or more oligosaccharides (48mg) of glucosamine–glucuronic acid–glucose, (2:2:1) (oligosaccharide 2), followed by a much smaller peak which was added to peak 3.

Refractionation of peak 3. Material from tubes 86–95 (Fig. 1) was refractionated as above. Elution with water (212ml) removed the contaminating glucose after which the adsorbed oligosaccharides were eluted with m-H₂SO₄ (500ml). Of the two peaks 3(a) (16mg) and 3(b) (18mg), which were separated, the first liberated, on further hydrolysis (2M-H₂SO₄, 100°C, 5h) equimolar amounts of glucuronic acid and glucosamine, the second equimolar quantities of glucuronic acid and galactosamine.

Most of the contents of tubes 98–110 (peak 4, Fig. 1, 77mg), which showed mannamine–glucose, 2:1, was used for the isolation of mannamine. After

Fig. 1. Ion-exchange chromatography of hydrolysate of polysaccharide S IX

Polysaccharide S IX (1g) was hydrolysed ($M-H_2SO_4$, $100^\circ C$, 15h) and applied to a column (57cm $\times$ 2.6cm) of AG50W(X8; 200–400 mesh; H^+ form) equilibrated with water at $4^\circ C$. Elution was first with 350ml of water, thereafter with $M-HCl$. Samples (0.1 ml and 0.5 ml) of the fractions (6ml) were analysed for neutral hexose (E_{412}) (—), and, without further hydrolysis, total hexosamines (E_{530}) (----).

hydrolysis of 56mg (see below) mannosamine, glucose and an oligosaccharide 2 (4a, 8.7mg) with the same analysis as its parent were obtained.

Elson–Morgan analysis of polysaccharide S IX oligosaccharides

Cifonelli & Dorfman (1958) reported that the Elson–Morgan assay could be used to indicate the type of hexosamine linkage in poly- and oligosaccharides. With the Gatt & Berman (1966) modification of the Elson–Morgan reaction, oligosaccharides 2, 3(a), 3(b), 4 and 4(a) were all shown to contain 3-linked terminal reducing hexosamine residues. In peaks 2 and 3(a) these were glucosamine, in 3(b) galactosamine, in 4 and 4(a), mannosamine.

The amount of hexosamine in each oligosaccharide, determined colorimetrically after hydrolysis ($4M-HCl$, $100^\circ C$, 5h), was equalled by the value obtained on analysis of the unhydrolysed oligosaccharide with ninhydrin (Yemm & Cocking, 1955). This confirmed that none of the oligosaccharide hexosamines was *N*-acetylated in these products of partial hydrolysis.

Direct Gatt–Berman (1966) analysis of the intact oligosaccharides in each case (except 3b which gave slightly more) gave lower hexosamine values (50–

90%) than after hydrolysis, as expected. The relative values indicated that oligosaccharide 2 was a pentasaccharide terminated by glucosamine, 3(a) and 3(b) disaccharides terminated by glucosamine and galactosamine, respectively, and 4 and 4(a) trisaccharides terminated by mannosamine.

Identification of D-galactosamine hydrochloride in peak 3(b)

Peak 3(b) (4.5mg) was hydrolysed ($4M-HCl$, $100^\circ C$, 5h) and excess of HCl removed before filtration through sintered glass and passage through Dowex 1 (X4; HCO_3^- form) to adsorb the glucuronic acid. The filtrate was made up to 25.0ml and samples were assayed by g.l.c., the hexosamine assay and by Galactostat. Identical results (1.98–2.06mg of galactosamine hydrochloride) were given by all three assays (87% recovery).

Isolation of mannosamine hydrochloride from peak 4

Peak 4 (56mg) was hydrolysed ($3M-HCl$, $100^\circ C$, 5h), excess of HCl removed and the hydrolysate fractionated on a column (50cm $\times$ 1.5cm, 90ml) of AG50W (X8; H^+ form). Glucose and glucuronic acid

were eluted with water (400 ml). Elution with M-HCl followed. Fractions (5.4 ml) were analysed for hexosamines. A small oligosaccharide peak (see above) was followed by a large peak of mannosamine. The amino sugar was isolated, freed of excess of HCl and crystallized from moist ethanol. Two recrystallizations from moist acetone gave 15 mg of mannosamine-HCl browning at 155°C, decomp. 180°C (lit., browning at 150°C, decomp. 178–180°C), unchanged by admixture with D-mannosamine-HCl. L-Mannosamine-HCl browns at 145°C and decomposes at 175–177°C (Kuhn & Bister, 1957). The crystals gave a colour yield and variation of the Elson-Morgan chromophore characteristic of an equal weight of authentic D-mannosamine-HCl. No chromogen was formed by Glucostat or Galactostat.

Isolation of hexosamines from peak 5

Material from tubes 135–155 (Fig. 1) was isolated, excess of HCl removed and the crystals formed on concentration were recovered and recrystallized from moist ethanol. Analysis by g.l.c. showed only pure glucosamine hydrochloride. This was previously characterized by Rao & Heidelberger (1966). G.l.c. analysis of the mother liquor showed not only mannosamine and no glucosamine, but also an extra peak at 199°C which co-chromatographed with authentic galactosamine. Crystallization of the mother liquor by the addition of acetone gave a mixture of mannosamine and galactosamine hydrochlorides.

Discussion

Analysis of the Cotton effect of polysaccharide S IX by Dr. E. A. Kabat (cf. Beychok & Kabat, 1965) indicates that the amino sugars are α -linked. Because of the high optical rotation of polysaccharide S IX, $[\alpha]_D^{25} + 121^\circ$, it is probable that most of the linkages of the other sugars also have an α -configuration.

The principal acidic fraction, A3, obtained from polysaccharide S IX by mild hydrolysis, consisted of glucuronic acid and glucose 1:1 (w/w). On reduction with borohydride and hydrolysis it gave glucuronic acid and sorbitol, showing glucose to be at the reducing end. The equivalent weight, 355, and electrophoretic mobility on paper were characteristic of aldobiouronic acids. On oxidation by periodate fraction A3 reduced 3.05 mol of oxidant/mol and liberated 1.2 mol of formic acid. The reduced product of oxidation was hydrolysed and gave chromatographic spots corresponding to glyceric acid and arabitol. These results and the high optical rotation are consistent with the formulation of fraction A3 as 3-O-(α -D-glucuronopyranosidyl)-D-glucose. It is a potent inhibitor of precipitation in the polysaccharide

S IX anti-(pneumococcal type IX) serum system and in the cross-reaction of isolichenin with anti-(pneumococcal type IX) serum (Heidelberger, 1963) (Tables 2 and 3).

Table 2 shows fraction A3, 3-O-(α -D-glucuronopyranosidyl)-D-glucose, to be the only potent inhibitor of the homologous reaction among the substances tested in the current as well as earlier studies. This is readily explicable, especially as maltobiouronate and the isomeric glucoglucuronate were scarcely inhibitory. As expected, the cross-reaction, isolichenin anti-(pneumococcal type IX) serum, is more easily inhibited than the homologous reaction by a variety of sugars, but, again, fraction A3 is the best inhibitor. Possibly maltose and nigerose show inhibitory activity in the cross-reaction with isolichenin because a second glucose residue of each might fit into antibody determinants complementary to glucuronic acid. Such a possibility has already been proposed for the precipitation of anti-(pneumococcal type II) serum by glycogen (Goodman & Kabat, 1960). The equal inhibition of the cross-reaction between oat glucan and anti-(pneumococcal type VIII) serum by cellobiose and cellobiouronic acid has been accounted for on the same basis (Allen *et al.*, 1965).

With regard to the amino sugars in polysaccharide S IX, 18% of mannosamine, apparently the D-isomer, has now been found in addition to the previously identified D-glucosamine, as well as a small amount of D-galactosamine. It is possible that the last originates from a contaminating polysaccharide in polysaccharide S IX. If so, the contaminant is not C-polysaccharide, since the galactosamine is linked (in oligosaccharide 3b, Fig. 1) to glucuronic acid, which has not been found in C-substance.

Hydrolysis of polysaccharide S IX by dilute acid liberated a number of oligosaccharides which reveal portions of the structure of the polysaccharide. Glucuronic acid is (1→3)-linked not only to D-glucose (fraction A3) but also to glucosamine (fraction 3a, Fig. 1). In peak 3(a), as well as in the penta- (peak 2, Fig. 1) and tri-saccharides (peaks 4, 4a) mentioned above, there are residues of only a single hexosamine, making it unlikely that two different ones occur consecutively. In peaks 4 and 4(a) at least a portion of the mannosamine is linked to glucose. All of the hexosamine-containing oligosaccharides showed the behaviour expected of (1→3)-linked hexosamines when analysed as described by Gatt & Berman (1966).

It is thus evident that, with two or possibly three hexosamine residues in addition to glucuronic acid and glucose in polysaccharide S IX, its structure is very complex. As some additional structural features could be deduced as a result of oxidation of polysaccharide S IX with periodate, these will be discussed in the following paper (Das *et al.*, 1972).

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