Immunopolysaccharides. Part IV. Structural Studies on the Type II* Pneumococcus *Specific Polysaccharide.*

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Acidic hydrolysis of a methylated derivative of the Type II *pneumococcus* specific polysaccharide revealed the presence of 2 : 4-di-*O*-methyl-L-rhamnopyranose (7 parts), 2 : 3-di-*O*-methyl-D-glucose (1 part), 2 : 3-di-*O*-methyl-D-glucuronic acid (2 parts), and 2 : 3 : 4-tri-*O*-methyl-D-glucuronic acid (1 part). The polysaccharide possesses a highly branched chain structure with D-glucose forming a large proportion of the branch points. The serological relation of the Type II polysaccharide to certain dextrans and other polyglucoses may be explained by the common presence in them of glucose units having the same spatial geometry at branch points.

Although the " soluble specific substance " of Type II *pneumococcus* was the first of the bacterial capsular materials to be recognised as a polysaccharide by Heidelberger and Avery (*J. Exp. Med.*, 1923, **38**, 73; 1924, **40**, 301) its fine structure remained undetermined in spite of the inherent chemical interest of these substances and their great importance for the understanding of bacterial specificity and immunity to infectious disease. However, the molecular structure of the more readily accessible specific polysaccharide of Type III *pneumococcus* has been shown by Reeves and Goebel (*J. Biol. Chem.*, 1941, **139**, 511) to be that of a polycellobiuronic acid with its units held by 1→3 linkages.

The present report deals with preliminary structural investigations on the Type II substance made possible by a generous sample of material prepared by E. R. Squibb and Sons, New Brunswick, N.J., U.S.A., and supplied through the courtesy of Professor M. Heidelberger and the Surgeon General of the United States Army.

The Type II polysaccharide has the property of cross-reacting specifically with Type III antiserum and this may be considered to be due to the fact that the Type II material possesses glucose (Heidelberger, *Chem. Rev.*, 1929, **3**, 403) and glucuronic acid units. Elegant methods for isolating the polysaccharide have been devised by Heidelberger, Kendall, and Scherp (*J. Exp. Med.*, 1936, **64**, 559). Eight-day broth cultures are usually made and the polysaccharide is precipitated by addition of 95% alcohol to the autolysed broth culture. It is freed from protein by Sevag's method (*Biochem. Z.*, 1934, **273**, 419) and from glycogen by addition of saliva.

In earlier studies of its physical properties, Record and Stacey (*J.*, 1948, 1561) have shown that it forms solutions of high viscosity in water, that its molecular weight is of the order of 240,000, and that it has a cylindrical molecule.

The material provided was essentially free from protein and glycogen. It did, however, contain small amounts of deoxypentosenucleic acid and glucosamine-containing material. It was readily freed from these by fractional precipitation with cetyltrimethylammonium bromide (Jones, *Biochem. Biophys. Acta*, 1953, **10**, 607).

Further fractionation was carried out by gradual addition of ethyl alcohol to aqueous solutions in the usual way. The resulting main fraction, $[\alpha]_D + 60°$, was electrophoretically homogeneous in the pH range 5·0—8·0, and was considered suitable for chemical examination.

The infrared spectrum indicated the possible presence of (1→4)-α- and (1→6)-α-glycosidic linkages, these α-linkages also being indicated by the downward trend of the specific rotation on acidic hydrolysis. The absence of furanose sugars was indicated by the fact that prolonged treatment with 0·01N-sulphuric acid at 95° failed to liberate any components detectable on a chromatogram. Chromatographic analysis of a more vigorously hydrolysed solution revealed four components. Two spots corresponded to D-glucurone and D-glucuronic acid while two others corresponded to D-glucose and to L-rhamnose.

* Part III, preceding paper.

Glucose had been recognised by Heidelberger (*loc. cit.*), and L-rhamnose had also been isolated and identified (Stacey, *Quart. Rev.*, 1947, **1**, 217; Record and Stacey, *loc. cit.*; Beiser, Kabat, and Schor, *J. Immunol.*, 1952, **69**, 297). The acidic nature of the polysaccharide was due to the glucuronic acid component which could be detected in hydrolysates by its strong colour reaction with naphtharesorcinol. The equivalent weight of the polysaccharide was estimated by conductometric titration to be *ca.* 1000, a figure largely in agreement with early published figures.

Methylation was smoothly accomplished with sodium hydroxide and methyl sulphate in the usual way, though the highest methoxyl content which could be introduced was only 42·0%

Fractionation of the sodium salt of the methylated polysaccharide was achieved by precipitation from chloroform or aqueous solution by means of solvents and the major portion, an essentially homogenous material (OMe, 42·1%), was selected for methanolysis. This was carried out with 4% methanolic hydrogen chloride in sealed tubes at 100°. The resulting products were hydrolysed and then separated into four fractions by means of ion-exchange resins. The neutral methylated sugars were separated by partition chromatography. The first and major fraction was shown (as described by Butler, Lloyd, and Stacey, preceding paper) to consist of 2 : 4-di-*O*-methyl-L-rhamnopyranose and the second was identified as 2 : 3-di-*O*-methyl-D-glucopyranose.

The two acidic fractions each behaved as mixtures and appeared to contain aldobiuronic acid residues. They were combined and treated in a sealed tube with 5% methanolic hydrogen chloride at 100°.

Identification of the products by means of de-esterification, acid hydrolysis, and oxidation with bromine, etc., gave 2 : 3 : 4-tri-*O*-methyl-D-glucuronic acid (1 part), 2 : 3-di-*O*-methyl-D-glucuronic acid (2 parts), and 2 : 3-di-*O*-methyl-D-glucose (1 part). An assessment of the total amounts of the identified constituents revealed the approximate proportions as 2 : 4-di-*O*-methyl-L-rhamnopyranose (7 parts), 2 : 3 : 4-tri-*O*-methyl-D-glucuronic acid (1 part), 2 : 3-di-*O*-methyl-D-glucuronic acid (2 parts), and 2 : 3-di-*O*-methyl-D-glucose (1 part). Somewhat similar proportions were found by our former colleague Dr. P. W. Kent (*Chem. and Ind.*, 1952, 1176) using a scanning method.

In the present state of the investigation any attempts at writing a formula for the repeating unit of polysaccharides are considered to be premature, and formulation of structure must await the securing of higher yields of the methyl ether and detailed examination of oligosaccharide fragments.

It is clear, however, that the structure is highly branched with some of the glycosidically linked glucuronic acid residues forming the ends of chains and having, as is usual, the carboxyl groups free. The L-rhamnose residues are all present in a chain structure while the D-glucose residues form the branch points in the molecule.

This occurrence of glucose residues as branch points with other residues linked through the 1→4 and 1→6 positions, may, as already suggested (Heidelberger and Aisenberg, *Proc. Nat. Acad. Sci.*, 1953, **39**, 453; Stacey, *Biochem. Soc. Symp.*, 1953, No. 10, p. 74) provide the key to an understanding of the long known cross reactivity of certain dextrans with Type II anti-*pneumococcus* serum (Sugg, Hehre, and Neill, *J. Bact.*, 1942, **43**, 24; Neill and Abrahams, *Proc. Soc. Exp. Biol.*, 1951, **78**, 537) and help to explain the reactivity of the dextrans in other antisera, the more so as glucose has now been recognised as a component of the specific polysaccharides of Types IX and XII *pneumococci* (Heidelberger, Barker, and Stacey, *Science*, 1954, **120**, 781) in antisera to which dextrans and glycogens react strongly.

The present finding that the glucose in the Type II substance occurs as 1, 4, and 6 branch points also enabled Heidelberger and Aisenberg (*loc. cit.*) to predict from the quantitative theory of specific precipitation (Heidelberger and Kendall, *J. Exp. Med.*, 1935, **61**, 563) that glycogen and amylopectin, which also contain multiple 1, 4, and 6 branch points of glucose, would precipitate Type II antiserum and to demonstrate (*J. Exp. Med.*, 1954, **99**, 343) that these polysaccharides of widespread occurrence show much the same distribution of cross reactivity in Type II and other anti-*pneumococcus* sera as do the dextrans. Heidelberger has also found that tamarind-seed polysaccharide (jellose),

in which two of the three glucose residues in the repeating unit of galactose, glucose, and xylose are linked in the 1, 4, and 6 positions, causes heavy precipitation with Type II anti-*pneumococcus* serum (" Perspectives in Microbiology," Rutgers Univ. Press, 1954).

Experimental

Properties of the Polysaccharide.—The polysaccharide, prepared by essentially the method of Heidelberger, Kendall, and Scherp (*J. Exp. Med.*, 1936, **64**, 559), was provided in the form of its sodium salt as a white powder, insoluble in organic solvents, but soluble in water forming highly viscous solutions, $[\alpha]_D^{18}$ +50° (*c*, 1·0).

Colorimetric tests on the hydrolysed polysaccharide gave the following results: biuret test for protein and iodine test for glycogen, negative; Dische's diphenylamine reagent (*Mikrochem.*, 1930, **8**, 4) for nucleic acids and Ehrlich's test (Morgan and Elson, *Biochem. J.*, 1934, **28**, 988) for amino-sugars, weakly positive; Tollens's reagent (naphtharesorcinol) for uronic acids, positive. It contained N 1·2, P 0·78, and ash 8·7%.

Treatment with cetyltrimethylammonium bromide and electrophoretic examination of aqueous solutions at pH 6·0 showed that *ca.* 6% of deoxyribonucleic acid was present in the polysaccharide (Stacey, *Ann. Acad. Sci. Fennicae*, 1955, *A*, **11**, 60, 262).

Three fractions were isolated when absolute ethanol was gradually added to a 3% aqueous solution which had been acidified to pH 3·8 with acetic acid. Their properties are compared in the Table.

Properties and composition of polysaccharide fractions.

Fractions	Wt. (g.)	$[\alpha]_D^{18}$	N (%)	P (%)	Ash (%)
A	0·17	+66°	1·5	0·80	4·3
B	0·46	+49	1·1	0·82	7·6
C	0·38	+47	1·1	—	5·7

Measurement of the specific viscosity of aqueous solutions of each fraction at concentrations ranging from 0·3% to 0·1% indicated that they were reasonably homogeneous in regard to molecular weight. The fractions were mixed with a larger sample of polysaccharide, dissolved in water, and treated with cetyltrimethylammonium bromide according to the method of Jones, Dutta, and Stacey (*Biochim. Biophys. Acta*, 1953, **10**, 613; cf. Stacey, *loc. cit.*).

The polysaccharide was then precipitated twice with ethyl alcohol and isolated by drying from the frozen state. It had $[\alpha]_D$ +60° (equiv., 1030) and contained traces only of nitrogen and phosphorus. Its infrared spectrogram showed peaks at 933 and 837 cm.$^{-1}$, providing evidence of α-(1→6)- and α-(1→4)-linkages (Barker, Bourne, Stacey, and Whiffen, *J.*, 1954, 171). Its electrophoretic behaviour in the pH range 5·0—8·0 revealed only one component. By acidic hydrolysis with 2N-sulphuric acid at 95°, D-glucose, D-glucuronolactone, D-glucuronic acid, and L-rhamnose were identified on chromatograms as components of the polysaccharide.

Methylation of the Polysaccharide.—(*a*) A solution of the partly purified polysaccharide (10 g.) in water (50 c.c.) was methylated with 30% aqueous sodium hydroxide (300 c.c.) and methyl sulphate (100 c.c.) at 35—42° in the usual manner. Most of the product was isolated by virtue of its insolubility in the hot reaction mixture. The soluble material was recovered from a dialysed solution by concentration to dryness under reduced pressure. In subsequent treatments with these reagents, the partly methylated polysaccharide was kept in solution by the careful addition of acetone. When this was removed by heat, the crude product was precipitated and, after being dried, was purified by extraction with methanol followed by fractional precipitation with ether, and then by precipitation from water with a large excess of acetone. After seven methylations, the hard leathery material (12 g.) was separated into fractions having OMe 33·9—42·2% and ash 10—40%. (*b*) A solution of the partly methylated polysaccharide (21·2 g.; OMe, 37·0%; ash 33·4%) in absolute methanol (700 c.c.) and ether (100 c.c.) was centrifuged to remove inorganic impurities (3·6 g.) and treated with N-sulphuric acid until acid to Congo-red. After removal of a small deposit of sodium sulphate, the acidic polysaccharide (11·9 g.) was precipitated by addition of light petroleum (2 vols.), washed by decantation, and dried. It was then treated in methanol (500 c.c.) with ethereal diazomethane for 36 hr. Removal of the volatile components left a hard grey solid (11·8 g.) (Found: OMe, 38·0; ash, 14·6%). Methylation by both methods was continued until the bulk of the polysaccharide had a methoxyl content of 42·0% and fractional precipitation methods proved it was homogeneous. It was ash-free and had $[\alpha]_D^{18}$ +38° (*c*, 1·0 in MeOH).

Methanolysis of the Methylated Polysaccharide.—The sodium salt (2·2 g.) was heated with 4%

methanolic hydrogen chloride (90 c.c.) in a sealed tube at 100°. After 10 hr. the solution was neutralised with silver carbonate, filtered, and concentrated to a yellow syrup (1·97 g.), n^{19} 1·4650 (Found: OMe, 47·6; ash, 8·4%).

When lower concentrations of methanolic hydrogen chloride were used, scission was incomplete, whereas in 6% concentration, the reagent was too drastic and caused loss of methoxyl.

Hydrolysis of the Mixture of Methylated Glycosides.—The mixture (1·97 g.) was treated with N-sulphuric acid (50 c.c.) at 90°, the optical rotation changing as follows: $[\alpha]_D^{18}$ +34° (initial); +43° (1·5 hr.); +48° (4·5 hr.); +51° (6·5 hr.); +53° (10·5 hr.; equil.).

A syrup (1·8 g.) was isolated by the usual procedure, after the solution had been neutralized with barium carbonate. Separation on a chromatogram using the top layer of 4 : 1 : 5 *n*-butanol–ethanol–water as the mobile phase; and aniline hydrogen phthalate as the indicator showed that at least 4 components were present, *viz.*: N_1, R_F 0·68—0·70, brown spot; N_2, R_F 0·53—0·55, brown spot; U_1, R_F 0·21—0·23 (tailing), red spot; R_F 0·17—0·19 (tailing), red spot.

Separation of the Neutral and Charged Fractions.—(*a*) Amberlite resin IR-100(H) removed barium ions from the hydrolysate, and Deacidite, Amberlite IR-4B, or (most effectively) Amberlite IR-A400 removed hexuronate ions, which were later recovered by elution with aqueous ammonia or barium hydroxide. Thus, a sample (1·80 g.) of the mixed components was separated into a neutral fraction (1·19 g.) and an acidic fraction (0·50 g.) by the agency of Amberlite resins IR-100(H) and IR-A400. (*b*) The syrupy mixture of neutral sugars (0·836 g.) was separated by the method and apparatus described by Hough, Jones, and Wadman (*J.*, 1949, 2511). A 3 : 2 (v/v) mixture of light petroleum (b. p. 40—60°) and *n*-butanol was used as the developing solvent. Two fractions, N1 (0·559 g.) and N2 (0·120 g.) were recovered. The former was a colourless syrup, n^{22} 1·4670, R_G 0·86, $[\alpha]_D^{18}$ +7° (*c*, 1·0 in EtOH), which partially crystallised at 0° and with ether–light petroleum (b. p. 40—60°) gave 2 : 4-*dimethyl*-L-*rhamnose* (0·55 g.), m. p. 91—93° (Found: C, 50·0; H, 8·4; OMe, 32·3. $C_8H_{16}O_5$ requires C, 50·0; H, 8·4; OMe, 32·3%).

Treatment of fraction N1 (0·17 g.) with aniline (0·12 g.) in boiling ethanol (2 c.c.) for 4 hr. and removal of the solvent in a vacuum-desiccator yielded crystalline 2 : 4-*di*-O-*methyl*-N-*phenyl*-L-*rhamnopyranosylamine* (0·08 g., 33%), melting, after crystallisation from ether–light petroleum (b. p. 40—60°), at 141—142·5°, and having $[\alpha]_D^{18}$ +136° ⟶ +4° in 24 hr. (*c*, 0·5 in EtOH) (Found: C, 62·9; H, 7·7; N, 5·3; OMe, 23·1. $C_{14}H_{21}O_4N$ requires C, 62·9; H, 7·9; N, 5·2; OMe, 23·2%).

Further Methylation of Fraction N1.—A single methylation with silver oxide and methyl iodide partially converted 2 : 4-di-*O*-methylrhamnose (0·185 g.) into a crystalline *methyl* 2 : 4-*di*-O-*methyl*-L-*rhamnopyranoside* (20 mg.) which, separated from a syrupy product on a porous tile, had m. p. 98—100°, $[\alpha]_D^{20}$ +85° (*c*, 0·5 in MeOH) (Found: C, 52·1; H, 8·6; OMe, 44·3. $C_9H_{18}O_5$ requires C, 52·4; H, 8·8; 45·3%). After two further methylations of the residue a syrup (0·12 g.) having OMe 55·4% was isolated.

Hydrolysis of the Fully Methylated Glycosides from Fraction N1.—The above syrupy product (0·10 g.) was hydrolysed with 0·5N-sulphuric acid (10 c.c.), $[\alpha]_D$ changing from +53° (initial) to a constant value of +43° in 1 hr., after which the solution was neutralised with barium carbonate and concentrated to dryness and the residue repeatedly extracted with chloroform. Removal of the solvent by distillation left a reducing syrup (70 mg.) with R_G identical with that of 2 : 3 : 4-tri-*O*-methyl-L-rhamnose. The sugar was converted into an aniline derivative by the usual method. After recrystallisation from ethanol–light petroleum (b. p. 60—80°) this melted at 114—116°, alone or in admixture with the authentic aniline derivative of 2 : 3 : 4-tri-*O*-methyl-L-rhamnose.

Examination of Fraction N2.—The syrup (R_G 0·65) (OMe, 26·5%) was fully methylated by methanolic hydrogen chloride followed by silver oxide and methyl iodide. Hydrolysis of the syrupy product with 0·5N-sulphuric acid liberated a reducing sugar with a R_G 1·00. It was converted by aniline into the aniline derivative, m. p. 134°, of 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose.

In a separate experiment fraction N2 was shown by the usual method (Smith, *J.*, 1939, 1724) and chromatogaphically to consist largely of 2 : 3 di-*O*-methyl-D-glucopyranose.

Examination of the Barium Salts of the Methylated Uronic Acids.—The residue obtained by concentration of the neutralised eluate from the Amberlite IR-400 column was a yellow, water-soluble powder (0·50 g.) (Found: OMe, 23·0; Ba 23·5. Calc. for $C_8H_{13}O_{7\frac{1}{2}}Ba$: OMe, 21·4; Ba, 23·7%). Simultaneous esterification and glycoside formation were effected by heating the product (0·5 g.) under reflux with 2% methanolic hydrogen chloride (10 c.c.). A grey precipitate, which rapidly appeared, was filtered off after 12 hr. Distillation of the solvent from

the neutralised filtrate (Ag_2CO_3) left a brown glass (0·257 g.) (Found : OMe, 49·8. Calc. for $C_{10}H_{18}O_7$: OMe, 49·6%).

Treatment of this product with methanolic ammonia for 36 hr. at 0° gave methyl 2 : 3 : 4-tri-*O*-methylglucopyranosiduronamide (12 mg.), m. p. 183°.

A further sample of the main material (0·50 g.) recovered from the methanolic ammonia solution appeared to be an oligosaccharide : it was boiled for 5 hr. with 2N-sulphuric acid. The hydrolysate was neutralised with barium carbonate, and the filtered solution evaporated to dryness. Exhaustive extraction of the barium salt with chloroform gave a syrup (0·11 g.) which was identified chromatographically and by the method of Smith (*loc. cit.*) as 2 : 3-di-*O*-methyl-D-glucopyranose. The barium salts were oxidised as described by Smith (*J.*, 1940, 1035) and Lawson and Stacey (*J.*, 1954, 1925). From the products there was separated 2 : 3 : 4-tri-*O*-methylglucarolactone (0·13 g.), m. p. 107°. The syrupy residue was converted into the amide; 2 : 3-di-*O*-methyl-D-glucarodiamide (0·20 g.), m. p. 156°, crystallised.

The various experiments were repeated in order to get a quantitative assessment of the various substances present. It was estimated that 5·6 g. of methylated polysaccharide contained 2 : 4-di-*O*-methyl-L-rhamnopyranose (3·0 g.), 2 : 3-di-*O*-methyl-D-glucopyranose (0·90 g.), 2 : 3-di-*O*-methyl-D-glucuronic acid (1·95 g.), and 2 : 3 : 4-tri-*O*-methyl-D-glucuronic acid (1·05 g.). It is to be noted that these figures indicate more glucuronic acid than was estimated in early analyses, and much less glucose than the 30% indicated by the rhamnose and glucuronic acid estimations. This assessment will need to be repeated with more refined techniques.

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