Some Structural Features of Pneumococcus Type XXII Capsular Polysaccharide

By **Bishnu P. Chatterjee** and **Chintalacharuvu V. N. Rao,*** Department of Macromolecules, Indian Association for the Cultivation of Science, Calcutta 32, India

The capsular polysaccharide of Pneumococcus Type XXII (S XXII) was freed from impurities and found to contain phosphate (0·4%), erthyritol (3%), and D-glucose, D-galactose, L-arabinose, L-rhamnose, and D-glucuronic acid in the molar ratio 7·0 : 2·5 : 2·0 : 4·8 : 2·8. Mild acidic hydrolysis gave an aldobiouronic acid characterised as 3-*O*-α-D-glucuronopyranosyl-D-glucose. Graded hydrolysis of S XXII yielded, besides the aldobiouronic acid, three disaccharides, *viz.* 4-*O*-β-D-glucopyranosyl-D-glucose, 5-*O*-β-D-galactopyranosyl-L-arabinose, and 3-*O*-β-D-galactopyranosyl-L-arabinose, and a small amount of a trisaccharide containing galactose, arabinose, and rhamnose. The identification of these oligosaccharides revealed some of the structural details of the polysaccharide.

MANY plant polysaccharides containing α-1,4-linked glucose residues show cross-reactions in Pneumococcus Type XXII antiserum,[1,2] *e.g.* synthetic polyglucose, oyster glycogen, rabbit liver glycogen, alkali-purified amylopectin, and tamarind seed polysaccharide. Some minor cross-reactions like that given by hualtaco gum [3] are also reported. According to the quantitative precipitin theory of Heidelberger and Kendall [4] the cross-reactions with polyglucoses were ascribed to the presence of similarly linked glucose residues in these polysaccharides and the Pneumococcus Type XXII specific polysaccharide (S XXII). However no work on the structural elucidation of S XXII appears to have been carried out. Brown [5] has reported some of the physical characteristics of this material, and i.r. absorption bands at 6·20—7·10 μm are said [6] to indicate the presence of carboxylate ions. With these results in view a detailed investigation of the structure of S XXII was undertaken.

A virulent strain of Pneumococcus Type XXII was grown in modified casein hydrolysate medium [7] and phenol was added to kill the bacteria. The capsular material was separated from the cells and was precipitated with ethanol. The protein fraction was removed by Sevag's treatment.[8] After eight treatments no interfacial layer was observed. The material was further purified by repeated precipitation with ethanol. Then the polysaccharide was fractionally precipitated to yield three fractions. Fraction 2 was obtained in large quantity. All three fractions were tested for the presence of 'C' substance by Ouchterlony's technique [9] using homologous antiserum. In the case of fraction 2 a single band was obtained whereas the other two fractions each gave two bands. On electrophoresis [thin-layer silica gel plates and borate buffer (pH 9·5) or phosphate buffer (pH 6·5)] only fraction 2 gave a single band. Further investigations were carried out on this fraction (S XXII).

S XXII was found to contain D-glucose (35·3%), D-galactose (12·3%), L-arabinose (10%), L-rhamnose (23·8%), D-glucuronic acid (13·6%), and erythritol (3%).

Mild acidic hydrolysis of the polysaccharide gave a hydrolysate containing 3-*O*-α-D-glucuronopyranosyl-D-glucose, which was isolated by separation on an ion exchange resin followed by paper chromatography. The aldobiouronic acid structure was established by, *inter alia*, periodate oxidation studies on the methyl ester methyl glycoside.

S XXII was subjected to graded hydrolysis by heating with 0·1N-hydrochloric acid for 4 h at 95° to yield considerable amounts of oligosaccharides. The acid sugar portion of the hydrolysate was separated from the neutral one by adsorbing the former on a column of Dowex I-X4 (HCO_3^- form) resin. The neutral portion was found to contain, besides glucose, galactose, arabinose, rhamnose, and erythritol, four oligosaccharides. The mixture containing mono- and oligo-saccharides was separated on Whatman 3MM paper into its components and the individual oligosaccharides were identified. Table 2 shows the components of the mixture and their characteristics.

The acidic fraction was displaced from the anion-exchange resin and found to contain only one component, whose mobility on paper chromatograms was the same as that of the aldobiouronic acid isolated earlier. This was characterised in the usual way as 3-*O*-α-D-glucuronopyranosyl-D-glucose.

The three disaccharides isolated from S XXII and characterised were 4-*O*-β-D-glucopyranosyl-D-glucose, 5-*O*-β-D-galactopyranosyl-L-arabinose, and 3-*O*-β-D-galactopyranosyl-L-arabinose. Under various experimental conditions tried, a rhamnose-containing disaccharide could not be isolated. This may be due to the labile nature of α-glycosidic linkages through which this sugar is joined to other units in the polysaccharide. The only trisaccharide obtained contained galactose, arabinose, and rhamnose, but this could not be fully characterised because of its poor yield.

It is evident that S XXII contains 1,4-linked glucobiose residues; this portion or one glucose unit in it could be responsible for the cross-reactions between

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[5] R. Brown, *J. Immunol.*, 1939, **37**, 445.
[6] S. Levine, H. J. R. Stevenson, and P. W. Kabler, *Arch. Biochem. Biophys.*, 1953, **45**, 65.
[7] P. D. Hoeprich, *J. Bacteriol.*, 1955, **69**, 682; 1957, **74**, 587.
[8] M. G. Sevag, *Biochem. Z.*, 1934, **273**, 419.
[9] Ö. Ouchterlony, *Arkiv Kemi*, 1948, **26B**, 1.

Type XXII Pneumococcus antiserum and polyglucoses mentioned earlier. The other minor cross-reactions like those given by hualtaco gum and tamarind seed polysaccharide may be due to the presence of similarly linked galactose and/or rhamnose residues in these polysaccharides and S XXII. It is noteworthy that S XXII contains arabinose; no other type-specific polysaccharide of Pneumococcus, whose structure has been elucidated, contains this sugar. Arabinose residues are linked at either C-3 or C-5 to a galactose unit.

The specific rotation of S XXII (+98°) is not high in comparison with other α-linked polysaccharides like amylose, indicating that both α- and β-type linkages are present and that the yield of oligosaccharides containing different sugars depends upon the distribution of these two kinds of linkage in the macromolecule. Graded hydrolysis studies established the presence of some sugar groupings in the polysaccharide.

EXPERIMENTAL

Paper partition chromatography was carried out by the descending method. Whatman No. 1 papers were used for chromatographic identification of sugars and the 3 MM grade was employed for their isolation (up to 200 mg). The following solvent systems (v/v) were used: (A) ethyl acetate–pyridine–water (8 : 2 : 1), (B) butan-1-ol–acetic acid–water (4 : 1 : 5; upper layer), (C) ethyl acetate–pyridine–acetic acid–water (5 : 5 : 1 : 3), (D) ethyl acetate–acetic acid–water (9 : 2 : 2). Spray reagents used were (a) saturated aqueous aniline oxalate, (b) alkaline silver nitrate solution, (c) the Hanes–Isherwood reagent for phosphate esters.

Unless otherwise stated all evaporations were carried out *in vacuo* at 35—40°. All quoted specific rotations are equilibrium values.

Total hexose was estimated with L-cysteine [10] at 380 and 414 nm, uronic acid with carbazole [11] at 535 nm, arabinose with L-cysteine–H_2SO_4 [12] at 390 and 420—426 nm, and rhamnose with L-cysteine–H_2SO_4 [13] at 396 and 426 nm for polysaccharide solutions of appropriate concentration. D-Glucose and D-galactose were determined by use of glucostat and galactostat reagents (Worthington Biochemical Corporation, Freehold, New Jersey), respectively, and erythritol was estimated by the periodate–chromotropic acid method [14] applied to the hydrolysate. Completion of hydrolysis was determined by the iodometric method.[15] Consumption of oxidant during oxidation by periodate was estimated spectrophotometrically [16] and liberated formic acid was estimated by titration with sodium hydroxide (Methyl Red).

Crystalline *N*-*p*-nitrophenylglycosylamines were prepared [17] by refluxing the sugars in methanol with *p*-nitroaniline in methanol containing a trace amount of concentrated hydrochloric acid for 2 h. The yellow crystals obtained on cooling were recrystallised twice from methanol and dried. The crystalline tosyl derivative of erythritol was prepared [18] by keeping a mixture of toluene-*p*-sulphonyl chloride and an ice-cold solution of erythritol in anhydrous pyridine for 24 h at room temperature. The crude derivative separated on pouring the solution into ice-cold water; it was washed with water and ethanol, crystallised from acetone–ethanol, and dried.

Molecular weights of the oligosaccharides were determined by the alkaline hypoiodite method.[19]

Preparation and Properties of S XXII.—A strain of Pneumococcus Type XXII (kindly supplied by Dr. H. P. Bernheimer, Downstate Medical Centre, State University of New York), was passed through mice three times to enhance its virulence. Modified casein hydrolysate medium (10 × 2 l) was inoculated with this virulent strain and was then incubated at 37° for 96 h. Phenol was added (1%) and the solution was kept overnight at room temperature. The solution was then centrifuged (Sharples centrifuge) at 40,000 rev. min^{-1} to remove cell debris and the effluent was concentrated to one-tenth of its volume. It was dialysed against distilled water for 4 days. The solution in the dialysis bag was concentrated to *ca.* 500 ml, and ethanol was added to precipitate the capsular material. The precipitate (3 g) was dissolved in 5% sodium acetate buffer (75 ml; pH 6·05) and the solution was kept overnight at 5°. The supernatant was decanted and the residue was washed with the same buffer. The washings were mixed with the main solution and then subjected to Sevag's treatment.

After eight treatments no interfacial layer was observed. The milky interfacial layers from all the treatments were combined and then washed with a small volume of acetate buffer. The washings were added to the main solution.

The solution containing the polysaccharide material was diluted with water so that the final concentration of sodium acetate was 3%. To this solution at 0°, chilled propan-2-ol (1·2 l) was added with stirring. The polysaccharide precipitated out and was collected by centrifugation. Further addition of propan-2-ol to the supernantant gave no precipitate. The material was redissolved in aqueous 3% sodium acetate (200 ml) and adjusted to pH 6·05, and the polysaccharide was reprecipitated by adding ethanol (1 l). This process was repeated four times. Finally the polysaccharide material in buffered solution (200 ml) was fractionally precipitated by gradual addition of ethanol. Three fractions were collected. Fraction 1 separated after adding 450 ml of ethanol and was isolated at the centrifuge, washed several times with ethanol and acetone, and then dried (P_2O_5); yield 30 mg. From the supernatant fractions 2 and 3 were precipitated by addition of 850 and 1000 ml of ethanol, respectively; yields: fraction 2, 500 mg; fraction 3, 25 mg. Each fraction was tested for the presence of 'C' substance by Ouchterlony's technique using Pneumococcus Type XXII antiserum (No. 566, kindly supplied by Professor M. Heidelberger). Fractions 1 and 3 gave two bands, one corresponding to 'C' substance, whereas a single sharp band was found with fraction 2. Each fraction was subjected to electrophoresis on thin-layer silica gel plates in borate buffer of pH 9·5 with a potential gradient of 18 V cm^{-1} and in phosphate buffer of pH 6·5 with a potential gradient of 20 V cm^{-1}. After 2 h the plates were removed, dried in air,

[10] Z. Dische, L. B. Shettles, and M. Osnos, *Arch. Biochem.*, 1949, **22**, 169.
[11] Z. Dische, *J. Biol. Chem.*, 1947, **167**, 189.
[12] M. Heidelberger, J. Adams, and Z. Dische, *J. Amer. Chem. Soc.*, 1956, **78**, 2853.
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[14] M. Lambert and A. C. Naish, *Canad. J. Res.*, 1950, **28B**, 83.
[15] F. L. Barker and H. F. E. Halton, *Biochem. J.*, 1920, **14**, 754.
[16] J. S. Dixon and D. Lipkin, *Analyt. Chem.*, 1954, **26**, 1092.
[17] F. Weygand, W. Perkow, and P. Kuhner, *Chem. Ber.*, 1951, **84**, 594.
[18] J. K. Hamilton and F. Smith, *J. Amer. Chem. Soc.*, 1956, **78**, 5907.
[19] E. L. Hirst, L. Hough, and J. K. N. Jones, *J. Chem. Soc.* 1949, 928.

and then sprayed with concentrated sulphuric acid. A single spot was detected in the case of fraction 2 (migration 0·5 cm towards positive electrode in phosphate buffer and 1·5 cm towards negative electrode in borate buffer) whereas fractions 1 and 3 gave more than one band. Fraction 2 was taken to be homogeneous and was free from ' C ' substance. Subsequent investigations were carried out on this fraction, which had the following properties: moisture, 0·2%; ash (as Na), 1·8%; $[\alpha]_D^{30}$ +98° (*c* 1·0 in H_2O); P, 0·4; N, 0%; OMe, 0·6%.

Hydrolysis of the Polysaccharide.—S XXII (100 mg) was hydrolysed by heating with 2N-sulphuric acid (10 ml) at 100° for 10 h. The solution was neutralised ($BaCO_3$) and filtered, and the filtrate was concentrated to a small volume. Paper chromatographic examination in solvents (B) and (C) showed the presence of glucose, galactose, arabinose, rhamnose, glucuronic acid, and erythritol; an aldobiouronic acid was also detected. The mixture was separated on 3 MM paper and the individual sugars were characterised by their specific rotations and by formation of crystalline derivatives (Table 1).

TABLE 1

Characterisation of sugars

Sugar	$[\alpha]_D^{30}$ (°)	Cryst. solvent; m.p. and mixed m.p. (°C)	Derivative	M.p. (°C) of derivative
D-Galactose	+79	MeOH; 112—118 (lit.,[a] 118—120)		217—218 (lit.,[b] 219)
D-Glucose	+50	EtOH–MeOH; 145 (lit.,[a] 148—150)		181—182 (lit.,[b] 184)
L-Arabinose	+109	MeOH; 154—156 (lit.,[a] 158)	*p*-Nitroanilide	200—201 (lit.,[b] 202)
L-Rhamnose	+8	MeOH; 90 (lit.,[a] 92)		218 (lit.,[c] 220)
D-Glucuronic acid	+34	MeOH; 160 (lit.,[a] 165)		127 (lit.,[d] 129—130)
Erythritol		EtOH; 120—121 (lit.,[e] 120—122°)	Tosyl	163—165 (lit.,[e] 165—166)

[a] Heilbron's ' Dictionary of Organic Compounds,' vols. 1, 3, and 5, Eyre and Spottiswoode, London. [b] Ref. 17. [c] T. Miyazaki and T. Yadome, *Carbohydrate Res.*, 1971, **16**, 153. [d] J. K. Hamilton, F. Smith, and D. R. Spriestersbach, *J. Amer. Chem. Soc.*, 1957, **79**, 443. [e] Ref. 18.

The content of each sugar in S XXII was estimated as: glucose, 35·3%; galactose, 12·3%; arabinose, 10·0%; rhamnose, 23·8%; glucuronic acid, 13·6%; erythritol, 3·0%.

S XXII (100 mg) was hydrolysed with 0·1N-sulphuric acid (5 ml) at 100° for 10 h. The solution, after the usual treatment, was passed through a column of Amberlite IR 120 (H^+) resin and the acidic portion was adsorbed on a column of Dowex 1-X4 (HCO_3^-) resin. The Dowex column was washed with water till the washings gave a negative Molisch test. It was eluted with 0·1N-sulphuric acid and the eluate was neutralised. After the usual treatment and paper chromatographic examination, the acid fraction was found to contain glucuronic acid and an aldobiouronic acid.

The acid sugars were separated on Whatman No. 3 MM paper and the aldobiouronic acid (29 mg) was isolated in homogeneous state; $[\alpha]_D^{30}$ +110° (lit.,[20] +106°). On complete hydrolysis with N-sulphuric acid it yielded glucose and glucuronic acid; a trace of glucuronolactone was also detected on paper chromatograms. The ratio of the amounts of these two sugars was estimated as 1 : 1.

The aldobiouronic acid (10 mg) was converted into its methyl ester methyl glycoside in the usual way. To the dry syrup (3 mg) dissolved in tetrahydrofuran (5 ml) a suspension of lithium aluminium hydride (10 mg) in the same solvent (5 ml) was added slowly. The mixture was kept overnight at room temperature, then refluxed for 5 h on a water-bath. Water was added and the precipitate was filtered off. The filtrate was deionised with Amberlite IR 120 (H^+) and Amberlite IR 45 (OH^-) resins and then concentrated to a small volume. After hydrolysis paper chromatographic examination in solvents (A) and (B) showed a single spot corresponding to glucose. A part of the acid (2 mg) was reduced with sodium borohydride (10 mg). After the usual treatment followed by hydrolysis, paper chromatographic examination in solvents (B) and (C) with aniline oxalate as spray reagent showed a single spot corresponding to glucuronic acid.

The methyl ester methyl glycoside of the aldobiouronic acid consumed 2·1 mol. equiv. of periodate, liberating 1·1 mol. equiv. of formic acid, in 6 h. The periodate-oxidised material was hydrolysed; paper chromatographic examination then showed a spot corresponding to glucose and no spot of uronic acid.

Graded Hydrolysis of S XXII.—The polysaccharide (160 mg) was dissolved in 0·1N-hydrochloric acid (20·0 ml) and heated at 95° on a water-bath for 4 h. The hydrolysate was then neutralised with silver carbonate and centrifuged. The supernatant was concentrated and freeze-dried; yield 150 mg. Paper chromatographic examination of the hydrolysate in various solvents showed five slow-moving spots besides those of monosaccharides present in S XXII.

The mixture (140 mg) was passed successively through columns of Amberlite IR 120 (H^+) and Dowex 1-X4 (HCO_3^-) resin. The Dowex column was washed with water (100 ml) and the washings were added to the eluate. The solution, containing neutral sugars, was concentrated to a small volume and then freeze-dried; yield 100 mg. This material on paper chromatographic examination in solvents (B) and (C) showed spots corresponding to glucose, galactose, arabinose, rhamnose, and erythritol, and four slow-moving materials. The mixture was separated on Whatman No. 3 MM paper in solvent (B) and individual oligosaccharides were isolated as homogeneous fractions.

TABLE 2

Oligosaccharides obtained by graded hydrolysis of S XXII

Fraction no.	Name of the sugars	R_{gal} *	$[\alpha]_D^{30}$(°)	Yield (mg)
i	Mixture of glucose, galactose, arabinose, rhamnose, and erythritol			30
ii	3-*O*-β-D-Galactopyranosyl-L-arabinose	0·58	+50	20
iii	5-*O*-β-D-Galactopyranosyl-L-arabinose	0·44	−10	15
iv	4-*O*-β-D-Glucopyranosyl-D-glucose	0·36	+36	25
v	A trisaccharide	0·21	+125	8
vi	3-*O*-α-D-Glucurono-pyranosyl-D-glucose	0·45	+110	35

* R_{gal} Values are with respect to D-galactose in solvent (B).

[20] J. D. Higginbotham, A. Das, and M. Heidelberger, *Biochem. J.*, 1972, **126**, 225.

Identification of Neutral Oligosaccharides.—Oligosaccharide I, 3-*O*-β-D-galactopyranosyl-L-arabinose, had $[\alpha]_D^{30}$ +50° (*c* 0·4 in H_2O) (lit.,[21] +53°), R_{gal} 0·58 in solvent (B), *M* 309. Hydrolysis of a sample (2 mg) gave galactose and arabinose. Reduction with sodium borohydride followed by hydrolysis and paper chromatographic examination revealed that arabinose was the reducing end group. The methyl glycoside of the disaccharide was subjected to periodate oxidation at 3° in the dark. In 2·5 h the disaccharide reduced 2·2 mol. equiv. of oxidant liberating 1·1 mol. equiv. of formic acid. The periodate-oxidised material on hydrolysis gave arabinose and other smaller expected aldehydes.

Oligosaccharide II, 5-*O*-β-D-galactopyranosyl-L-arabinose, had $[\alpha]_D^{30}$ −10° (*c* 0·3 in H_2O) (lit.,[22] −13°; lit.,[23] −18°), R_{gal} 0·44 in solvent (B), *M* 310. The disaccharide was shown to be composed of galactose and arabinose. Galactose was identified as the non-reducing sugar by reduction of the disaccharide with sodium borohydride followed by hydrolysis. The methyl glycoside of the disaccharide reduced 3·1 mol. equiv. of periodate, liberating 1·2 mol. equiv. of formic acid, in 2·5 h. The periodate-oxidised material on complete hydrolysis did not give any sugar. The structure of the oligosaccharide is not fully settled since the analytical data also fit the 4-*O*-β-D-galactopyranosyl-L-arabinose structure.

Oligosaccharide III, 4-*O*-β-D-glucopyranosyl-D-glucose, had $[\alpha]_D^{30}$ +36° (*c* 0·3 in H_2O) (lit.,[24] +34·9°), R_{gal} 0·36 in solvent (B), *M* 340. The methyl glycoside of this disaccharide consumed 3·2 mol. equiv. of periodate, liberating 1·1 mol. equiv. of formic acid in 3·5 h.

Oligosaccharide IV (8 mg) had $[\alpha]_D^{30}$ +125° (*c* 0·2 in H_2O), R_{gal} 0·21 in solvent (B). Hydrolysis of a sample (1 mg) yielded galactose, arabinose and rhamnose. A portion (2 mg) of the material was reduced with sodium borohydride and then hydrolysed. Chromatography in solvent (B) with aniline oxalate as spray reagent showed spots corresponding to galactose and arabinose. The molecular weight was 454 (calculated for a trisaccharide containing galactose, arabinose, and rhamnose, 458). Further characterisation could not be attempted owing to shortage of material. The oligosaccharide seems to be a trisaccharide consisting of galactose, arabinose, and rhamnose, the last being at the reducing end.

Identification of 3-O-α-D-*Glucuronopyranosyl-*D-*glucose.*—The Dowex resin column was washed with water (1 l) till free from neutral sugars and then eluted with 0·1M-sulphuric acid (50 ml). The column was then washed with water (20 ml) and the washings were added to the eluate. The combined solution was neutralised and filtered, and the filtrate was concentrated to 10 ml and then freeze-dried; yield 35 mg. Paper chromatographic examination in solvents (C) and (D) showed a single spot, R_{gal} 0·45 in solvent (D), $[\alpha]_D^{30}$ +110° (*c* 0·5 in H_2O) (lit.,[20] +106°). The aldobiouronic acid was characterised as described earlier.

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[24] V. E. Gilbert, F. Smith, and M. Stacey, *J. Chem. Soc.*, 1946, 622.