The Type-Specific Substance from *Pneumococcus* Type 33B

By M. J. WATSON*

Microbial Chemistry Research Laboratory, Department of Organic Chemistry,
University of Newcastle upon Tyne, Newcastle upon Tyne NE1 7RU, U.K.

(Received 3 December 1973)

1. The type-specific substance, S. 33B, from *Pneumococcus* type 33B contains P, 2.89; hexose, 51; total sugar, 69; galactosamine, 18; and D-glucose, 20%. 2. After degradation with alkali, followed by enzymic dephosphorylation, S. 33B yielded a hexasaccharide. 3. The hexasaccharide was assigned the structure *O*- β -D-glucopyranosyl-(1 $\rightarrow$ 5)-*O*- β -D-galactofuranosyl-(1 $\rightarrow$ 3)-*O*-2-acetamido-2-deoxy- β -D-galactopyranosyl-(1 $\rightarrow$ 4)-*O*-[α -D-galactopyranosyl-(1 $\rightarrow$ 2)]- α -D-galactopyranosyl-(1 $\rightarrow$ 2)-ribitol. 4. Phosphate residues in S. 33B are located on the hydroxyl groups at position 5 of ribitol units and on the hydroxyl groups at position 6 of hexopyranose residues.

Several capsular materials of the *Pneumococcus* have been shown to contain ribitol phosphate (Shabarova *et al.*, 1962). The chemistry of one of these type-specific substances, S. 33B, from *Pneumococcus* type 33B is examined in more detail in the present paper.

Type 33B *Pneumococcus* is known as such in the Danish system of nomenclature (Kauffmann *et al.*, 1940), but in the American system (Eddy, 1944) it is designated type 42. In a preliminary study of the capsular material (Brown & Robinson, 1943) the organism is referred to as type 30. In this paper, the Danish system has been used. The problem of nomenclature is discussed by Kauffman *et al.* (1960).

In their investigations Brown & Robinson (1943) reported the following analytical data for S. 30 preparation no. 1: P, 2.91; N, 2.22; total acetyl, 11.43; reducing sugars, 47.14%; [α]_D +77.6°; and ash, 8.84%.

The identity of the S. 33B preparation was confirmed by the immunological reaction with S. 33B antiserum. The presence of pneumococcal C-substance, a common contaminant of capsular materials, was shown by the technique of double-diffusion with S. 33B and anti-C serum to constitute approx. 5% of the preparation. When S. 33B was analysed for phosphate, hexose, galactosamine, total sugar and D-glucose the following values were obtained: P, 2.89; hexose, 51; total sugar, 69; galactosamine, 18; D-glucose, 20%. The analyses were carried out in a manner similar to those for the capsular materials S. 10A (Rao *et al.*, 1966a) and S. 34 (Roberts *et al.*, 1963). *O*-Acetyl groups were shown to be present by the method of Frèrejacque (1955). Acid hydrolysis of S. 33B, in agreement with earlier work (Shabarova

et al., 1962), yielded glucose, galactose, galactosamine, anhydriitol, ribitol phosphates and P_i. Only a trace of ribitol was present. These products were detected by paper chromatography with the periodate-Schiff reagents for α -glycols (Baddiley *et al.*, 1956), the acid molybdate spray for phosphoric esters (Hanes & Isherwood, 1949), the alkaline AgNO₃ reagents for sugars and polyols (Trevelyan *et al.*, 1950), the aniline phthalate reagent for reducing sugars (Partridge, 1949) and the ninhydrin reagent for hexosamines (Consden & Gordon, 1948).

The degradation of S. 33B with alkali was carried out as described for S. 29 (Rao *et al.*, 1969). Serological activity was lost and two major groups of products containing phosphorus of R_{ribitol} values 0.13 and 0.26 respectively in solvent system A [propan-1-ol-aq. NH₃ (sp.gr. 0.88)-water (6:3:1, by vol.)] (Hanes & Isherwood, 1949) and a minor phosphorus-free product were detected by paper chromatography. When the mixture was treated with alkaline phosphomonoesterase two non-reducing compounds were liberated, which were eluted from paper chromatograms and separated by means of Dowex 50 (H⁺ form) resin into neutral and basic residues. The total yield was 25–30% of the intact polymer. Both compounds were distinguishable from each other by paper chromatography, were homogeneous and gave purple colours rapidly with the periodate-Schiff reagents showing the presence of CHOH-CH₂OH end groups (Hardy & Buchanan, 1963). The neutral oligosaccharide had R_{ribitol} 0.41 and the basic oligosaccharide R_{ribitol} 0.36 in solvent A. The neutral residue also had R_{ribitol} 0.26 in ethyl acetate-pyridine-acetic acid-water (5:5:1:3, by vol.) (Fischer & Nebel, 1955). On hydrolysis with acid both oligosaccharides gave glucose, galactose, galactosamine, ribitol and anhydriitol. Also

* Present address: Department of Clinical Biochemistry, The Royal Victoria Infirmary, Newcastle upon Tyne NE1 4LP, U.K.

the basic compound liberated a ninhydrin-positive reducing disaccharide, which is consistent with the relative stability towards acid of glycosides of 2-amino-2-deoxy sugars compared with their *N*-acetyl derivatives (Foster *et al.*, 1957; Watson *et al.*, 1972). The i.r. spectrum of the neutral oligosaccharide showed characteristic bands at 1640 and 1555 cm^{-1} (NH-CO-CH₃ group) and had an optical rotation $[\alpha]_D^{25} + 51.3^\circ$. The galactose/glucose ratio in the compound was determined by the method of Wilson (1959) and found to be 2.9:1. Like S. 10A, S. 29 and S. 13 (Watson *et al.*, 1972), the neutral oligosaccharide is the *N*-acetyl derivative of the basic oligosaccharide. Further, the qualitative and quantitative analysis of the neutral compound and S. 33B lead to a hexasaccharide structure for the neutral oligosaccharide containing three galactose residues, one of glucose, one of *N*-acetylgalactosamine and one of ribitol.

Although the usual procedure applied to ribitol phosphate polymers of the pneumococci of alkaline hydrolysis followed by phosphate treatment (Roberts *et al.*, 1963; Rao *et al.*, 1966a, 1969; Watson *et al.*, 1972) yields phosphorus-free oligosaccharide repeating units, in the present instance the yield of phosphorus-free products was low and organic phosphates remained. A quantitative determination showed that after alkaline hydrolysis 44–49% of the total phosphorus was released as P_i on treatment with phosphomonoesterase. It appears that a proportion of phosphodiester linkages in S. 33B are resistant to alkali hydrolysis, most probably because of the inaccessibility of neighbouring hydroxyl groups, which participate in cyclic phosphate formation and subsequent cleavage (Brown, 1963).

Methylation of the neutral hexasaccharide (14 mg) with methyl iodide and silver oxide in dimethylformamide (Kuhn *et al.*, 1955), followed by methanolysis, gave 1,2,3,5-tetra-*O*-methylribitol and the methyl glycosides of 2,3,4,6-tetra-*O*-methylgalactose, 2,3,4,6-tetra-*O*-methylglucose and 2,3,6-tri-*O*-methylgalactose in approximately equal amounts. The methyl ethers were detected by g.l.c. as described by Rao *et al.* (1969). No methylated galactosamine derivative was detected under the conditions used for g.l.c.

Partial acid hydrolysis of the neutral hexasaccharide (12 mg) in 66% (v/v) formic acid (Roberts *et al.*, 1963) yielded *N*-acetylgalactosamine, identified by the Morgan–Elson reagent for *N*-acetylhexosamines (Partridge, 1948), a trace amount of ribitol and of galactose, disaccharides D₁ (3 mg) and D₂, trisaccharides T₁ (2 mg) and T₂ (2 mg) and an oligosaccharide mixture from which tetrasaccharide O₁ was isolated. These were separated by preparative paper chromatography in solvent system B [butan-1-ol–pyridine–water (6:4:3, by vol.)] (Jeanes *et al.*, 1951). Disaccharide D₁ gave a strong reaction with aniline phthalate and alkaline AgNO₃. With the

periodate–Schiff reagents it gave a blue–purple colour and had R_{ribitol} 0.66 in solvent B. Acid hydrolysis of disaccharide D₁ yielded glucose and galactose in equal amounts as judged by spot intensities on paper chromatograms with the aniline phthalate spray reagent. When reduced with borohydride and hydrolysed with acid, glucose and a hexitol (presumably galactitol) were detected, showing that the disaccharide is a glucosyl-galactose. Methylation studies on the hexasaccharide, which show that glucose occurs as a non-reducing end group, confirms the order of the units in disaccharide D₁. The very high mobility of disaccharide D₁, together with the methylation evidence for the possibility of a (1→5)-linked galactofuranose unit in the hexasaccharide, strongly suggests that disaccharide D₁ is 5-*O*-glucosyl-galactose. Treatment of disaccharide D₁ with almond emulsin β -glucopyranosidase liberated glucose, whereas yeast α -glucopyranosidase did not hydrolyse the disaccharide. The linkage in disaccharide D₁ has therefore a β configuration. Trisaccharide T₁ had R_{ribitol} 0.51 in solvent B and yielded glucose, galactose and galactosamine on acid hydrolysis. It reacted with alkaline AgNO₃ and gave a blue–purple colour with the periodate–Schiff reagents. With the Morgan–Elson reagent trisaccharide T₁ gave a very strong purple colour indicating a (1→3)- or (1→6)-linked *N*-acetylgalactosamine as a reducing end group. Periodate-oxidation studies, discussed below, show that the *N*-acetylgalactosamine unit in the hexasaccharide is (1→3)- or (1→4)-linked. Since trisaccharide T₁ gave a positive reaction with alkali followed by Ehrlich's reagent (Morgan & Elson, 1934), the linkage is (1→3), for (1→4)-linked *N*-acetylhexosamines fail to react with this reagent (Kuhn *et al.*, 1954). With benzidine–trichloroacetic acid reagent (Bacon & Edelman, 1951), trisaccharide T₁ gave a strong brown colour similar to the one reported for *O*- β -D-galactopyranosyl-(1→3)-*N*-acetyl-D-glucosamine (Côté & Morgan, 1956). Different colour reactions are given by (1→4)- and (1→6)-linked *N*-acetyl amino sugars; the former appear to give a golden-yellow colour (Côté & Morgan, 1956) and the latter a brownish red (Watkins, 1958). The behaviour of trisaccharide T₁ to benzidine–trichloroacetic acid reagent lends support to the previous evidence that the *N*-acetyl galactosamine is (1→3)-linked. Disaccharide D₂ present in low proportion had R_{ribitol} 0.38 in solvent B. It gave a weak brown colour with aniline phthalate, a moderately weak reaction with alkaline AgNO₃ and a strong yellow colour with the periodate–Schiff reagents, showing the presence of a (1→2)-linkage (Hardy & Buchanan, 1963). Acid hydrolysis of disaccharide D₂ yielded galactose. When disaccharide D₂ was treated with almond emulsin (containing α - and β -galactopyranosidase) galactose was released. A similar treatment

in the presence of silver oxide, which inhibits the action of the α -galactosidase (Roberts *et al.*, 1963), failed to hydrolyse disaccharide D₂. Disaccharide D₂ is evidently *O*- α -galactopyranosyl-(1 $\rightarrow$ 2)-galactose. Trisaccharide T₂ had R_{ribitol} 0.29 in solvent B, yielded galactose and ribitol on acid hydrolysis, gave a purple colour rapidly with the periodate-Schiff reagents and was non-reducing.

When the hexasaccharide (4mg) was oxidized with periodate, the aldehyde product reduced with borohydride and subsequently hydrolysed in acid (Smith degradation) (Goldstein *et al.*, 1965) the products, detected by paper chromatography with appropriate spray reagents, were galactose, galactosamine, threitol and glycerol.

The methylation studies, partial hydrolysis with formic acid and the results of periodate oxidation lead to structures (I) and (II) for the neutral and basic hexasaccharides respectively. The formation of the hydrolysis products can now be explained. Disaccharide D₁ arises from units A and B of compound (I) by cleavage of the acid-labile furanosyl linkage. Disaccharide D₂ arises from units F and D, the same fragment being present in trisaccharide T₂, which comprises units F, D and ribitol unit E. Trisaccharide T₂ is formed by the ready hydrolysis of the *N*-acetylgalactosaminyl linkage. The other product of hydrolysis of this linkage is trisaccharide T₁ from units A, B and C. As the furanosyl and *N*-acetylgalactosaminyl linkages are of comparable stability towards formic acid hydrolysis it was possible to isolate tetrasaccharide O₁ comprising units C, D, E and F. Tetrasaccharide O₁ had R_{ribitol} 0.16 in solvent

B, gave a purple colour rapidly with the periodate-Schiff reagents and was non-reducing. On acid hydrolysis tetrasaccharide O₁ yielded galactosamine, galactose and ribitol in the approximate proportions 1:2:1 as estimated by intensity of spots on paper chromatograms. The Smith-degradation products are also accommodated in structure (I). Glycerol arises from units A and F respectively and from ribitol, threitol from galactofuranose unit B, and galactosamine from the *N*-acetylgalactosamine unit C. Galactose arises from the third galactose unit D. This unit must therefore be 2,3- or 2,4-disubstituted. Evidence in favour of 2,4-disubstitution comes from the chromatographic behaviour of the ninhydrin-positive glycoside arising from acid hydrolysis of the basic hexasaccharide (II). The glycoside appears to be a galactosaminyl-galactose and its chromatographic mobility in solvent B ($R_{\text{galactose}}$ 0.19) suggests that it is (1 $\rightarrow$ 4)-linked. The (1 $\rightarrow$ 6)-linked disaccharide from S. 29 had $R_{\text{galactose}}$ 0.14 and the (1 $\rightarrow$ 3)-linked disaccharide from S. 10A had $R_{\text{galactose}}$ 0.31. These disaccharides, as expected, run slower and faster respectively than the (1 $\rightarrow$ 4)-linked units. The linkages in structures (I) and (II) are assigned the configurations shown on account of the optical rotation of the neutral hexasaccharide $[\alpha]_D^{18} + 51.3^\circ$ and on the action of almond emulsin on disaccharides D₁ and D₂. The ribitol unit is linked at C-2 in the *D*-configuration for biosynthetic reasons discussed below.

Although S. 33B does not appear to comprise a simple repeating unit linked through phosphodiester bridges (Roberts *et al.*, 1963; Watson *et al.*, 1972)

it is possible to locate the position of phosphate groups in S. 33B by periodate-oxidation studies. Further, other aspects of the chemistry of the intact polymer are clarified by these investigations.

In an oxidation of S. 33B (10.1 mg) a quantitative determination was made of the sugars remaining after Smith degradation. The values were: hexose, 24; total sugar, 41; galactosamine, 16; D-glucose, 11%. Threitol, glycerol, ethylene glycol, glycerol phosphates and ethylene glycol phosphate were also among the products after this sequence. This result shows that approximately half of the glucose and of the galactose in S. 33B is (1→3)-linked and/or involved as unoxidizable branch points and that N-acetyl-galactosamine is (1→3)- and possibly, in part, (1→4)-linked. No anhydrosorbitol or sorbitol phosphates were detected after oxidation, showing that sorbitol in S. 33B is destroyed by periodate. Threitol was confirmed by paper electrophoresis in sodium arsenite at pH 9.6 (Frahn & Mills, 1959). Ethylene glycol phosphate will arise from D-sorbitol units substituted at C-5 with a phosphate group, and oxidizable between C-3 and C-4. This result is in agreement with the view that sorbitol phosphate residues originate from CDP-sorbitol (Rao *et al.*, 1966b; Baddiley, 1970). Glycerol phosphate will arise from hexopyranose residues bearing a phosphate group on the hydroxyl at C-6 and which are oxidizable between C-3 and C-4. Smith degradation of the alkali-hydrolysis products of S. 33B yielded sorbitol phosphates and anhydrosorbitol, which is consistent with 2,5-disubstituted D-sorbitol in the polymer (Rao *et al.*, 1966b).

S. 33B is the fifth capsular polysaccharide containing sorbitol phosphate and galactofuranose to be reported. There now appears to be a class of pneumococcal capsular materials containing not only sorbitol phosphate, but also galactofuranose residues. This group comprises S. 10A (Rao *et al.*, 1966a,b), S. 13 (Watson *et al.*, 1972), S. 29 (Rao *et al.*, 1969), S. 33B and S. 34 (Roberts *et al.*, 1963; Dixon *et al.*, 1966; Chittenden *et al.*, 1968; Roy & Glaudemans, 1968), of which S. 34 is the only substance not containing an amino sugar.

I thank Dr. Rachel Brown for gifts of S. 33B, Dr. Erna Lund, of the Statens Seruminstitut, Copenhagen, for providing S. 33B and S. 13 antisera, Dr. D. E. Brundish for C-substance determinations, and Professor J. Baddiley and Professor J. G. Buchanan for their guidance and advice during the investigations. The work was supported by a grant from the Science Research Council and was carried out during a tenure of a Science Research Council Studentship.

- Bacon, J. S. D. & Edelman, J. (1951) *Biochem. J.* **48**, 114–126
- Baddiley, J. (1970) *Accounts Chem. Res.* **3**, 98–105
- Baddiley, J., Buchanan, J. G., Handschumacher, R. E. & Prescott, J. F. (1956) *J. Chem. Soc. London* 2818–2823
- Brown, D. M. (1963) *Advan. Org. Chem.* **3**, 75–157
- Brown, R. & Robinson, L. K. (1943) *J. Immunol.* **47**, 7–13
- Chittenden, G. J. F., Roberts, W. K., Buchanan, J. G. & Baddiley, J. (1968) *Biochem. J.* **109**, 597–602
- Consden, R. & Gordon, A. H. (1948) *Nature (London)* **162**, 180–181
- Côté, R. H. & Morgan, W. T. J. (1956) *Nature (London)* **178**, 1171–1172
- Dixon, J. R., Buchanan, J. G. & Baddiley, J. (1966) *Biochem. J.* **100**, 507–511
- Eddy, B. E. (1944) *U.S. Pub. Health Rep.* **59**, 449–451
- Fischer, F. G. & Nebel, H. J. (1955) *Hoppe-Seyler's Z. Physiol. Chem.* **302**, 10–19
- Foster, A. B., Horton, D. & Stacey, M. (1957) *J. Chem. Soc. London* 81–86
- Frahn, J. L. & Mills, J. A. (1959) *Aust. J. Chem.* **12**, 65–89
- Frèrejacque, M. (1955) *C. R. Acad. Sci.* **240**, 1804–1805
- Goldstein, I. J., Hay, G. W., Lewis, B. A. & Smith, F. (1965) *Methods Carbohydr. Chem.* **5**, 361–370
- Hanes, C. S. & Isherwood, F. A. (1949) *Nature (London)* **164**, 1107–1112
- Hardy, F. E. & Buchanan, J. G. (1963) *J. Chem. Soc. London* 5881–5885
- Jeanes, A., Wise, C. S. & Dimler, R. J. (1951) *Anal. Chem.* **23**, 415–420
- Kauffman, F., Mørch, E. & Schmith, K. (1940) *J. Immunol.* **39**, 397–426
- Kauffmann, F., Lund, E. & Eddy, B. E. (1960) *Int. Bull. Bacteriol. Nomencl. Taxon.* **10**, 31–40
- Kuhn, R., Gauhe, A. & Baer, H. H. (1954) *Chem. Ber.* **87**, 1138–1141
- Kuhn, R., Trischmann, H. & Löw, I. (1955) *Angew. Chem.* **67**, 32
- Morgan, W. T. J. & Elson, L. A. (1934) *Biochem. J.* **28**, 988–995
- Partridge, S. M. (1948) *Biochem. J.* **42**, 238–250
- Partridge, S. M. (1949) *Nature (London)* **164**, 443
- Rao, E. V., Buchanan, J. G. & Baddiley, J. (1966a) *Biochem. J.* **100**, 801–810
- Rao, E. V., Buchanan, J. G. & Baddiley, J. (1966b) *Biochem. J.* **100**, 811–814
- Rao, E. V., Watson, M. J., Buchanan, J. G. & Baddiley, J. (1969) *Biochem. J.* **111**, 547–556
- Roberts, W. K., Buchanan, J. G. & Baddiley, J. (1963) *Biochem. J.* **88**, 1–7
- Roy, N. & Glaudemans, C. P. J. (1968) *Carbohydr. Res.* **8**, 214–218
- Shabarova, Z. A., Buchanan, J. G. & Baddiley, J. (1962) *Biochim. Biophys. Acta* **57**, 146–148
- Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950) *Nature (London)* **166**, 444–445
- Watkins, W. M. (1958) *Nature (London)* **181**, 117–118
- Watson, M. J., Tyler, J. M., Buchanan, J. G. & Baddiley, J. (1972) *Biochem. J.* **130**, 45–54
- Wilson, C. M. (1959) *Anal. Chem.* **31**, 1199–1201