The Type-Specific Substance from *Pneumococcus* Type 10A(34)

THE PHOSPHODIESTER LINKAGES

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1. The phosphate groups in the type-specific substance S. 10A from *Pneumococcus* type 10A (34) were shown to join the hydroxyl group at position 1 or 5 of ribitol and the hydroxyl group at position 5 or 6 of a D-galactofuranosyl residue in the next repeating unit. 2. A partial formula of the type-specific substance was derived.

In the preceding paper (Rao, Buchanan & Baddiley, 1966) the structure of the dephosphorylated repeating unit of the type-specific substance S.10A from *Pneumococcus* type 10A (34) was derived. The degradation in alkali that was used to obtain the repeating unit had caused the hydrolysis of phosphodiester linkages in S.10A itself. We now describe experiments that determine the position of these linkages and lead to a structure of the type-specific substance.

EXPERIMENTAL

Materials and methods. These are described in the preceding paper (Rao *et al.* 1966).

Hydrolysis with acid. S.10A (4mg.) was heated with 2N-HCl (0.4ml.) in a sealed tube at 100° for 3hr. The acid was removed by evaporation *in vacuo* over solid KOH and the residue subjected to chromatography as a band on Whatman no. 4 paper in solvent A; the products were detected with the molybdate reagent for phosphates and with aniline phthalate. Product 1 ($R_{\text{galactose}} 0.33$) contained phosphate and gave a brown colour with aniline phthalate. Product 2 ($R_{\text{galactose}} 0.41$) gave a blue colour with the molybdate reagent but did not react with aniline phthalate. Products 1 and 2 were separately eluted from the paper with water and the solutions were treated with alkaline phosphatase (1mg.) and $(\text{NH}_4)_2\text{CO}_3$ (3mg.). After a drop of toluene had been added the mixtures were kept at 37° for 16hr. The enzymic hydrolysates were passed through small columns of Dowex 2 (CO_3^{2-} form) resin, the eluates evaporated to dryness *in vacuo* and the residues chromatographed in solvent B. Product 1 yielded galactose whereas product 2 gave ribitol.

Oxidation of S.10A with periodate. (1) Quantitative determination. A solution (3.0ml.) of S.10A containing 4.07mg. of the substance (3.27 $\mu\text{g.}$ atoms of phosphorus) was mixed with 0.02M-sodium metaperiodate (3.0ml., 60 μmoles) and reduction of periodate was determined spectrophotometrically. After 1hr. 3.2mol.prop. and after 120hr. 9.1mol.prop. of periodate were reduced for each phosphorus. No further reduction of periodate was observed. A molecule of structure (X) should reduce

2mol.prop. of periodate very rapidly (corresponding to the ribitol and exocyclic glycol of the galactofuranose) followed by 2mol.prop. more slowly (internal glycol systems of both galactofuranosyl units) and 4mol.prop. on slow overoxidation of the galactofuranose unit not bearing the phosphate group (8mol.prop. in all).

(2) Formaldehyde production. S.10A (10.2mg.) was dissolved in water (10ml.) and phosphorus determined on 0.1ml. and 0.2ml. samples of the solution. Formaldehyde was determined on 0.1ml. and 0.2ml. samples after oxidation with periodate (Roberts, Buchanan & Baddiley, 1963). 0.9mol.prop. of formaldehyde was produced for each phosphorus.

S.10A (6.5mg.) was treated with N-NaOH (0.8ml.) at 100° for 3hr. The hydrolysate was passed through a column of Dowex 50 (NH_4^+ form) resin (3ml.) and the eluate was evaporated to dryness *in vacuo*. The residue was dissolved in water (10ml.) and samples were analysed for phosphorus and for formaldehyde after oxidation with periodate: 1.9mol.prop. of formaldehyde was produced for each phosphorus.

Degradation of S.10A with periodate. (1) The substance (7.8mg.) was dissolved in water (0.5ml.) and the solution cooled to 2°. To this solution was added 0.1M-sodium metaperiodate (0.3ml.) and the mixture was kept at 2° for 30min. Ethylene glycol was added to destroy excess of periodate and the products were reduced with NaBH_4 (10mg.). The solution was passed through a column of Dowex 50 (H^+ form) resin and the eluate freeze-dried. Borate was removed from the residue by evaporation several times with hot methanol. The residue was hydrolysed with 2N-HCl (0.8ml.) at 100° for 3hr. The hydrolysate was passed through Dowex 2 (CO_3^{2-} form) resin and the eluate was evaporated to dryness under reduced pressure. The residue was chromatographed as a band in solvent B; galactose and arabinose were eluted and determined (Found: galactose/arabinose ratio, 2.2:1).

(2) S.10A (6.4mg.) was dissolved in water (0.5ml.) and mixed with 0.1M-sodium metaperiodate (0.5ml.). The mixture was kept at 2° for 40hr. Excess of periodate was destroyed by the addition of ethylene glycol, NaBH_4 (15mg.) was added and the mixture was kept overnight. After the addition of a drop of acetic acid the solution was passed through a column of Dowex 50 (H^+ form) resin, the

column washed with water and the eluate freeze-dried. Borate was removed from the residue by evaporation several times with hot methanol. The residue was hydrolysed with 2*N*-HCl (0.6 ml.) at 100° for 3 hr. and then diluted to 10 ml. with water. Phosphorus and galactose were determined on samples (Found: P/galactose ratio, 1:2.07).

The hydrolysate was evaporated *in vacuo* and the residue examined by paper chromatography in solvent *B*, aniline phthalate being used for detection. Galactose but no arabinose or galactose phosphate was detected.

(3) S.10A (10.5 mg.) was treated with 0.1*M*-sodium metaperiodate (3 ml.) and kept at room temperature in the dark for 5 days. Then NaBH₄ (50 mg.) was added and the solution was kept at room temperature overnight. The products were treated in the usual manner and hydrolysed with 2*N*-HCl at 100° for 3 hr. The hydrolysate was neutralized to pH 6 and its volume adjusted to 10 ml. with water. Galactose and phosphorus were determined on samples of the solution (Found: P/galactose ratio, 1:1.86). A control experiment, carried through all the treatments, showed that products derived from periodate had no effect on the determination.

(4) S.10A (10 mg.) was treated with 0.1*M*-sodium metaperiodate (3 ml., about 20 mol.prop.) for 72 hr. at room temperature in the dark. Then NaBH₄ (40 mg.) was added and the mixture was kept for 16 hr. at room temperature. Acetic acid (a few drops) was added and the solution was passed through a column of Dowex 50 (H⁺ form) resin (5 ml.) and the eluate (50 ml.) was evaporated to dryness under reduced pressure. Boric acid was removed by evaporation several times with methanol. The residue was hydrolysed with 2*N*-HCl (1 ml.) at 100° for 3 hr. and the acid removed by evaporation *in vacuo*. A portion of the residue was chromatographed in solvents *A* and *B*. The products, identified by comparison with authentic substances, were galactose, galactosamine, threitol, glycerol and ethylene glycol. Two organic phosphates (R_{ribitol} 0.50 and 0.57 in solvent *A*) were detected by the molybdate spray. They each gave a purple colour with periodate-Schiff reagents and were indistinguishable from the products of acid treatment of authentic threitol phosphate (see below). When each was eluted from a chromatogram irrigated with solvent *A* and dephosphorylated with alkaline phosphatase and the products were chromatographed in solvents *B* and *E*, threitol was the product in both cases.

Hydrolysis of D-threitol phosphate with acid. The cyclohexylammonium salt (4 mg.) was hydrolysed with 2*N*-HCl (0.4 ml.) at 100° for 3 hr. and the acid removed by evaporation *in vacuo*. A small sample of the residue was chromatographed in solvent *A*. Two products (R_{ribitol} 0.51 and 0.57), which gave a purple colour rapidly with periodate-Schiff reagents and a blue colour with the molybdate reagent, were formed in addition to a trace of threitol. The remaining residue was chromatographed as a band on Whatman no. 4 paper in solvent *A* and the two phosphates were eluted with water. The solutions were treated with alkaline phosphatase. Inorganic phosphate was removed by passing the solutions through columns of Dowex 2 (CO₃²⁻ form) resin and the eluates were evaporated to dryness. The residues were chromatographed in solvents *B* and *E*. The product in each case was threitol.

Degradation of the alkali-hydrolysis products of S.10A

with periodate. S.10A (7 mg.) was hydrolysed with *N*-NaOH (1.2 ml.) at 100° for 3 hr. The hydrolysate was passed through a column of Dowex 50 (NH₄⁺ form) resin (2.5 ml.). The eluate (25 ml.) was evaporated under reduced pressure and the residue treated with 0.1*M*-sodium metaperiodate (3 ml.). The mixture was kept at room temperature for 5 days in the dark and then reduced with NaBH₄ (50 mg.). The product was hydrolysed with 2*N*-HCl and the hydrolysate, after removal of the acid, was chromatographed in solvents *A*, *B* and *E*. The hydrolysis products were galactose, galactosamine, threitol, glycerol, ethylene glycol, anhydrosorbitol, ribitol phosphates and threitol phosphates.

DISCUSSION

When the capsular substance S.10A was hydrolysed with acid, phosphates of ribitol and galactose were among the products. It therefore seemed probable that the ribitol residue in one repeating unit (I, where GalNAc represents *N*-acetylgalactosamine) was joined through a phosphodiester linkage to a galactose residue in the next. Whereas the hexasaccharide (I) yielded 2.6 mol.prop. of formaldehyde when oxidized with periodate, S.10A yielded only 0.9 mol.prop. under the same conditions. When the oxidation mixture from S.10A was reduced with borohydride and the products were hydrolysed with acid threitol phosphates and ethylene glycol but no ribitol phosphates were produced. Since neither threitol nor ethylene glycol was produced by a similar degradation of the dephosphorylated repeating unit (I) a terminal hydroxyl group of a ribitol residue must bear a phosphate group joined in turn to the hydroxyl group at position 5 or 6 of a galactofuranose residue, as in structure (II), yielding the phosphodiester (III) by degradation.

On the available evidence it is not possible to distinguish between the hydroxyl groups at positions 5 and 6 on the galactofuranose residue as the site of attachment of the phosphate group; in structure (II) position 6 has been arbitrarily preferred. The galactofuranose and ribitol parts of the phosphodiester will be considered in turn.

Of the two galactofuranosyl residues it is the one attached to the hydroxyl at position 3 of a galactopyranosyl residue that bears the phosphate group. This follows from studies involving oxidation with periodate. In the accompanying paper (Rao *et al.* 1966) it was reported that prolonged oxidation of the neutral hexasaccharide (I) with periodate

resulted in the destruction of all but one of the galactose residues in the molecule. When S.10A was treated under similar conditions 1.86 mol. prop. of galactose remained unoxidized. It was therefore clear that the phosphate group prevented over-oxidation of the galactofuranose residue attached to the 3-hydroxyl group of galactopyranose. The periodate reduced (9.1 mol. prop.) is also in agreement with this formulation.

The phosphate group is probably attached to the D-5-position of the ribitol residue rather than the D-1-position. A mode of biosynthesis from CDP-ribitol (Baddiley, Buchanan, Carss & Mathias, 1956; Baddiley, Buchanan & Carss, 1957) is envisaged, which would leave the phosphate group in this position. In this case, the oligosaccharide sequence is attached to the D-2-position of the ribitol, a situation that also exists in S.34 (W. K.

Roberts, J. G. Buchanan & J. Baddiley, unpublished work). It is hoped to prove the location of the phosphate group on the ribitol by a chemical synthesis of 2-O- α -D-galactopyranosyl-D-ribitol. A partial structure (X) can now be written for S.10A in which the only unsettled points are the configurations of the glycosidic linkages and a decision between positions 5 and 6 for the point of attachment of the phosphate group on the galactofuranose residue.

Several other observations confirm this structure. Whereas the neutral hexasaccharide (I) could be degraded to give galactose and arabinose in the ratio 2:2.08 (Rao *et al.* 1966), oxidation of S.10A itself with periodate under similar conditions, followed by reduction with borohydride and hydrolysis with acid, gave galactose and arabinose in the ratio 2:2:1. The maximum value of 3:1 is not

achieved as hydrolysis of the phosphodiester is not unidirectional and some galactose phosphate is produced; this is removed by resin treatment and so cannot be included in the ratio. When the time of oxidation was extended, no arabinose could be detected after subsequent treatment with borohydride and acid, and the phosphorus/galactose ratio was 1:2.07. Quantitative aspects of the periodate oxidation of S.10A are described in the Experimental section.

The course of hydrolysis of S.10A in alkali is illustrated by the partial formula (II). Since both the galactofuranose and the ribitol moiety possess a neighbouring hydroxyl group that is necessary for lability towards alkali, it would be expected that hydrolysis of the phosphodiester would not be unidirectional (Rao, Roberts, Buchanan & Baddiley, 1964). It has already been shown (Rao *et al.* 1966) that hydrolysis of S.10A in alkali leads to the formation of diphosphates and phosphorus-free products as well as monophosphates. In detail, the molecule (II) should be hydrolysed by route A, leading by way of the cyclic phosphate (IV) to the phosphates (V) or (VI), or by route B by way of (VII) to the monophosphates (VIII) or (IX). Two consequences of this have been verified experimentally. The crude product of hydrolysis of S.10A with alkali has been treated with periodate; 1.9mol.prop. of formaldehyde was produced for each phosphorus, compared with 0.9mol.prop. from S.10A and 2.6mol.prop. from the neutral hexasaccharide. When the oxidation mixture was treated with borohydride and the products were subjected to hydrolysis with acid, the products included ribitol phosphates and anhydribose. These must clearly arise from a fragment of struc-

ture (IX) present in the original alkali hydrolysate (when S.10A itself is subjected to this oxidative sequence all the ribitol is destroyed). Threitol phosphates and ethylene glycol were also produced, showing that the other compounds (V), (VI) and (VIII) were present in the hydrolysate.

It is now emerging that there is a class of capsular compounds from the *Pneumococcus* containing both galactofuranose and ribitol residues. Roberts *et al.* (1963) showed that the repeating unit of S.34 contained two galactofuranose residues. We have found (E. V. Rao, J. M. Tyler, J. G. Buchanan & J. Baddiley, unpublished work) that S.10, S.13 and S.29 also contain both ribitol and galactofuranose. The biosynthesis and immunology of these substances should prove of interest.

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