Oxidation of the Capsular Polysaccharide of Pneumococcal Type IX by Periodate

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1. The pneumococcal type IX polysaccharide (polysaccharide S IX) has been oxidized by sodium metaperiodate and reduced by sodium borohydride. Of the constituent monosaccharides, *N*-acetylglucosamine and *N*-acetylmannosamine remain unaltered, whereas 40% of the glucose and 90% of the glucuronic acid are oxidized. 2. The effect of oxidation and subsequent reduction on the precipitation of polysaccharide S IX in anti-(pneumococcal) sera is described and interpreted in structural terms. 3. Oligosaccharides produced by oxidation, reduction and hydrolysis with dilute acid have been isolated and partially characterized. 4. The results in this paper and the preceding one (Higginbotham *et al.*, 1972) are used to postulate a possible structure for polysaccharide S IX.

In the preceding paper (Higginbotham *et al.*, 1972) we have shown that the immunologically specific capsular polysaccharide of pneumococcal type IX (polysaccharide S IX) contains *N*-acetylmannosamine, probably the D- form, and a small amount of *N*-acetyl-D-galactosamine in addition to the previously recognized D-glucose, D-glucuronic acid and *N*-acetyl-D-glucosamine. We now present results on the oxidation of polysaccharide S IX and use the findings, together with those of partial hydrolysis, to give a possible approximation of the structure of this complex substance.

Experimental and Results

Materials and methods were essentially as given previously (Heidelberg, 1963; Rao & Heidelberg, 1966; Higginbotham *et al.*, 1972).

Reduction of periodate by polysaccharide S IX

Polysaccharide S IX, sample 143A₁ (Rao & Heidelberg, 1966) (0.4 g) was oxidized in 150 ml of 0.1 M-sodium acetate buffer, pH 4, at 4°C with 100 ml of 0.037 M-sodium metaperiodate. Reduction of periodate, which almost ceased in 10 days, was 0.40 mol/180 g (average mol.wt. of the anhydro-sugar constituents), about one-third greater than without buffer for 3 days at 4°C (Rao & Heidelberg, 1966). At room temperature, without buffer, reduction of periodate ceased at 0.34 mol/180 g after 4 days (experiment by Dr. P. Chakravarty).

Periodate oxidized-reduced polysaccharide S IX

Polysaccharide S IX, 143A₁ (2 g), was dissolved in 250 ml of 0.1 M-sodium acetate buffer, pH 4, and mixed

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at 4°C with 4 g of sodium metaperiodate in the same volume of buffer. Toluene was added. After 17 days in the dark at 4°C, 10 ml of ethylene glycol was added with stirring. After 1 h the solution was dialysed, reduced with 3.2 g of NaBH₄ added in two portions during 3 h, and left overnight at 4°C. Excess of borohydride was decomposed with ice-cold 3 M-H₂SO₄ added to pH 2. After 1 h, the mixture was neutralized at 0°C with M-NaOH, dialysed and evaporated *in vacuo* to 15 ml. A small precipitate was filtered off and washed with 1% LiCl; the filtrate and washings were evaporated *in vacuo* to about 20 ml and precipitated with 80 ml of ethanol. The precipitate was triturated with ethanol and acetone and dried *in vacuo* over P₂O₅ (yield 1.54 g). This product precipitated far less antibody (Table 1) than did the earlier, less-completely oxidized, preparation (Rao & Heidelberg, 1966).

The cyanine dye complex (Edstrom, 1969) with oxidized-reduced polysaccharide S IX showed an extinction maximum at 550 nm instead of at 642 nm as did the complex with polysaccharide S IX. As both products presumably have the same number of ionizable carbonyl groups, the spectral shift is evidently influenced by other factors in oxidized-reduced polysaccharide S IX.

Hydrolysis (4 M-HCl, 100°C, 5 h) of oxidized-reduced polysaccharide S IX (2 mg) and analysis by g.l.c. showed that it had retained its full complement of *N*-acetylglucosamine and *N*-acetylmannosamine and contained 6% of anhydro-*N*-acetylglactosamine. Colorimetric analysis for glucose in oxidized-reduced polysaccharide S IX showed that 40% had been destroyed by oxidation, and analysis by the carbazole method indicated that 90% of the glucuronic acid had been oxidized.

Oxidized-reduced polysaccharide S IX was also hydrolysed with M-H₂SO₄ at 100°C for 19 h and neutralized with BaCO₃. Chromatography in solvent

a (Higginbotham *et al.*, 1972) showed spots corresponding to erythritol (R_{Glc} 1.145), glucose, hexosamine and erythreic acid (R_{Glc} 0.67). Benzidine-periodate stained erythritol well and gave a light-green-yellow colour with erythreic acid and glyceric acid. Solvent *c* gave better separation of glucose and erythritol (R_{Glc} 2.67) but the spot due to erythreic acid (R_{Glc} 2.4) was close to that of erythritol. The erythreic acid standard was prepared by oxidation of D-erythrose (Sigma Chemical Co., St Louis, Mo., U.S.A.) with bromine in the presence of $BaCO_3$ and was isolated by sequential elution through Amberlite IR-120 (H^+ form) and Dowex 1 (X4; HCO_3^- form). On chromatography with solvent *a*, $R_{erythritol}$ was 0.7, $R_{erythrose}$ 0.64; with solvent *c*, $R_{erythritol}$ 0.98, $R_{erythrose}$ 0.77.

Oligosaccharides formed by mild acid hydrolysis of oxidized-reduced polysaccharide S IX

The polysaccharide (1.39g) was dissolved in 0.05M- H_2SO_4 at room temperature and dialysed against 0.05M- H_2SO_4 for 24h. Dialysis was repeated five times. The dialysates were neutralized with $BaCO_3$, concentrated *in vacuo*, combined and deionized on Amberlite IR-120 (H^+ form). The solution, at pH2, was evaporated *in vacuo* to about 50ml and subjected to autohydrolysis on the boiling-water bath for 10min. When chromatographed on Whatman 3MM paper for 116h with solvent *b*, the product was resolved into components D1, D2, D3 and D4 in order of increasing mobility. Fraction D1 (about 60mg), after hydrolysis (2M-HCl, 100°C, 8h) and g.l.c., showed glucosamine, mannosamine, glucose and erythritol with small amounts of what appeared to be erythreic acid, glyceric acid and glycerol. Component D1 was separable when rechromatographed in solvent *b* (Higginbotham *et al.*, 1972) into three fractions, one of which contained mannosamine as the only hexosamine. Component D2, with R_{Glc} 0.3 in solvent *b*, 0.15 in solvent *c* (Higginbotham *et al.*, 1972), gave spots on hydrolysis (M- H_2SO_4 , 100°C, 19h) corresponding to glucose, erythritol, hexosamine and erythreic acid.

Component D3 (220mg) was dissolved in water, filtered, deionized again with the mixed-bed resin Bio-Rad AG501-8X(D), and concentrated *in vacuo* to about 1ml. On addition of a drop of ethanol and storage in a desiccator the product crystallized. It had R_{Glc} 0.54 in solvent *b* and 0.46 in solvent *c*. With staining reagent A (Higginbotham *et al.*, 1972), it gave a white patch; with reagent B, a compact spot. Reduction with $NaBH_4$ caused no changes. Hydrolysis with 0.5M- H_2SO_4 at 100°C for 20h gave chromatographic spots corresponding to glucose, hexosamine and erythritol. Component D3 contained 34.4% of anhydroglucose and its nitrogen content indicated 47% of anhydro-N-acetylhexosamine. Mild hydro-

lysis with Dowex 50W (X2) on a boiling-water bath slowly released glucose, followed by erythritol and finally N-acetylhexosamine. Simultaneously, a spot corresponding to the principal component of fraction D4 increased in intensity (see below).

Component D3 (150mg) was dissolved in water and separated into two portions, D3(*a*) and D3(*b*), by successive precipitations with ethanol. Hydrolysis (2M-HCl, 100°C, 6h) of a portion of fraction D3(*a*) and analysis by g.l.c. showed glucose, mannosamine, glucosamine, and erythritol in 2:2:1:1 molar ratios. Colorimetric analysis of intact material gave 38% anhydroglucose and no glucuronic acid. Since erythritol and erythreic acid were not separable by g.l.c. (both eluted at 169°C with the programme: 1 min at 150°C, 7°C/min up to 200°C) the distinction was made by passage through Dowex 1 (X4; HCO_3^- form) and elution with water (erythritol) and aq. 0.5M- NH_3 (erythreic acid). Hydrolysis (2M-HCl, 100°C, 7.5h) of fraction D3(*b*) and analysis as above showed glucose, mannosamine and erythritol in the molar ratios 2:2:1, with no glucuronic acid. The hexosamine residues in both fractions were probably N-acetylated, as fraction D3 had not been adsorbed on passage through deionizing resin.

Component D4 (moist weight 199mg), rechromatographed on paper with solvent *b*, gave three fractions, D4(*a*), D4(*b*) and D4(*c*), in the order of increasing mobility. D4(*a*) (3.6mg) was not studied. D4(*b*) (56mg) and D4(*c*) (11mg) were only partially separated. Hydrolysis of the former (3M-HCl, 100°C, 5.5h) gave mannosamine, glucose and erythritol, 2:1:1, ratios in accord with the observed scission of glucose from component D3 when heated with Dowex 50W (X2) and formation of a component with the mobility of D4(*b*).

Table 1. *Precipitation of polysaccharide S IX and oxidized-reduced polysaccharide S IX in anti-(pneumococcal) sera*

Abbreviations: H, horse; R, rabbit; C, absorbed with pneumococcal C-polysaccharide.

Antiserum ... Polysaccharide	Antibody N precipitated (μ g/1.0ml of antiserum)		
	IIH513	IXH623C	IXR91 ₂
S II	3600		
S IX	0	1655	5340
Oxidized-reduced			
S IX, 90 μ g			155
120 μ g			163
200 μ g		739	
240 μ g			154
400 μ g		747	

A summary of the analyses of oligosaccharides obtained in the various hydrolyses of polysaccharide S IX and oxidized-reduced polysaccharide S IX is given in Table 2.

Discussion

When polysaccharide S IX was oxidized more completely than previously (Rao & Heidelberger, 1966) and then reduced, all of the *N*-acetylhexosamines resisted oxidation, whereas nearly one-half of the glucose and almost all of the glucuronic acid were oxidized. Table 1 shows that one or both of the latter sugars are important determinants of the specificity of pneumococcal type IX. Their modification by oxidation and reduction decreases precipitation in the equine serum by 55% and in the antiserum raised in the rabbit by 97%, effects much greater than shown by the earlier, less-fully oxidized, preparation. Table 1 also shows that polysaccharide S IX does not precipitate anti-(pneumococcal type II) serum, a type in which non-reducing end-groups of D-glucuronic acid are a principal determinant of specificity. Therefore the residues of glucuronic acid that are oxidized in polysaccharide S IX are not non-reducing end groups, nor are the oxidized units of glucose linked through positions 1 and 6 or 1, 4 and

6, as multiples of such units also precipitate anti-(pneumococcal type II) serum (Heidelberger & Adams, 1956). That the oxidized residues of both sugars are (1→4)-linked in polysaccharide S IX is indicated by the appearance of erythreic acid and erythritol in the hydrolytic products of oxidized-reduced polysaccharide S IX.

The amount of oxidation observed (0.4 mol/180 g) was slightly greater than expected (0.33 mol) for 40% destruction of glucose and 90% of glucuronic acid if each residue reduced only 1 mol of periodate/mol of sugar. The slight excess is probably due to overoxidation of the polysaccharide and oxidation of any non-reducing end-groups. However, an oxidation with periodate in the absence of buffer at 25°C for 4 days resulted in a stable final reduction of 0.34 mol of periodate/180 g.

Some of the periodate-resistant glucose residues are those found (1→3)-linked by glucuronic acid (fraction A3; Higginbotham *et al.*, 1972). The remainder occur, probably (1→3)-linked, in fractions D3(a), D3(b) and D4(b) (Table 2).

All of the D fractions, obtained by dialysis of oxidized-reduced polysaccharide S IX against 0.05M-H₂SO₄, were terminated at the original reducing end by erythritol, not erythreic acid, and were therefore linked to position 4 of glucose, not

Table 2. Analysis of oligosaccharides obtained from polysaccharide S IX

For experimental details see the text. Values represent molar ratios. +, Present by paper chromatography; -, absent by paper chromatography.

Treatment	Oligo-saccharide	<i>N</i> -Acetyl-glucosamine	<i>N</i> -Acetyl-mannosamine	Glucose	Glucuronic acid	
Hydrolysis with 0.1M- and 0.15M-H ₂ SO ₄	A1	+	+	+	+	
	A2	1	1	4	3	
	A3	0	0	1	1	
	A4	0	0	0	1	
		Glucosamine	Mannosamine	Glucose	Glucuronic acid	
Hydrolysis with M-H ₂ SO ₄	1	0	0	1	1	
	2	2	0	1	2	
	3(a)	1	0	0	1	
	3(b)	1 (Galactosamine)	0	0	1	
	4	0	2	1	0	
	4(a)	0	2	1	0	
		<i>N</i> -Acetyl-glucosamine	<i>N</i> -Acetyl-mannosamine	Glucose	Glucuronic acid	Erythritol
Oxidation with periodate; reduction with NaBH ₄ and treatment with 50mM-H ₂ SO ₄	D1	Present by g.l.c.			Absent	Present
	D2	+	+	+	—	+
	D3(a)	1	2	2	0	1
	D3(b)	0	2	2	0	1
	D4(b)	0	2	1	0	1

Fig. 1. Possible tentative structure for polysaccharide S IX

glucuronic acid, in the polysaccharide. Fraction D3(b) (Table 2) was converted by loss of glucose into a product with the properties of fraction D4(b), so that glucose was probably at the non-reducing end of fraction D3(b). Fraction D4(b) is similar to the acid-produced oligosaccharides 4 and 4(a) (Table 2) and probably originates from the same sequence in the polysaccharide. D3(a) and D3(b) (Table 2) differ only by an extra glucosamine residue in the former, and therefore glucosamine is probably at the non-reducing end of D3(a).

Elson-Morgan analysis of the acid-produced oligosaccharides (Higginbotham *et al.*, 1972) showed that all of the reducing hexosamine groups were (1→3)-linked. Their resistance to oxidation by periodate is therefore in accordance with the colorimetric indications.

The finding of galactosamine raises difficulties. 6% of anhydro-*N*-acetyl galactosamine was found in oxidized-reduced polysaccharide S IX, about 2% in oligosaccharide 3(b) (Table 2) plus peak 5 (cf. Higginbotham *et al.*, 1972), whereas only 0.6% was found in hydrolysates of polysaccharide S IX. It is evident that hydrolysis with strong acid either destroys galactosamine or liberates it only partially. Hydrolysis seems more favourable in two stages, as in the formation of oligosaccharide 3(b), whereas

still larger amounts are liberated after oxidation of glucose and glucuronic acid residues, as in oxidized-reduced polysaccharide S IX. It is uncertain whether *N*-acetylgalactosamine is a constituent of polysaccharide S IX or of an otherwise undetected impurity.

From the results given in the two papers a possible tentative structure for polysaccharide S IX may be postulated (Fig. 1). In this, —A3— etc. indicate the oligosaccharides studied, and on which the structural representation is based. As far as is known, all sugars are in the D-form.

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