The Specific Substance from *Pneumococcus* Type 34

THE CONFIGURATION OF THE GLYCOSIDIC LINKAGES

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1. The specific compound from *Pneumococcus* type 34 was isolated from capsular material by ion-exchange chromatography. This separated it from a substance with chemical and serological properties corresponding to those reported for C-substance. 2. The configuration of the two galactofuranosyl linkages in the repeating unit of S.34 was determined and the configurations previously assigned to the other glycosidic linkages were confirmed. 3. The dephosphorylated deacetylated repeating unit is thus *O*- β -D-galactofuranosyl-(1 $\rightarrow$ 3)-*O*- α -D-glucopyranosyl-(1 $\rightarrow$ 2)-*O*- β -D-galactofuranosyl-(1 $\rightarrow$ 3)-*O*- α -D-galactopyranosyl-(1 $\rightarrow$ 2)-ribitol.

It has been shown that a number of type-specific substances from pneumococci contain ribitol phosphate residues and thus possess a formal structural resemblance to the teichoic acids (Shabarova, Buchanan & Baddiley, 1962); detailed structural features of several of these substances have been described (Rebers & Heidelberger, 1959, 1961, 1963; Roberts, Buchanan & Baddiley, 1963; Rao, Roberts, Buchanan & Baddiley, 1964; Rao, Buchanan & Baddiley, 1966*a,b*). The structure of a dephosphorylated repeating unit in S.34 was believed to be *O*-D-galactofuranosyl-(1 $\rightarrow$ 3)-*O*- α -D-glucopyranosyl-(1 $\rightarrow$ 2)-*O*-D-galactofuranosyl-(1 $\rightarrow$ 3)-*O*- α -D-galactopyranosyl-(1 $\rightarrow$ 2)-ribitol (Roberts *et al.* 1963), in which the configuration of the two galactofuranosyl linkages had not been established. Although this unit was obtained in a pure state by hydrolysis of the polymer with alkali and removal of resulting phosphomonoester groups with a phosphatase, additional evidence was required to provide a complete structure for S.34 itself. This was necessary since, whereas the purified unit contained neither nitrogen nor acetyl groups, both of these had been observed in the capsular material from this *Pneumococcus* (Brown & Robinson, 1943). Thus the capsular material contained 1.71% of nitrogen, 11.4% of acetyl and had a phosphorus/hexose ratio 1:2.8. On the other hand, a polymer built from the unit described by Roberts *et al.* (1963) in which adjacent units are joined through phosphodiester linkages would contain neither nitrogen nor acetyl and would have a phosphorus/hexose ratio 1:4. It seemed likely that the capsular material contained an additional sub-

stance that would account for all of the nitrogen, 25% of the phosphorus and possibly some of the acetyl. To establish further details of the structure of S.34 it was necessary to prepare pure material containing none of the suspected nitrogenous contaminant.

This paper describes an ion-exchange chromatographic method for the purification of S.34 and a stepwise degradative procedure for determining the configuration of its glycosidic linkages.

EXPERIMENTAL

Material. The capsular material from *Pneumococcus* type 34 was supplied by Dr Rachel Brown. It is described by Brown & Robinson (1943) as the soluble specific substance of type 34 (Buckley strain), preparation no. 1. Intestinal phosphomonoesterase was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A.

Analytical methods. Phosphorus was determined by the method of Chen, Toribara & Warner (1956), *O*-acetyl by the method of Hestrin (1949) and hexoses by the phenol-H₂SO₄ procedure (Dubois, Gilles, Hamilton, Rebers & Smith, 1956). Periodate was measured spectrophotometrically (Dixon & Lipkin, 1954; Aspinall & Ferrier, 1957) and formaldehyde by a modification of the chromotropic acid method (Roberts *et al.* 1963). Amino compounds were analysed with the Technicon Autoanalyzer. Optical rotations were measured with an ETP-NPL automatic polarimeter, type 143A, with cells of path length 1 cm.

Paper chromatography. This was carried out on Whatman no. 1 or no. 4 paper. The following solvent systems were used: *A*, propan-1-ol-aq. NH₃ (sp.gr. 0.88)-water (6:3:1, by vol.) (Hanes & Isherwood, 1949); *B*, butan-1-ol-pyridine-water (6:4:3, by vol.) (Jeanes, Wise & Dimler, 1951). The products were detected by the periodate-Schiff reagents for α -glycols (Baddiley, Buchanan,

Handschemacher & Prescott, 1956), molybdate reagents for phosphoric esters (Hanes & Isherwood, 1949) and alkaline AgNO_3 for sugars and polyols (Trevelyan, Procter & Harrison, 1950).

Serological examination. The activity of the type-specific substance was determined by the method of Ouchterlony (1948) by Dr G. T. Mills, and the species-specific substance was examined by Dr Jean M. Tyler by the same method.

Chromatography of S.34 on DEAE-cellulose. A column (20 cm. $\times$ 2 cm.) of DEAE-cellulose (HCO_3^- form) was prepared and a solution of capsular material (20 mg.) in water (3 ml.) was applied to the top of the column. Elution was carried out slowly at 4° with a solution (100 ml.) of $(\text{NH}_4)_2\text{CO}_3$ running in a linear gradient from 0 to 0.5 M. Fractions (5 ml.) were collected automatically and the phosphorus and hexose content of each fraction were determined. Fractions 10–13 and 17–35 formed two peaks in the phosphorus elution curve. Appropriate fractions were combined, $(\text{NH}_4)_2\text{CO}_3$ was removed by dialysis and the solutions were freeze-dried. The residue (3 mg.) corresponding to the first peak gave a phosphorus/hexose ratio 1:1 and that (12 mg.) corresponding to the second peak a phosphorus/hexose ratio 1:3.9. Material corresponding to the first peak had C-activity and a little S.34-activity, whereas that corresponding to the second peak reacted with type 34 antiserum but was devoid of C-activity. A typical preparation of material corresponding to the second peak was analysed for phosphorus, hexose, O-acetyl and reduction of periodate with the resultant formation of formaldehyde. The phosphorus:hexose:O-acetyl:periodate:formaldehyde proportions were 1:3.80:0.44:2.50:1.43.

Removal of O-acetyl groups from purified S.34. Material (20 mg.) corresponding to the second peak in the chromatographic procedure was dissolved in water (1.5 ml.), mixed with aq. NH_3 (sp.gr. 0.88) (1.5 ml.) and kept at 37° for 24 hr. The NH_3 was removed by aeration and the solution was freeze-dried. Samples were analysed as before to give the phosphorus:hexose:O-acetyl:periodate:formaldehyde proportions 1:3.81:0.0:3.18:2.15.

Analysis of the minor component from capsular material. A sample (2 mg.) of the minor component with C-activity was hydrolysed with N-HCl at 100° for 3 hr. in a sealed tube. The hydrolysate was evaporated to dryness *in vacuo* and the residue was examined by paper chromatography with solvent A; alanine, serine, glucosamine and phosphate were detected. More vigorous hydrolysis (6N-HCl at 100° for 12 hr.) and examination for amino compounds in the Autoanalyzer revealed glutamic acid, alanine, glucosamine, galactosamine, lysine, serine, muramic acid and two compounds that were probably galactosamine 6-phosphate and a muramic acid phosphate (Liu & Gotschlich, 1963).

Preparation of the tetraglycosylglycerol (II) from S.34. The deacetylated specific substance (20 mg.) that had been purified by the above chromatographic procedure was dissolved in 0.01 M-sodium metaperiodate solution (10 ml.) and the solution was kept at room temperature in the dark for 80 hr. Then NaBH_4 (20 mg.) was added and after 24 hr. at room temperature 2 drops of acetic acid were introduced. The solution was passed through a column of Dowex 50 (H^+ form) resin (20 ml.) and freeze-dried. The residue was hydrolysed in a sealed tube with N-NaOH (1.5 ml.) at 100° for 3 hr. The hydrolysate was passed through a column of Dowex 50 (NH_4^+ form) resin (20 ml.) and evaporated to

dryness *in vacuo*. The residue was dissolved in water (2 ml.) and a few drops of the solution were examined by paper chromatography in solvent A. An organic phosphate with the properties expected for ethylene glycol phosphate was detected. The remainder of the solution was passed through a column of Dowex 2 (HCO_3^- form) resin (20 ml.), the filtrate evaporated to dryness *in vacuo* and a sample of the residue examined by chromatography in solvent A. Only one product, $R_{\text{arabinose}} 0.65$, was detected; it had $[\alpha]_D + 27^\circ$ (c 1.8 in water).

Stepwise degradation of the tetraglycosylglycerol (II). The above product (20 mg.) was dissolved in 0.02 M-sodium metaperiodate solution (10 ml.) and after 80 hr. in the dark at room temperature 0.02 M-ethylene glycol solution (10 ml.) was added. After 2 hr. at room temperature a 1% solution of 1,1-dimethylhydrazine (3 ml.) was added, the pH being adjusted to 6 with acetic acid; a red colour developed during 16 hr. at 37°. The solution was shaken with small portions of chloroform until no more colour was extracted and was then passed through a column of Dowex 50 (NH_4^+ form) resin (10 ml.) and one of Dowex 2 (HCO_3^- form) resin (10 ml.). The filtrate was freeze-dried, leaving a residue with $[\alpha]_D + 46^\circ$ (c 1.3 in water). A sample (3 mg.) of this product was hydrolysed in N-HCl at 100° for 3 hr. and the products were examined by chromatography in solvent B; glucose, galactose, arabinose and glycerol were detected.

The triglycosylglycerol (III) (15 mg.) from the above degradation was subjected to the same sequence of oxidation and reaction with 1,1-dimethylhydrazine as has been described for the tetraglycosylglycerol; the product, which now contained only arabinose, galactose and glycerol, had $[\alpha]_D + 3^\circ$ (c 2.0 in water). The above arabinosylgalactosylglycerol (15 mg.) was subjected to the same degradative sequence as before and the product, galactosylglycerol, had $[\alpha]_D + 157^\circ$ (c 1.3 in water); similar degradation of this gave only glycerol, identified chromatographically with solvent B.

Hexa-O-acetyl-O- α -D-galactopyranosylglycerol. A sample (8 mg.) of 2-O- α -D-galactopyranosylglycerol from the degradation was mixed with anhydrous sodium acetate (6 mg.) and acetic anhydride (1 ml.). The solution was heated under reflux for 3 hr., cooled, poured into water (5 ml.) and the resulting crystalline precipitate filtered off. The needles had m.p. 99° and this was not depressed when a sample was mixed with authentic material, m.p. 101° (Putman & Hassid, 1954; Austin, Hardy, Buchanan & Baddiley, 1965). The infrared spectra of the two samples in KBr disks were identical.

DISCUSSION

In view of the homogeneity of the 'reduced pentasaccharide' obtained by the action of alkali and phosphatase on S.34 (Roberts *et al.* 1963) it seemed likely that the natural polymer itself was composed of these units joined together through phosphodiester linkages with probably an unspecified number of O-acetyl residues attached to hydroxyl groups. Nevertheless, it was necessary to remove a suspected impurity containing nitrogen, phosphorus, hexose and probably acetyl residues before reliable experiments could be carried out on the position of the phosphate groups and on the

configuration of the glycosidic linkages. Unsuccessful attempts to separate S.34 from the nitrogenous polymer included fractional precipitation with organic solvents, precipitation with Cetavlon and gel diffusion with Sephadex G-75 and G-200 (carried out in association with Dr W. K. Roberts). A successful separation of S.34 from a nitrogenous fraction was achieved on a column of DEAE-cellulose by using gradient elution with ammonium carbonate solution at 4°. The phosphorus/hexose ratio in the main component was in good agreement with a structure in which four hexose residues and ribitol are attached through a phosphate as a repeating unit in the polymer; analysis also indicated that about 0.5 *O*-acetyl residue occurs in the repeating unit; the preparation showed a good specific reaction with type 34 antiserum and no C-activity was observed.

The nature of the minor fraction from the capsular material has not been investigated in detail. It contained amino acids, glucosamine, galactosamine, phosphate and unidentified components. It gave a serological reaction for C-substance but also had some type 34 activity. The identified components are consistent with those observed in preparations of C-substance (Liu & Gotschlich, 1963).

The pentasaccharide isolated by Roberts *et al.* (1963) from S.34, and believed to represent the dephosphorylated deacetylated repeating unit, has the structure (I), in which the stereochemistry of the ribitol was not established and the configuration of the galactofuranosyl residues was not determined. In an attempt to study the configuration of the sugar linkages a degradation was envisaged whereby the changes in specific rotation caused by stepwise removal of sugar residues would be studied. Such a stepwise removal could be achieved by oxidation with periodate and elimination of the aldehydic fragments with phenylhydrazine (Barry, 1943) or preferably with 1,1-dimethylhydrazine (LeCocq & Ballou, 1964). In the oxidation of the pentasaccharide (I) with periodate difficulties would be encountered through unavoidable oxidation of both the ribitol and the galactofuranosyl residue within the oligosaccharide chain. However, Roberts *et al.* (1963) were able to degrade the pentasaccharide (I) to the glycerol derivative (II) by means of a controlled oxidation with periodate followed by reduction with borohydride. Compound (II) would be ideal for the subsequent stepwise degradation, but it was prepared instead from S.34 itself by deacetylation, oxidation with periodate, reduction with borohydride and hydrolysis with alkali. It is noteworthy that in S.34 the phosphodiester linkages joining units occur between the 2- or 3-positions of galactofuranosyl residues and the primary hydroxyl group of ribitol depicted

on the extreme right of formula (I) (Rao *et al.* 1964; J. R. Dixon, W. K. Roberts, J. G. Buchanan & J. Baddiley, unpublished work). Thus, when the deacetylated polymer was oxidized with periodate, formaldehyde was produced from C-6 of both galactofuranose residues (phosphate/formaldehyde ratio 1:2) and the ribitol was oxidized between positions 3 and 2(4), thereby breaking the polymer chain (phosphate/periodate reduced ratio 1:3). The product was reduced with sodium borohydride; by this means both *D*-galactofuranosyl residues are converted into *L*-arabinofuranosyl and, in view of the protecting phosphodiester group, there is no tendency for the terminal furanosyl residue to undergo further oxidation.

The phosphodiester linkage was hydrolysed in alkali. Since the phosphate group now joins an ethylene glycol residue to the 2- or 3-position on the terminal *L*-arabinofuranosyl residue, cyclization of the phosphate group occurred preferentially to give ethylene glycol cyclic phosphate rather than the sterically unfavourable cyclic phosphate involving vicinal *trans*-hydroxyl groups on a furanose ring (Rao *et al.* 1964). Hydrolysis was unidirectional, giving ethylene glycol phosphate and the phosphorus-free arabinosylglucosylarabinosylgalactosylglycerol (II). Only the terminal *L*-arabinofuranosyl residue in compound (II) contains an unsubstituted glycol system; consequently, when this was treated with periodate, and the product degraded with 1,1-dimethylhydrazine, the terminal arabinose was destroyed. The compound (II) had $[\alpha]_D + 27^\circ$ whereas the product, *O*- α -*D*-glucopyranosyl-(1 $\rightarrow$ 2)-*O*- α -*L*-arabinofuranosyl-(1 $\rightarrow$ 3)-*O*- α -*D*-galactopyranosyl-(1 $\rightarrow$ 2)-glycerol (III), had $[\alpha]_D + 46^\circ$. Thus the *L*-arabinofuranosyl residue was contributing in a negative sense to the rotation of (II). Now methyl α -*L*-arabinofuranoside has $[\alpha]_D - 125^\circ$ and so it follows that the terminal arabinofuranosyl residue in the compound (II) must have the α -configuration and hence the terminal *D*-galactofuranosyl residue from which it was derived in compound (I) must have the β -configuration.

The glucosyl residue was removed from the compound (III) by repetition of the oxidation with periodate and treatment with 1,1-dimethylhydrazine. The product, *O*-*L*-arabinofuranosyl-(1 $\rightarrow$ 3)-*O*- α -*D*-galactopyranosyl-(1 $\rightarrow$ 2)-glycerol (IV), had $[\alpha]_D + 3^\circ$. Thus the glucopyranosyl residue contributes a large positive rotation, an observation that confirms the previous evidence (Roberts *et al.* 1963) that the glucosyl linkage has an α -configuration. Repetition of the procedure removed the second *L*-arabinofuranosyl residue and gave *O*- α -*D*-galactopyranosyl-(1 $\rightarrow$ 2)-glycerol (V) with $[\alpha]_D + 157^\circ$, the hexa-acetate of which was identical with an authentic sample of synthetic material; natural

floridoside has $[\alpha]_D + 165^\circ$ (Putman & Hassid, 1954). The removal of the L-arabinofuranosyl residue has thus produced a large positive increase in the specific rotation and it is concluded that this glycosidic linkage must have had an α -configuration; it follows that the second galactofuranosyl residue in the unit (I) must have a β -configuration. The identification of floridoside as a degradation product confirms the α -configuration of the linkage between galactose and ribitol in the original unit (I). Periodate followed by 1,1-dimethylhydrazine on the galactosylglycerol (IV) gave glycerol, and no sugar derivatives had survived this final oxidation.

The analytical values for O-acetyl content and periodate oxidation show that the O-acetyl groups are so located that oxidation with periodate and production of formaldehyde are prevented to the same extent. The most probable location of these groups is on C-6 (or C-5) of some of the galactofuranosyl residues.

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