The Type-Specific Substance from *Pneumococcus* Type 13

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1. The type-specific substance, S. 13, from *Pneumococcus* type 13 was subjected to hydrolysis with alkali, followed by enzymic dephosphorylation, to yield a pentasaccharide. 2. The pentasaccharide, corresponding to the dephosphorylated repeating unit of S. 13, was shown to be *O*- β -D-galactopyranosyl-(1 $\rightarrow$ 4)-*O*- β -D-glucopyranosyl-(1 $\rightarrow$ 3)-*O*- β -D-galactofuranosyl-(1 $\rightarrow$ 4)-*O*-2-acetamido-2-deoxy- β -D-glucopyranosyl-(1 $\rightarrow$ 2)-ribitol. 3. The phosphodiester linkages in S. 13 join the hydroxyl group at position 1 of ribitol and the hydroxyl group at position 4 of a galactopyranosyl residue in the next repeating unit. 4. Ester groups, presumably *O*-acetyl, are located on positions 2 or 3 of most glucopyranosyl residues in S. 13. 5. A partial structure for S. 13 is proposed.

Polymers containing ribitol phosphate are of widespread occurrence as components of bacterial cell walls and capsules (Archibald & Baddiley, 1966; Baddiley, 1970). In particular, the capsules of several types of *Pneumococcus* contain both ribitol phosphate and D-galactofuranose residues (Roberts *et al.*, 1963; Rao *et al.*, 1966*a,b*; Dixon *et al.*, 1966; Chittenden *et al.*, 1968; Dixon *et al.*, 1968; Roy & Glaudemans, 1968; Rao *et al.*, 1969).

A preliminary study of the type-specific substance, S. 13, from the capsule of *Pneumococcus* type 13 (Cooper *et al.*, 1932; Brown, 1939) showed that it yielded galactose, glucose, glucosamine, ribitol, anhydriitol, galactose phosphates, ribitol phosphates and inorganic phosphate on acid hydrolysis (Shabarova *et al.*, 1962). Through the generosity of Dr. Rachel Brown, who has provided us with further samples of S. 13, we have been able to demonstrate the presence of galactofuranose groups, and to derive an almost complete primary structure for the polymer. This work is described in the present paper.

No ambiguity arises in the nomenclature of type 13, since both Danish and American systems give the same type number (Kauffmann *et al.*, 1960). In this series of papers we have used Danish nomenclature throughout.

Discussion

The identity of the S. 13 preparation was confirmed by the immunological reaction with S. 13 antiserum.

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The analytical data for S. 13 suggested the presence of three hexose residues and one residue of *N*-acetylglucosamine for each phosphate group. *O*-Acetyl groups were also present in the preparation. Like other ribitol-containing capsular compounds examined, S. 13 was labile towards alkali. When hydrolysis was done under conditions similar to those for S. 29 (Rao *et al.*, 1969) only monophosphates were detected by paper chromatography. When these were dephosphorylated enzymically two compounds resulted, one neutral and the other basic, and were separated from each other by means of Dowex 50 (H⁺ form) resin. On paper chromatograms each was homogeneous and distinguishable from the other. Prolonged alkali hydrolysis of the neutral compound gave a product chromatographically indistinguishable from the basic compound, which showed that they differed from each other by an *N*-acetyl group. Acid hydrolysis of the neutral compound gave galactose, glucose, glucosamine, ribitol and anhydriitol. Further analysis showed the presence of two galactose residues, one glucose residue, one *N*-acetylglucosamine and one ribitol, confirming the analytical values of S. 13 itself. The two phosphate-free degradation products will be referred to as the neutral pentasaccharide and the basic pentasaccharide.

When the neutral pentasaccharide was oxidized with periodate, the aldehyde product reduced with borohydride and subsequently hydrolysed in acid (Abdel-Akher *et al.*, 1952; Goldstein *et al.*, 1965), the products were glucosamine, arabinose, erythritol and glycerol. The presence of arabinose showed that one galactose residue occurred in the furanose form with a substituent on position 2 or 3 (Roberts *et al.*, 1963). The significance of the other products will emerge later.

Since a galactofuranose unit was present in the

molecule it was expected that partial acid hydrolysis of the neutral pentasaccharide would cause preferential cleavage of its glycosidic linkage. In keeping with this, a reducing trisaccharide, T, was a major product, together with disaccharides D₁, D₂ and D₃, galactose, ribitol and *N*-acetylglucosamine. Disaccharides D₁ and D₂ both yielded glucose and galactose in roughly equimolar quantities on acid hydrolysis and were present in small amounts in the original partial hydrolysate. Disaccharide D₁ was indistinguishable from lactose on paper chromatography, and borohydride reduction yielded a product with the chromatographic properties of lactitol. Disaccharide D₂ proved to be a glucosyl galactose since its product of borohydride reduction gave glucose and a hexitol (presumably galactitol) on acid hydrolysis. Disaccharide D₂ was indistinguishable from 3-*O*- β -D-glucopyranosyl-D-galactose (a gift from Dr. H. M. Flowers), and the products of borohydride reduction were also identical in their chromatographic behaviour.

Trisaccharide T, on acid hydrolysis, yielded galactose and glucose in the approximate ratio of 2:1. Borohydride reduction of trisaccharide T gave a product which, on acid hydrolysis, yielded galactose, glucose and a hexitol (presumably galactitol). The colour reactions of trisaccharide T and its reduction product were very similar to those of disaccharide D₂. The structures for saccharides D₁, D₂ and T can be accommodated in the structure (I) for T. Disaccharide D₁ (lactose) is accounted for by A and B, and disaccharide D₂ by B and C. The relatively high yield of the trisaccharide T is explained by unit C being in the furanose form in the intact neutral pentasaccharide.

Acid hydrolysis of disaccharide D₃ yielded glucosamine and ribitol. It was non-reducing and had the same mobility on paper chromatography as that reported for 2-*O*-(*N*-acetyl- β -D-glucosaminyl)-L-ribitol (II) (Baddiley *et al.*, 1962). These results are consistent with the structure (III) for the neutral pentasaccharide.

The oxidative degradation of the neutral pentasaccharide can now be explained as follows. Arabinose arises from galactose unit C, erythritol from glucose unit B and glycerol from galactose unit A and ribitol unit E. Since the glucosamine survives the periodate oxidation the linkage of C to the *N*-acetylglucosamine D must be to the C-3 or C-4 hydroxyl group of D. That it is on the C-4 hydroxyl was shown by similar degradation of the basic pentasaccharide (IV), where it was found that the glucosamine residue was oxidized, resulting in an increased yield of erythritol.

The relatively high proportion of free ribitol and of *N*-acetylglucosamine formed during the partial hydrolysis of the neutral pentasaccharide is accounted for by hydrolysis of the labile *N*-acetylglucosaminyl

linkage. In contrast, vigorous acid hydrolysis of the basic pentasaccharide (IV) yielded galactose and glucose in the approximate ratio 2:1, and a disaccharide D₄. Disaccharide D₄ was relatively stable to acid hydrolysis, yielding only a small amount of glucosamine and anhydribose after hydrolysis with 2M-hydrochloric acid at 100°C for 6h. It had chromatographic properties and colour reactions very similar to 2-*O*- α -D-glucosaminyl-L-ribitol, a sample of which was available (Hardy *et al.*, 1963). Treatment of disaccharide D₄ with nitrous acid yielded ribitol and 2,5-anhydromannose, and these results point to (V) as its structure. Mild acid hydrolysis of the basic pentasaccharide gave a trisaccharide indistinguishable from T, together with disaccharide D₄. These results are all consistent with structure (IV) for the basic pentasaccharide. The formation of disaccharide D₄ (V) is to be expected because of the stability of the glucosaminyl linkage towards acid (Moggridge & Neuberger, 1938; Foster *et al.*, 1957; Rao *et al.*, 1966a, 1969).

β -Linkages have been assigned throughout because of the high negative rotation ($[\alpha]_D -36.2^\circ$) of the neutral pentasaccharide. Procedures involving the action of almond emulsin β -glucopyranosidase have confirmed the configuration of two of the linkages. The enzyme preparation contained both α - and β -galactopyranosidase, but the action of the α -galactosidase may be inhibited by silver oxide (Roberts *et al.*, 1963). The neutral pentasaccharide yielded galactose, together with a non-reducing tetrasaccharide, both in the presence and absence of silver oxide, showing that unit A is β -linked to B in formula (III). Trisaccharide T was hydrolysed completely to galactose and glucose by the enzyme, showing the presence of a β -linkage between units B and C in formula (I) and hence in formula (III). The fact that glucose is not released from the neutral pentasaccharide (III) by the action of the enzyme is most probably a steric effect due to the furanose form of unit C in formula (III) but not in formula (I).

The initial observation that S.13 yields ribitol phosphates and galactose phosphates on acid hydrolysis indicated strongly that a ribitol residue of one repeating unit was linked to a galactose unit in the next by a phosphodiester group. The precise mode of attachment was shown by the behaviour of S.13 during hydrolysis in alkali, and by periodate-oxidation studies on the polymer.

The formation of monophosphates when S.13 was treated with alkali showed that the hydrolysis occurred uni-directionally. Subsequent acid hydrolysis of the products yielded phosphates of galactose, but no ribitol phosphates, in contrast with the acid hydrolysis of S.13 itself. The galactose phosphates were shown to be an equilibrium mixture of the 3- and 4-phosphate, which would arise during acid treatment of galactose 3- or 4-phosphate (Chittenden *et al.*,

Scheme 1. Degradation of neutral and basic pentasaccharides with periodate, borohydride and acid in sequence

1968). When the monophosphates derived from the action of alkali on S. 13 were subjected to the sequence periodate oxidation, borohydride reduction and acid hydrolysis the products were galactose phosphates and threitol phosphates together with the expected phosphate-free products. This may be explained if the pentasaccharide monophosphates contain the two units (VI) and (VII). The accepted mechanism for alkali hydrolysis of phosphodiester (Brown, 1963)

requires the intermediate formation of a cyclic phosphate, wherever possible, by participation of a vicinal hydroxyl group, and subsequent hydrolysis to a mixture of isomeric phosphomonoesters. The present results may be interpreted in terms of partial structure (VIII) for S.13. In (VIII), X and Y represent the oligosaccharide components as dictated by structure (III). Two points emerge from this scheme. First, the alkali hydrolysis is uni-directional, because the group

Y in formula (VIII) prevents cyclic phosphate formation involving a ribitol hydroxyl group. Under conditions of acid hydrolysis S.13 yields some ribitol phosphates as well as galactose phosphates because of hydrolysis of the group Y and competitive participation of the ribitol hydroxyl group in the phosphodiester cleavage. This general problem has been discussed by Chittenden *et al.* (1968) with reference to S.34. Secondly, these results do not discriminate between the two possible positions of phosphate linkage to the galactose, as expressed in formula (VIII). This point was settled by oxidative degradation of S.13 itself, which is now represented by (IX). The products were threitol and its phosphates, glycerol and its phosphates, glucose, glucosamine, arabinose and a trace of erythritol. The presence of threitol and its phosphates and the absence of galactose phosphates showed that the phosphodiester linkage was to the C-4 hydroxyl group of galactose unit A in formula (IX). The glycerol and glycerophosphates are derived from the ribitol unit, acid hydrolysis of the phosphodiester linkage being random, as in S.13 itself. The phosphate group is located on the L-1 (in preference to the D-1) position of the ribitol because of its presumed mode of biosynthesis from cytidine diphosphate ribitol, which contains the L-ribitol 1-phosphate moiety (Rao *et al.*, 1966b; Baddiley, 1970).

The fact that the glucose unit B has largely survived the periodate oxidation of S.13 (only a very small amount of erythritol was detected) showed that it was protected in some way. The protecting group is alkali-labile, since the glucose residues in the pentasaccharide monophosphates are oxidizable, and is very probably an *O*-acetyl group. The structure (IX) was confirmed by quantitative periodate oxidation.

S.13 reduced 3.8mol.prop. of periodate, corresponding to unit A (1mol), unit C (1mol) and unit E (2mol), with the formation of 1.8mol.prop. of formaldehyde, derived from units C and E (each 1mol.-prop.). Deacetylated S.13 (X) reduced 4.5mol.prop. of periodate, the increase being due to oxidation of the glucose unit B. The precise location of the *O*-acetyl group in S.13 is unknown. A similar problem with S.34 (Dixon *et al.*, 1968) was solved immunologically (Roy & Glaudemans, 1968). The particular S.13 antiserum available to us was equally reactive towards deacetylated S.13, preventing the application of the method in this case.

Experimental

Materials

The specific substance (S.13) from *Pneumococcus* type 13 was a generous gift from Dr. Rachel Brown. It is described as the soluble specific substance of type 13, preparation no. 3 (Brown, 1939). Type 13 rabbit antiserum (dated 1938) was kindly given to us by Dr. Erna Lund. Intestinal phosphomonoesterase and almond β -glucosidase (known to contain several glycosidases; Pigman, 1944) were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Chromatography

Paper chromatography was done on Whatman no. 1 or no. 4 paper, with the following solvent systems: A, propan-1-ol-aq. NH_3 soln. (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 *et al.*, 1951); C, butan-1-ol-ethanol-water-

aq. NH_3 soln. (sp.gr. 0.88) (40:10:49:1, by vol.; organic phase) (Hirst *et al.*, 1949); D, butan-1-ol-pyridine-water-benzene (5:3:3:1, by vol.; organic phase) (Bull, 1962); E, ethyl acetate-pyridine-acetic acid-water (5:5:1:3, by vol.) (Fischer & Nebel, 1955); F, propan-2-ol-conc. HCl -water (65:17:18, by vol.) (Smith & Wyatt, 1951).

Compounds were detected by: the periodate-Schiff reagents for α -glycols (Baddiley *et al.*, 1956), the acid molybdate spray for phosphoric esters (Hanes & Isherwood, 1949), the alkaline AgNO_3 reagents for sugars and polyols (Trevelyan *et al.*, 1950), the aniline phthalate reagent for reducing sugars (Partridge, 1949), the ninhydrin reagent for hexosamines (Consden & Gordon, 1948) and the Morgan-Elson reagent for *N*-acetylhexosamines (Partridge, 1948). When experiments were carried out on materials eluted from paper chromatograms appropriate determinations on equivalent paper extracts were made.

Analytical methods

Phosphate was determined by the method of Chen *et al.* (1956). Total hexose was determined by the phenol- H_2SO_4 method (Dubois *et al.*, 1956), and glucosamine by the method of Randle & Morgan (1955). Periodate was measured spectrophotometrically (Dixon & Lipkin, 1954; Aspinall & Ferrier, 1957), the correction factor 0.14 being used to allow for the extinction of IO_3^- , and formaldehyde by a modification of the chromotropic acid method (Roberts *et al.*, 1963).

Characterization of S.13

Immunological study. The substance in solution (1-4 mg/ml) was examined by the Ouchterlony technique by using type 13 antiserum. It was highly reactive, giving only one line of precipitation. De-*O*-acetylated S.13, described below, reacted similarly. No immunological reaction was detected with a

sample of S.13 that had been hydrolysed with aq. NaOH as described below.

With the same technique the preparation was tested for the presence of C-substance (How *et al.*, 1964; Brundish & Baddiley, 1968). Two samples (0.2mg and 1.0mg), dissolved in 0.15M-NaCl (1.0ml), were examined by double-diffusion against two horse anti-pneumococcal sera (antisera types I and VII) with a high anti-(C-substance) content. It appeared that the S.13 preparation contained about 10–15% of C-substance. Nevertheless, on acid hydrolysis (see below) no dark solid separated and no galactosamine was detected (cf. Brundish & Baddiley, 1968), showing that the amount of C-substance was insufficient to interfere with analytical procedures.

Optical rotation. Brown (1939) gives $[\alpha]_D -28.4^\circ$ (water).

E_{260} . The E_{260} of a 0.1% solution of S.13 measured against water was 0.04.

Paper electrophoresis. Paper electrophoresis of S.13 was done on Whatman no. 1 paper in a Shandon high-voltage electrophoresis apparatus. The 0.04M-veronal-HCl buffer, pH 8.6, was used for 7 h at a voltage gradient of 8 V/cm. The material moved 8 cm towards the anode and gave a purple colour rapidly with the periodate-Schiff reagents and a blue colour with molybdate reagent.

Infrared spectrum. The i.r. spectrum (KBr disc) showed weak but characteristic bands at 1640 and 1565 cm^{-1} (NH-CO-CH₃ group) and a weak band at 1732 cm^{-1} (O-CO-CH₃ group).

Quantitative analysis. S.13 (4.7mg) was dissolved in 2M-HCl (3.1ml) and heated at 100°C for 3 h in a sealed tube. The cooled hydrolysate was carefully neutralized to pH 6.8 with dil. NaOH and the volume suitably adjusted. Total phosphorus and hexosamine were determined on samples (Found: P, 3.04%). Brown (1939) found 3.23% (Found: anhydrohexosamine 17.0%; phosphate/hexosamine molar ratio, 1:1.07).

A similar neutralized hydrolysate of S.13 (135 $\mu\text{g}/\text{ml}$) was analysed for total hexose (Found: anhydrohexose, 42.0%; phosphate/hexose molar ratio, 1:2.65).

Acid hydrolysis. S.13 (10mg) was hydrolysed with 2M-HCl (1 ml) in a sealed tube at 100°C for 3 h. The acid was removed by evaporation *in vacuo* over KOH and part of the residue was examined by paper chromatography in solvents A and B. The products were detected as follows: periodate-Schiff reagents showed ribitol, anhydribose, ribitol phosphates and galactose phosphates; molybdate reagent showed ribitol phosphates, galactose phosphates and P_i; alkaline AgNO₃ showed ribitol, galactose, glucose, glucosamine and galactose phosphates; aniline phthalate showed galactose, glucose, glucosamine and galactose phosphates; ninhydrin showed glucosamine.

The remainder of the residue was chromatographed as a band in solvent A and the phosphates were eluted with water. Treatment with alkaline phosphatase (0.5mg) in 0.03M-(NH₄)₂CO₃ (0.5ml) overnight at 37°C yielded galactose and ribitol. The products were detected by chromatography in solvent B. The proportion of galactose was much greater than that of ribitol as estimated by spot intensities with the periodate-Schiff reagents and alkaline AgNO₃.

O-Acetyl groups. Samples (0.3–0.6mg) of S.13 were examined for O-acetyl groups by the method of Frèrejacque (1955) and gave a positive reaction. The O-acetyl groups were removed by dissolving S.13 (30mg) in water (2ml) and adding methanol (1ml). Aq. NH₃ soln. (sp.gr. 0.88, 0.75ml) was then added and the mixture was left overnight at 21°C. The solution was dialysed against water, freeze-dried and samples (0.3–0.6mg) were examined for O-acetyl groups; none was detected. Samples were also examined immunologically as described above.

Isolation and composition of the neutral and basic pentasaccharides

Hydrolysis of the specific substance with alkali. S.13 (120mg) was dissolved in 1M-NaOH (9ml) and the solution heated in a sealed tube for 1.5 h at 100°C. The hydrolysate was passed through a column of Dowex 50 (NH₄⁺ form) resin (20ml) and eluted with water. The eluate (200ml) was evaporated to a small volume and then freeze-dried. The residue (118mg) was examined by chromatography in solvent A. One major product, with R_{ribitol} value 0.26, was detected, which gave a purple colour rapidly with the periodate-Schiff reagents and a blue colour with the molybdate reagent. No P_i was detected.

Enzymic dephosphorylation of the products of alkali hydrolysis. The alkali-hydrolysis products (100mg) were dissolved in water (14ml) and treated with (NH₄)₂CO₃ (40mg) and alkaline phosphatase (9mg). A few drops of toluene were added and the mixture was incubated at 37°C for 18 h. The enzymic hydrolysate was passed through a column of Dowex 2 (CO₃²⁻ form) resin (12ml) and eluted with water. The eluate (150ml) was evaporated *in vacuo* and freeze-dried. The residue was dissolved in water (6ml), passed through a column of Dowex 50 (H⁺ form) resin (7ml) and the column washed quickly with water, followed by aq. 1M-NH₃ soln. The neutral eluate (70ml) and the basic eluate (70ml) were collected separately and each was evaporated *in vacuo* and freeze-dried. The neutral residue (58mg) and the basic residue (5mg) were each examined by chromatography in solvent A and found to be homogeneous. Attempts were made to crystallize the neutral residue from aq. ethanol but were not successful.

Alkaline hydrolysis of the neutral oligosaccharide. The neutral oligosaccharide (20mg) was hydrolysed in

1M-NaOH (2 ml) at 100°C for 8h. The hydrolysate was passed through a column of Dowex 50 (NH₄⁺ form) resin (6ml) and eluted with water. The eluate (60ml) was evaporated *in vacuo* to dryness, the residue dissolved in water (2ml) and passed quickly through a column of Dowex 50 (H⁺ form) resin (4ml), and eluted with water (40ml) followed by aq. 1M-NH₃ soln. (40ml). Both eluates were evaporated *in vacuo* and freeze-dried. The neutral residue (15mg) and the basic residue (5mg) were chromatographically homogeneous when examined in solvent A. They had the same mobilities and chromatographic properties as the neutral and basic residues obtained above.

Neutral pentasaccharide. The neutral pentasaccharide gave a purple colour rapidly with the periodate-Schiff reagents, R_{ribitol} 0.55 in solvent A, $[\alpha]_D^{18}$ -36.2° (c 1.0 in water).

Basic pentasaccharide. The basic pentasaccharide gave a purple colour rapidly with the periodate-Schiff reagents and a pink colour with ninhydrin; R_{ribitol} was 0.46 in solvent A.

Acid hydrolysis of the neutral pentasaccharide. The neutral pentasaccharide (3mg) was hydrolysed with 2M-HCl (0.3ml) at 100°C for 3h. The acid was removed *in vacuo* over KOH and the residue examined by paper chromatography in solvents A and B, by using the periodate-Schiff reagents, alkaline AgNO₃, aniline phthalate and ninhydrin for detection. The products of hydrolysis were glucose, galactose, glucosamine, ribitol and anhydrosorbitol.

Galactose/glucose ratio in the neutral pentasaccharide. The galactose/glucose ratio was determined by the method of Wilson (1959). Samples of lactose (3mg) and the neutral pentasaccharide (3mg) were each hydrolysed with 2M-HCl for 3h at 100°C. After removal of the acid *in vacuo* over KOH, the residues were chromatographed as a series of spots in solvent B. The ratio of galactose to glucose was 2.04:1.

Degradation of the pentasaccharides with periodate

Degradation of the neutral pentasaccharide with periodate, followed by borohydride. The neutral pentasaccharide (4mg) was treated with 0.1M-NaIO₄ (1.0ml) and the solution was kept at 21°C for 72h in the dark. Then NaBH₄ (20mg) was added and the solution kept overnight at 21°C. A few drops of acetic acid were added, the solution was passed through a column of Dowex 50 (H⁺ form) resin (2ml) and the eluate (20ml) was evaporated to dryness *in vacuo*. Boric acid was removed by distillation three times with methanol, and the residue was hydrolysed with 2M-HCl (0.4ml) at 100°C for 3h. The acid was removed *in vacuo* over KOH and the residue chromatographed in solvents B and C. The products, detected with the periodate-Schiff reagents, alkaline AgNO₃ and aniline phthalate, were glucosamine, arabinose,

erythritol and glycerol. The glycerol spot was approximately twice as intense as the erythritol spot, judged by reaction with the periodate-Schiff reagents.

Degradation of the basic pentasaccharide with periodate followed by borohydride. The basic pentasaccharide (2mg) was treated with 0.1M-NaIO₄ (0.5ml) and the solution was kept at 21°C for 72h in the dark. Then NaBH₄ (10mg) was added and the solution was kept overnight at 21°C. A few drops of acetic acid were added, the solution was passed through a column of Dowex 50 (H⁺ form) resin (1ml) and the column eluted with water (10ml). The eluate was evaporated to dryness *in vacuo* and boric acid removed by distillation with methanol. The residue was hydrolysed with 2M-HCl at 100°C for 3h, the acid removed *in vacuo* and the hydrolysate examined by paper chromatography in solvents B and C. Glycerol, erythritol and arabinose were detected; the amounts of erythritol and glycerol were approximately equal, as judged by spot intensities. The Dowex 50 column was eluted with aq. 1M-NH₃ soln. and the evaporated eluate subjected to acid hydrolysis as before. No products were detected by means of the periodate-Schiff reagents, alkaline AgNO₃ or ninhydrin, showing that the glucosamine moiety in the basic pentasaccharide had been oxidized by periodate.

Acid degradations of the oligosaccharides

Partial acid hydrolysis of the neutral pentasaccharide. The neutral pentasaccharide (18mg) was hydrolysed with formic acid (66%, v/v; 1.8ml) at 100°C for 25min in a sealed tube. The hydrolysate was evaporated to dryness *in vacuo*, the residue treated with a few drops of dil. aq. NH₃ soln. and again evaporated to dryness *in vacuo*. Part of the residue was examined by paper chromatography in solvent B; the products detected were ribitol, *N*-acetylglucosamine, galactose (a trace), disaccharides D₁, D₂ and D₃ and an oligosaccharide mixture. Disaccharides D₁ and D₂ were present in small amount, disaccharide D₃ being the major product. The remainder of the hydrolysate was chromatographed as a band in solvent B and disaccharides D₁, D₂ and D₃ were eluted with water. A trisaccharide T, the major component of the oligosaccharide mixture, was also eluted with water.

On paper chromatography in solvents B and D disaccharide D₁ had R_{lactose} 1.0 and gave strong reactions with alkaline AgNO₃ and aniline phthalate. Acid hydrolysis of disaccharide D₁ with 2M-HCl at 100°C for 3h, followed by paper chromatography in solvent B, yielded galactose and glucose in equal amounts as judged by spot intensities.

Disaccharide D₁ was dissolved in a few drops of water, NaBH₄ (3mg) added and the solution kept at 21°C overnight. A few drops of acetic acid were

added and the solution was passed through a small column of Dowex 50 (H^+ form) resin. The residue was evaporated to dryness *in vacuo* and boric acid removed by distillation (three times) with methanol. A small sample of lactose was similarly treated; both products had the same mobility and gave identical reactions when examined by paper chromatography in solvent B.

Disaccharide D_2 was indistinguishable from 3-*O*- β -D-glucopyranosyl-D-galactose (Flowers, 1967) in its mobility and chromatographic properties. They both had $R_{lactose}$ 1.2 and $R_{galactose}$ 0.62 in solvent B, gave the same colour reactions with the periodate-Schiff reagents and gave strong reactions with alkaline $AgNO_3$ and aniline phthalate. Disaccharide D_2 was hydrolysed with 2M-HCl and yielded galactose and glucose in equal amounts as judged by spot intensities on paper chromatograms.

Small samples of disaccharide D_2 and 3-*O*- β -D-glucopyranosyl-D-galactose were each reduced with $NaBH_4$ (3mg) and the reduction products examined by paper chromatography in solvent B. Each had the same mobility, $R_{galactose}$ 0.79 in solvent B, and gave identical colour reactions with the periodate-Schiff reagents. The spots were initially purple in colour, then had a yellow 'halo', and finally became deep-brownish yellow after several hours (Hardy & Buchanan, 1963). Acid hydrolysis of both reduction products with 2M-HCl yielded glucose and a hexitol.

Disaccharide D_3 had $R_{galactose}$ 0.85 and $R_{ribitol}$ 0.65 in solvent B and $R_{glucosamine}$ 1.25 and $R_{ribitol}$ 0.83 in solvent E. Disaccharide D_3 gave a purple colour rapidly with the periodate-Schiff reagents; it was non-reducing, giving a weak reaction with alkaline $AgNO_3$, becoming more intense on steaming the chromatogram (Brundish *et al.*, 1965), and showed no reaction with aniline phthalate. A sample of disaccharide D_3 was hydrolysed with 2M-HCl at 100°C for 3h and the products, examined by paper chromatography, were glucosamine, ribitol and anhydriibitol.

Trisaccharide T had $R_{lactose}$ 0.44 in solvent B. It gave a yellow colour with the periodate-Schiff reagents and a strong reaction with alkaline $AgNO_3$ and aniline phthalate. Acid hydrolysis of trisaccharide T yielded galactose and glucose in the approximate ratio 2:1, as judged by spot intensities on paper chromatograms.

A sample of trisaccharide T was reduced with $NaBH_4$ in the usual manner and the product examined by paper chromatography in solvent B. It had $R_{galactose}$ 0.48, gave a purple colour rapidly, followed by a yellow 'halo' with the periodate-Schiff reagents, and gave a weak reaction with alkaline $AgNO_3$, becoming more intense on steaming the chromatogram. Acid hydrolysis of the reduction product yielded galactose, glucose and a hexitol detected by chromatography in solvent B.

When treated with almond β -glucosidase, with the

products examined by paper chromatography, trisaccharide T yielded galactose and glucose. Hydrolysis was complete and the ratio of galactose to glucose was approx. 2:1 as judged by spot intensities on the chromatograms.

Acid hydrolysis of the basic pentasaccharide. The basic pentasaccharide (4mg) was hydrolysed with 1M-HCl (0.7ml) at 100°C for 2h. The hydrolysate was passed through a column of Dowex 2 (CO_3^{2-} form) resin (1.5ml) and the eluate (20ml) evaporated *in vacuo* and freeze-dried. Part of the residue was examined by chromatography in solvent B; the products detected were galactose and glucose in the approximate ratio 2:1 and a disaccharide D_4 . The remainder of residues was chromatographed as a band in solvent B and D_4 eluted with water.

Disaccharide D_4 had R_F 0.58 in solvent A and $R_{galactose}$ 0.72 and $R_{ribitol}$ 0.53 in solvent B. It gave a purple colour rapidly with the periodate-Schiff reagents, a weak reaction with alkaline $AgNO_3$ becoming intense on steaming the chromatogram, and a pink colour with ninhydrin. Samples of disaccharide D_4 were hydrolysed with 2M-HCl at 100°C for 3 and 6h. The hydrolysates were evaporated *in vacuo* and the residues examined by paper chromatography. No products of hydrolysis were detected after 3h, but after 6h small amounts of glucosamine and anhydriibitol were detected, together with unhydrolysed disaccharide D_4 .

The chromatographic properties of disaccharide D_4 were compared with the disaccharide 2-*O*- α -D-glucosaminyl-L-ribitol (Hardy *et al.*, 1963) and were found to be very similar. In solvent F, D_4 had R_F 0.51 and the disaccharide R_F 0.53. A small sample of disaccharide D_4 was treated with 0.02M-HCl (0.1ml) and $NaNO_2$ (10mg) in water (0.2ml) and the mixture heated at 50°C in a sealed tube for 2h. The solution was passed through a small column of Dowex 50 (NH_4^+ form) resin; the eluate was boiled for 10min and then evaporated *in vacuo* and the residue examined by paper chromatography in solvent B. The two products detected were ribitol and a compound chromatographically indistinguishable from 2,5-anhydromannose (Baddiley *et al.*, 1962; Hardy *et al.*, 1963).

Partial acid hydrolysis of the basic pentasaccharide. The basic pentasaccharide (2mg) was hydrolysed with formic acid (66%, v/v; 0.2ml) at 100°C for 15min. After evaporation to dryness *in vacuo* and destruction of the formyl ester with aq. NH_3 soln. the residue was chromatographed in solvent B. Trisaccharide T and disaccharide D_4 were among the products detected.

Action of almond emulsin on the neutral pentasaccharide. The neutral pentasaccharide (1mg) was dissolved in water (0.1ml) and treated with almond β -glucosidase solution (2%, w/v; 0.05ml). The mixture was kept at 37°C for 18h, and then it was evaporated to a small volume and the products were

examined by paper chromatography in solvent B. Two major products, galactose and a tetrasaccharide, were detected. The tetrasaccharide had $R_{\text{galactose}}$ 0.55; it reacted weakly with alkaline AgNO_3 , the spot becoming intense on steaming the chromatogram, and did not react with aniline phthalate. A small amount of unhydrolysed pentasaccharide ($R_{\text{galactose}}$ 0.23) was also detected.

A second series of experiments was done in the presence of silver oxide. A solution of almond β -glucosidase (2%, w/v; 0.5ml) was mixed with a saturated aq. silver oxide soln. (0.25ml) and kept in ice-water for 10min. The enzyme preparation was then warmed to room temperature and a sample (0.08ml) added to a solution of the neutral pentasaccharide (1mg) dissolved in water (0.1ml). The mixture was incubated at 37°C for 18h, evaporated to dryness *in vacuo* and the residue examined by chromatography in solvent B. Galactose, the tetrasaccharide ($R_{\text{galactose}}$ 0.55) and unhydrolysed neutral pentasaccharide ($R_{\text{galactose}}$ 0.23) were detected. Samples (1mg) of methyl α - and β -galactopyranoside were treated in a similar manner. Galactose was partly liberated from the β -anomer, whereas no galactose was liberated from the α -anomer.

Phosphodiester linkage

Acid hydrolysis of the pentasaccharide phosphates and identification of the galactose phosphates. The alkali-hydrolysis products of S.13 (6mg) were hydrolysed with 2M-HCl (0.6ml) at 100°C for 3h. The acid was removed *in vacuo* and part of the residue examined by chromatography in solvents A and B. The products detected were ribitol, anhydribose, galactose, glucose, glucosamine and two organic phosphates (1 and 2).

Phosphate 1 had $R_{\text{galactose}}$ 0.36 in solvent A and gave a slowly developing yellow colour (5–10min) with the periodate-Schiff reagents, whereas phosphate 2, $R_{\text{galactose}}$ 0.28 in solvent A, gave a slowly developing blue spot (30–40min). With aniline phthalate both phosphates gave a brown spot. Phosphates 1 and 2 were identical with galactose 4-phosphate and 3-phosphate respectively in their chromatographic properties.

A mixture of galactose 3- and 4-phosphate was obtained by acid hydrolysis of a sample of galactose 3-phosphate (Chittenden *et al.*, 1968) with 2M-HCl for 3h at 100°C.

The remainder of the residue was chromatographed as a band in solvent A, and phosphates 1 and 2 were eluted with water. The eluates were freeze-dried, the residues dissolved in a few drops of 0.03M-(NH_4)₂CO₃ soln. and treated with alkaline phosphatase overnight at 37°C. The solutions were evaporated *in vacuo* and the residues examined in solvent B. Both phosphates yielded galactose.

Degradation of S.13 with periodate, followed by borohydride. S.13 (12mg) was treated with 0.1M-NaIO₄ (3ml) and the solution kept at 21°C for 72h in the dark. Then NaBH₄ (50mg) was added to the still-viscous solution and the solution was kept overnight at 21°C. A few drops of acetic acid were added, the solution was passed through a column of Dowex 50 (H⁺ form) resin (6ml) and the eluate was evaporated *in vacuo*. Boric acid was removed by distillation three times with methanol, and the residue was hydrolysed with 2M-HCl (1.2ml) at 100°C for 3h. The acid was removed *in vacuo* and part of the residue was examined by chromatography in solvents A, B and C. The products detected were glucose, glucosamine, arabinose, threitol, glycerol and a trace of erythritol. Two organic phosphates were also detected: phosphate 1 had $R_{\text{galactose}}$ 0.56 in solvent A and phosphate 2 had $R_{\text{galactose}}$ 0.49. Each gave a purple colour rapidly with the periodate-Schiff reagents.

The remainder of the residue was chromatographed as a band in solvent A and the organic phosphates were eluted with water. The eluates were evaporated to dryness *in vacuo*, the residues dissolved in a few drops of 0.03M-(NH_4)₂CO₃ soln. and treated with phosphatase at 37°C for 18h. In each case threitol was detected. Glycerol in a small quantity was also detected in the dephosphorylation mixture of phosphate 1. Evidently glycerol phosphate has a similar mobility to phosphate 1 and chromatography in solvent A could not separate them. This was confirmed directly by chromatography of phosphate 1 and authentic α -glycerophosphate in solvent A.

The mobilities of phosphates 1 and 2 were compared with the products of acid hydrolysis of threitol 1-phosphate cyclohexylammonium salt (a gift from Dr. D. H. Rammner). The material (2mg) was hydrolysed with 2M-HCl (0.2ml) at 100°C for 3h and the acid removed *in vacuo*. The residue was examined by chromatography, which revealed two organic phosphates with $R_{\text{galactose}}$ 0.54 and 0.48 respectively in solvent A. Each phosphate gave a purple colour rapidly with the periodate-Schiff reagents.

Degradation of the pentasaccharide phosphates with periodate, followed by borohydride. The products of alkali hydrolysis of S.13 (4mg) were treated with 0.1M-NaIO₄ (1.4ml) and left at 21°C for 4 days in the dark. Then NaBH₄ (20mg) was added and the solution was left overnight at 21°C and treated in the usual manner. Hydrolysis with acid liberated galactose, arabinose, glucosamine, threitol, erythritol and glycerol, which were detected by paper chromatography in solvents A, B and C. Galactose phosphates were revealed by the molybdate and aniline phthalate reagents and threitol phosphates with the molybdate and periodate-Schiff reagents.

Quantitative periodate oxidation studies on S.13 and the de-O-acetylated substance. A solution (2.0ml) of S.13 (4.16mg) was mixed with 0.02M-NaIO₄

(2.0ml) and the reduction of periodate was determined spectrophotometrically. After 1 h 2.5 mol. prop., after 48 h 3.4 mol. prop., and after 120 h 3.8 mol. prop. of periodate was reduced for each phosphate group and no further reduction was observed.

Similarly a solution (2.0ml) of de-*O*-acetylated S.13 (4.62mg) was mixed with 0.02M-NaIO₄ (2.0ml) and the reduction of periodate measured. After 1 h 2.6 mol. prop., after 48 h 3.9 mol. prop., and after 120 h 4.5 mol. prop. of periodate was reduced for each phosphate group and no further reduction was observed.

In another experiment S.13 (7.47mg) was dissolved in water (25ml). Samples (1.0, 0.8, 0.6, 0.4 and 0.3ml) of this solution were diluted to 2.0ml, mixed with 0.1M-NaIO₄ (0.5ml) and kept at 21°C in the dark for 2h; 1.76 mol. prop. of formaldehyde was produced for each phosphate group.

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