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STRUCTURAL STUDIES ON THE SPECIFIC TYPE-14 PNEUMOCOCCAL POLYSACCHARIDE

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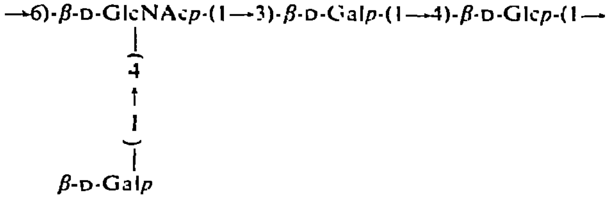
ABSTRACT

The structure of the *Pneumococcus* type-14 capsular polysaccharide has been reinvestigated by using methylation analysis, different specific degradations, and n.m.r. spectroscopy. It is concluded that the polysaccharide is composed of tetrasaccharide repeating-units having the structure:



$$\begin{array}{l}\rightarrow 6)\text{-}\beta\text{-D-GlcNAc}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Gal}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow\\ \qquad\qquad 4\\ \qquad\qquad \uparrow\\ \qquad\qquad 1\\ \qquad \beta\text{-D-Gal}p\end{array}$$

INTRODUCTION

The pneumococcal capsular polysaccharides are the principal, type-specific, immunological determinants of these micro-organisms[1]. Chemical studies have shown relationships between capsular structures and their immunological specificity[1], and the type-14 capsular material (S 14) has been extensively studied in both these respects[1,2]. It is composed of D-glucosyl, D-galactosyl, and 2-acetamido-2-deoxy-D-glucosyl residues; on the basis of methylation analysis, enzymic hydrolysis with induced enzymes, and the isolation of various oligosaccharides, both a dodecasaccharide and a simpler hexasaccharide repeating-unit have been proposed for its structure (reviewed in Refs. 1 and 2). However, because these suggestions are inconsistent, and in view of studies of the reaction of the S-14 polysaccharide with lectins[3-5] and its importance as a model substance in various biological systems, we have reinvestigated its structure by modern methods.

RESULTS AND DISCUSSION

The S-14 capsular polysaccharide was free from contamination with protein, nucleic acid, and C-substance, and had $[\alpha]_D$ +5°. Sugar analysis of a polysaccharide that contains 2-acetamido-2-deoxy-D-glucosyl residues is complicated by the fact that

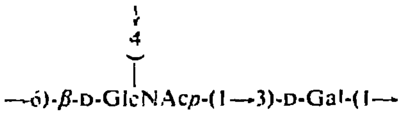
N-deacetylation, giving resistant glucosaminyl linkages, competes with the acid hydrolysis of glycosidic linkages. Analysis of a hydrolysate, as alditol acetates[6], indicated the presence of D-glucose and D-galactose, in the ratio 1:1.6, and 2-acetamido-2-deoxy-D-glucose. When the analysis was performed as devised by Dmitriev *et al.*[7], with deamination of the products of acid hydrolysis by reaction with nitrous acid, D-glucose, D-galactose, and 2,5-anhydro-D-mannose, also analysed as alditol acetates, were obtained in the proportions 1:1.6:0.5. Finally, *N*-deacetylated S 14 was prepared by treatment with sodium hydroxide–sodium thiophenolate in aqueous methyl sulfoxide[8]; on acid hydrolysis, this material yielded D-glucose and D-galactose in the ratio 1:0.8. In the latter analysis, the glycosidic linkage of the 2-amino-2-deoxy-D-glucosyl residue was not significantly hydrolysed, and, accordingly a 2-amino-2-deoxy-D-glucopyranosyl-D-galactose moiety (see below) was also isolated from the hydrolysate.

The n.m.r. spectrum of S 14 showed, *inter alia*, signals for the methyl protons of the *N*-acetyl group (δ 2.04) and for anomeric protons (δ 4.3–4.9) in the ratio 3:4. For the anomeric protons, no individual assignments could be made, but, since the coupling constants were high ($J_{1,2}$ ~7 Hz) and the chemical shifts were in the higher part of the field region observed for anomeric protons, these data suggest that all of the sugar residues are pyranosidic and β-linked. The low optical rotation of S 14 further supports this conclusion.

A methylation analysis of S 14, with determination of the partially methylated sugars by g.l.c.–m.s. of their alditol acetates[9–12], gave 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,4,6-tri-*O*-methyl-D-galactose, and 2,3,6-tri-*O*-methyl-D-glucose in the proportions 0.8:1:1. The presence of 2-deoxy-3-*O*-methyl-2-(*N*-methylacetamido)-D-glucose was also observed, but quantitation of this compound, or of other hexosamine derivatives examined during this work, was not attempted because of difficulties in obtaining reproducible response factors on g.l.c.

The general conclusion, from biosynthetic considerations, that many bacterial polysaccharides are composed of simple oligosaccharide repeating-units supported, at this stage, interpretation of the above results as being consistent with the presence of a tetrasaccharide repeating-unit in S 14. The results suggest one terminal D-galactosyl group, one D-galactosyl residue linked through O-3, one D-glucosyl residue linked through O-4, and one branching 2-acetamido-2-deoxy-D-glucosyl residue linked through both O-4 and O-6.

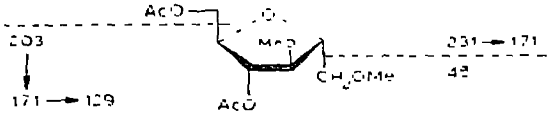
The products obtained after acid hydrolysis of *N*-deacetylated S 14 were reduced with sodium borohydride and *N*-acetylated. Fractionation on Sephadex G-15 gave a disaccharide alditol which proved to be β-linked from a consideration of its low optical rotation, $[\alpha]_{578}$ −22°, and n.m.r. spectrum (which contained a doublet in the anomeric region at δ 4.51, $J_{1,2}$ 8 Hz); on hydrolysis and subsequent reduction with sodium borodeuteride, the material gave 2-acetamido-2-deoxy-D-glucitol-*1-d* and D-galactitol. The sequence hexosaminyl-hexitol was also evident from the mass spectrum of the methylated disaccharide alditol and thus, in conjunction with the methylation analysis of the original S 14, the structural unit **1** was shown to be present in the polysaccharide:



$$\rightarrow 6)\text{-}\beta\text{-D-}\overset{\overset{\downarrow}{4}}{\text{GlcNAc}p}\text{-}(1\rightarrow 3)\text{-D-Gal-}(1\rightarrow$$

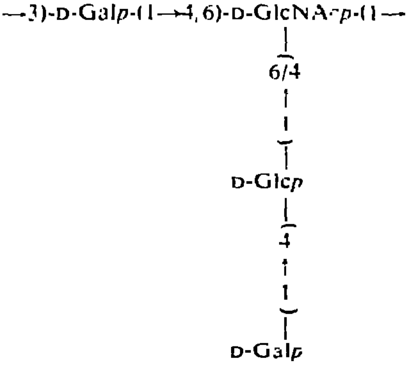
1

Deamination of *N*-deacetylated S 14 with nitrous acid, followed by reduction with sodium borohydride and fractionation of the product on Sephadex G-25, yielded a component eluted in the tetrasaccharide region. The component had $[\alpha]_D$ +16° and, in the anomeric region of its n.m.r. spectrum (δ 4.05–4.52), there were overlapping doublets with high coupling constants (J ~7 Hz); these results are again consistent with the presence of β linkages. On acid hydrolysis, the tetrasaccharide yielded D-galactose, D-glucose, and 2,5-anhydro-D-mannitol in the proportions 2.2:1:0.9. Methylation analysis gave 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,3,6-tri-*O*-methyl-D-glucose, and 2,5-anhydro-1,3-di-*O*-methyl-D-mannitol in the proportions 2.2:1:1.1. Methylation analysis of oligosaccharides containing 2,5-anhydro-D-mannitol residues has been reported, and allows identification of the partially methylated 2,5-anhydrohexitol, as its acetate, by g.l.c.–m.s.[13]. Some pertinent fragments in the mass spectrum of the present derivative are indicated in the formula **2**. They are formed by fission between C-1 and C-2, and C-5 and C-6, respectively, followed by β-elimination of acetic acid or methanol. When, as the result of the β-elimination of methanol, an *O*-acetyl group is linked to an unsaturated carbon, ketene is consecutively eliminated.



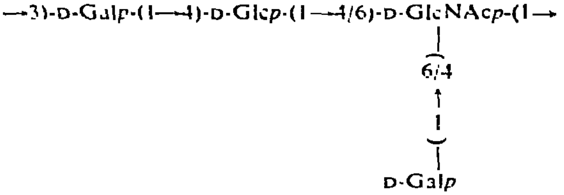
2

The results of the deamination confirmed those of the methylation analysis and partial hydrolysis, but were consistent with the possible sequences shown in **3a** and **3b** for the repeating unit of S 14:



$$\rightarrow 3)\text{-D-Gal}p\text{-}(1\rightarrow 4,6)\text{-D-}\underset{\substack{|\\ 6/4\\ \uparrow\\ 1\\ |\\ \text{D-Glc}p\\ |\\ 4\\ \uparrow\\ 1\\ |\\ \text{D-Gal}p}}{\text{GlcNAc}p}\text{-}(1\rightarrow$$

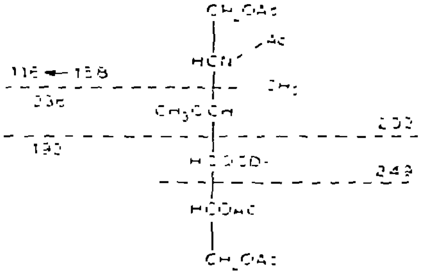
3a



$$\rightarrow 3)\text{-D-Gal}p\text{-}(1\rightarrow 4)\text{-D-Glc}p\text{-}(1\rightarrow 4/6)\text{-D-}\underset{\substack{|\\ 6/4\\ \uparrow\\ 1\\ |\\ \text{D-Gal}p}}{\text{GlcNAc}p}\text{-}(1\rightarrow$$

3b

To establish the order of the sugars, the terminal galactosyl residue was modified and eliminated, so enabling identification of its site of attachment to the polysaccharide. S 14 was oxidised first with D-galactose oxidase[14] and then with hypoiodite[15], thus transforming the terminal β-D-galactopyranosyl group into a β-D-galactopyranosyluronic acid group. Sugar and methylation analyses of this product showed that the oxidation had been essentially complete. The modified S 14 was subjected to a uronic acid degradation[16], involving methylation according to Hakomori[17], followed by treatment of the permethylated material with base and subsequently with acid under mild conditions. This treatment eliminated the terminal D-galacturonic acid group, and the position to which it was linked was exposed as a free hydroxyl group. Remethylation of the product with trideuteriomethyl iodide, hydrolysis, and analysis of the sugars formed, by g.l.c.-m.s. of their alditol acetates, showed the presence of 2,4,6-tri-*O*-methyl-D-galactose and 2,3,6-tri-*O*-methyl-D-glucose in the ratio 0.9:1. 2-Deoxy-3,4-di-*O*-methyl-2-(*N*-methylacetamido)-D-glucose, with a trideuteriomethyl group at O-4, was also obtained, together with a trace of 2-deoxy-3-*O*-methyl-2-(*N*-methylacetamido)-D-glucose from unoxidised polysaccharide. The di-*O*-methyl derivative of the amino sugar demonstrated that the terminal D-galactopyranosyl group in S 14 is linked to O-4 of the 2-acetamido-2-deoxy-D-glucosyl residue. The mass spectrum of the 2-deoxy-di-*O*-methyl-2-(*N*-methylacetamido)-D-glucitol derivative (4), *e.g.*, the primary fragments *m/e* 249, 236, 202, and 192, indicated in the formula, demonstrated unambiguously that the trideuteriomethyl group was located at O-4 and not at O-6.



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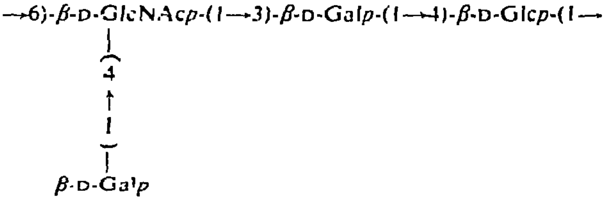
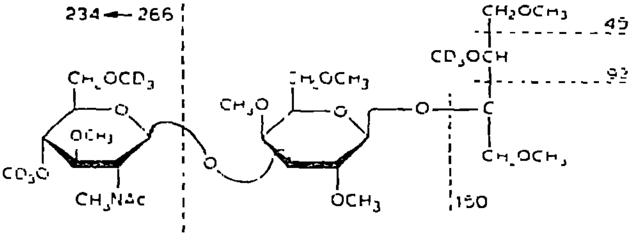
From the results given above, the complete tetrasaccharide repeating-unit (5) of S 14 may be deduced.



$$\begin{array}{l}\rightarrow 6)\text{-}\beta\text{-D-GlcNAc}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Gal}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow \\ \quad\quad 4 \\ \quad\quad \uparrow \\ \quad\quad 1 \\ \beta\text{-D-Gal}p\end{array}$$

5

In order to further confirm this structure, S 14 was subjected to a modified Smith-degradation[18], in which the polyalcohol obtained after periodate oxidation-borohydride reduction was methylated before the mild hydrolysis with acid. The degraded material was then remethylated, using trideuteriomethyl iodide. From the suggested repeating-unit (**5**), the main product should have structure **6**. In agreement with this structure, the mass spectrum of **6** showed, *inter alia*, fragments given by both terminals, the methylated 2-acetamido-2-deoxy-D-glucosyl and the erythritol moieties, respectively, with the expected number of trideuteriomethyl groups. 2-Deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-D-glucose, with trideuteriomethyl groups at O-4 and O-6, 2,4,6-tri-*O*-methyl-D-galactose, and 1,3,4-tri-*O*-methyl-D-erythritol, with a trideuteriomethyl group at O-3, were detected in an acid hydrolysate, providing further confirmation for the repeating-unit structure (**5**).



6

Several of the structural elements in the previously proposed[1,2] structures for the oligosaccharide repeating-unit of S 14 are also present in structure **5**. The absence of disaccharide side-chains and of a terminal α-D-galactopyranosyl group in the revised structure is, however, significant in connection with reactions between S 14 and antibodies or lectins[1,3-5]. Of particular interest are immunological studies with horse anti-type 14 pneumococcal serum[1,19], in which it was shown that cross reactivities of blood-group substances with the serum were strongly inhibited by 2-acetamido-2-deoxy-4-*O*-β-D-galactopyranosyl-D-glucose and only slightly inhibited by the corresponding (1→3)- or (1→6)-linked disaccharides. In a similar system[19], a tetrasaccharide from human milk, lacto-*N*-neotetraose (**7**), also inhibited strongly. Both these observations are in agreement with the structure **5** for S 14.

β-D-Gal*p*-(1→4)-β-D-GlcNAc*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc

7

EXPERIMENTAL

General methods. — Concentrations of solutions were performed under reduced pressure at bath temperatures below 40°. A Perkin-Elmer 990 instrument equipped with a flame-ionisation detector was used for g.l.c. Separations were performed on glass columns (180 × 0.15 cm, with 100/120 mesh Gas Chrom Q as support material) containing (*a*) 3% of OV-225 (at 210° for alditol acetates and at 190° for partially methylated alditol acetates); (*b*) 3% of OV-17 (at 190° for amino sugar derivatives); and (*c*) 3% of OV-1 (for permethylated oligosaccharide derivatives). Peak areas were measured with a Hewlett-Packard 3370 B electronic integrator. For some separations of partially methylated alditol acetates, a Hewlett-Packard 5830A g.l.c. instrument was used with a SP-1000 W.C.O.T. column (25 m × 0.25 mm) (LKB Products, Bromma, Sweden) at 210°. G.l.c.–m.s. was performed with a Varian MAT 311-SS 111 m.s.–computer system at an ionisation potential of 70 eV. N.m.r. spectra were recorded in the PFT mode with a Varian XL-100 instrument at 85° on solutions of material in D_2O containing an internal standard of sodium 1,1,2,2,3,3-hexadeuterio-4,4-dimethyl-4-silapentane-1-sulfonate. I.r. spectra (using KBr discs) and u.v. spectra (on aqueous solutions) were recorded on Perkin-Elmer 257 and Beckman DK 2 instruments, respectively. Optical rotations were determined on a Perkin-Elmer 141 instrument with 100-mm semi-micro cells. Gel filtrations were performed on Sephadex G-15 and G-25 columns with water as irrigant, and elutions were monitored by a Waters R 403 differential refractometer.

Material — S 14, lot 116R, was prepared by E. R. Squibb & Sons, New Brunswick, N.J., U.S.A. A solution of the polysaccharide in water (5 mg/ml) had no significant u.v. absorbance at either 260 or 280 nm, showing it to be free from both nucleic acids and protein. The absence of both ribitol and 2-acetamido-2-deoxy-D-galactose from sugar analyses of S 14 showed the material to be free from C-substance[20], a common contaminant of pneumococcal capsular polysaccharides[1,2]. The polysaccharide had $[\alpha]_D^{2}$ +5° (*c* 0.5, water) and ν_{max}^{KBr} ~1630 cm^{-1} (*N*-acyl region), no evidence for *O*-acyl groups (~1735 cm^{-1}) was found. The n.m.r. spectrum of the polysaccharide was recorded on a solution (10 mg/ml) in D_2O and 85°, and enabled determination of the acetamido:neutral sugar ratio (see Results and Discussion).

Sugar and methylation analyses. — Methylation of polysaccharides and oligosaccharides was carried out according to the method of Hakomori[17]; polysaccharides were recovered by dialysis, and oligosaccharides by partitioning between water and chloroform, when they were extracted into the organic phase. As previously described, sugars and methylated sugar derivatives were analysed by g.l.c.–m.s. as their alditol acetates[6,9-12]. To avoid loss of amino sugars, Dowex 50 was not used after the reduction step in these analyses, but excess of sodium borohydride was destroyed by the addition of 50% aqueous acetic acid. After acetylation in acetic anhydride–pyridine and evaporation of the reagents, the residue was dissolved in water, and the alditol acetates were extracted into chloroform. Partially methylated and trideuteriomethylated alditol acetates gave mass spectra in which fragments containing trideuteriomethoxyl groups were recognised by the shift of three mass-units[10].

Determination of amino sugars. — The quantitation of 2-acetamido-2-deoxy-D-glucose in S 14 was carried out after its conversion into 2,5-anhydro-D-mannitol[7]. *N*-Deacetylated polysaccharide (2 mg, for preparation see below) was hydrolysed with 0.25M H_2SO_4 (16 h, 100°), the acid neutralised with $BaCO_3$, and the solution filtered and freeze-dried. A solution of the residue in water (0.5 ml) was treated with 33% aqueous acetic acid (0.5 ml) and 5% aqueous sodium nitrite (0.5 ml) for 1 h at 20°, deionised with Dowex 50(H$^+$) resin, and freeze-dried, and the residue was analysed after conversion into alditol acetates.

N-*Deacetylation of S 14.* — This was performed essentially as described by Erbing *et al.*[8] The polysaccharide (50 mg) was dissolved in water (1 ml) and methyl sulfoxide (10 ml), and sodium hydroxide (400 mg) and thiophenol (100 mg) were added. The solution, in a serum vial, was stirred for 8 h at 90°, neutralised with 2M HCl, dialysed, filtered, and freeze-dried (yield, greater than 80%). The degree of *N*-deacetylation was nearly 100%, as determined by comparing the intensity of the *N*-acetyl signal (δ 2.04) with those of the anomeric protons (δ 4.30–4.85) in the n.m.r. spectrum of the product.

Hydrolysis of N-*deacetylated polysaccharide and isolation of a disaccharide alditol.* — Sugar analysis of *N*-deacetylated polysaccharide showed a galactose–glucose ratio of 0.8:1, compared to 1.6:1 for native material, indicating a resistant 2-acetamido-2-deoxy-D-glucose–D-galactose linkage. A disaccharide containing these two sugars was isolated from the polysaccharide as follows. *N*-Deacetylated S 14 (20 mg) was hydrolysed with 0.25M H_2SO_4 (16 h, 100°), the acid was neutralised with barium carbonate, and the products were reduced with sodium borohydride. Excess of reducing agent was decomposed with 50% aqueous acetic acid, and the hydrolysate was evaporated with methanol and desalted on a small column of Sephadex G-15. Material was eluted as a broad, unresolved peak in the monosaccharide–disaccharide region, and the appropriate fractions were combined, and acetylated in acetic anhydride–pyridine (1:1) at 20° for 16 h followed by evaporation to dryness by codistillation with ethanol. The residue was partitioned between chloroform and water, and the organic phase was evaporated to dryness. The residue was *O*-deacetylated with a saturated solution of ammonia in methanol at 20° for 16 h. After evaporation to dryness, the material was dissolved in water and fractionated on a column of Sephadex G-15 to give peaks in the mono- and di-saccharide regions. The disaccharide alditol (2.7 mg) had $[\alpha]_D^{22}$ −22° (*c* 0.2, water), in the n.m.r. spectrum, it gave signals in the anomeric region at δ 4.51 ($J_{1,2}$ 8 Hz, 1 H), and *N*-acetyl region at δ 1.97 (3 H). Following hydrolysis, and reduction with sodium borodeuteride, the derived alditols were analysed and identified as 2-amino-2-deoxyglucitol (monodeuterated at C-1) and galactitol. When methylated, the disaccharide alditol gave a single peak on g.l.c. (OV-1 column at 240°), T_{LAc} 2.68 (retention time relative to permethylated lactitol). The mass spectrum showed, *inter alia*, the following fragments (relative intensities in brackets, and some assignments[10] in square brackets): *m/e* 43 (23), 45 (100), 56 (17), 59 (41), 71 (42), 73 (17), 75 (28), 87 (25), 88 (33), 89 (39), 98 (16), 101 (66), 115 (30), 129 (43) [aH_1], 133 (10), 142 (12), 154 (10), 171 (18), 182 (14), 196 (3) [aA_3], 219 (11), 228 (21) [aA_2], 235 (19) [bA_1], 260 (14) [aA_1], 295 (2) [abJ_1], 305 (2), and 337 (2).

Deamination of N-*deacetylated polysaccharide.* — *N*-Deacetylated S 14 (29 mg) in water (1.5 ml) was treated with equal volumes (2.5 ml each) of 33% aqueous acetic acid and 5% aqueous sodium nitrite for 1 h at 20°. The solution was treated with Dowex 50(H^+) resin and freeze-dried, and the residue was taken up in water and reduced overnight with sodium borohydride (50 mg). After deionisation with Dowex 50(H^+) resin, the solution was evaporated to dryness, and methanol (3 × 5 ml) was distilled from the residue, which was then dissolved in water and applied to a column of Sephadex G-25. The only carbohydrate-containing material was eluted as a single peak (13.2 mg) in the tetrasaccharide region. The compound had $[\alpha]_D^{22}$ +16° (*c* 0.6, water); in the n.m.r. spectrum, the signals in the anomeric region (δ 4.05–4.52) consisted of overlapping doublets ($J_{1,2}$ ~7 Hz). Part of the material (25%) was acetylated and then had R_F 0.78 (t.l.c., silica gel, ethyl acetate). Another portion (25%) was hydrolysed, and the sugars were analysed. The remainder of the material (50%) was subjected to methylation analysis, and the partially methylated derivatives were analysed as their alditol acetates. A 2,5-anhydro-D-mannitol derivative was identified from its mass spectrum which showed, *inter alia*, the following fragments: *m/e* 43 (100), 45 (30), 69 (15), 111 (32), 129 (26), 156 (5), 171 (0.5), 203 (2), and 231 (2). The compound showed[9] $T = 0.72$ (OV-225 column at 190°).

Oxidation of the terminal D-*galactose residue in S 14, and subsequent uronic acid degradation of the polysaccharide.* — S 14 (33 mg) in 0.02M sodium phosphate buffer (pH 7.0, 10 ml) was treated under toluene with D-galactose oxidase (KABI, Stockholm, Sweden) (500 units) and horseradish peroxidase (Sigma) (160 units)[14,15]. After 72 h at room temperature, iodine (85 mg) and sodium carbonate (110 mg) in water (17 ml) were added[15], and the solution was stirred for 4 h and then extracted (30 min with stirring) with an equal volume of 80% aqueous (w/w) phenol. After centrifugation, the phases were separated, and the aqueous layer was carefully removed and dialysed overnight against running tap-water. The modified polysaccharide (30 mg) was recovered by freeze-drying, and sugar analysis of a part (2 mg) showed galactose and glucose (0.72:1) and 2-acetamido-2-deoxy-D-glucose. The remainder of the polysaccharide was methylated with methyl iodide, and a portion (25%) was hydrolysed, and analysed after conversion of the partially methylated sugars into alditol acetates. 2,4,6-Tri-*O*-methyl-D-galactose, 2,3,6-tri-*O*-methyl-D-glucose, and 2-deoxy-3-*O*-methyl-2-(*N*-methylacetamido)-D-glucose were detected; the neutral sugar ethers were in the ratio 0.96:1. The absence of 2,3,4,6-tetra-*O*-methyl-D-galactose, the proportions of tri-*O*-methyl sugars, and the sugar analysis of unmethylated material showed that oxidation had been confined to the terminal galactose residue and was essentially complete. The remainder of the methylated material (75%) was dried over phosphorus pentaoxide, taken up in methanol–2,2-dimethoxypropane–dichloromethane (18:1:2, 20 ml), and the solution boiled under reflux for 40 min in the presence of toluene-*p*-sulfonic acid (5 mg), giving a clear, yellow solution. Freshly cut sodium (250 mg) was added, boiling was continued for 2 h, the solution was neutralised with 50% aqueous acetic acid and poured into water (50 ml), and the product was extracted into chloroform (3 × 25 ml). The combined extracts were washed with water (25 ml) and evaporated to dryness, and the residue was hydrolysed at 100° for 1 h in 50% aqueous acetic acid. After dialysis, the solution was freeze-dried, and the residue was remethylated using trideuteriomethyl iodide, hydrolysed, and analysed as partially methylated alditol acetates The retention time on g.l.c. and the mass spectrum of the *O*-methyl-*O*-trideuteriomethyl-D-glucosamine derivative were identical with those of an authentic sample of 2-deoxy-3,4-di-*O*-methyl-2-(*N*-methylacetamido)-D-glucitol except that, in the mass spectrum, shifts of three mass units were seen in fragments containing the trideuteriomethoxyl group. The following fragments were observed, *inter alia*: *m/e* 43 (100), 45 (8), 56 (11), 74 (45), 87 (12), 90 (16), 98 (42), 99 (19), 102 (30), 116 (99), 128 (8), 132 (28), 142 (28), 158 (39), 176 (6), 192 (6), 197 (3), 202 (10), 236 (4), 249 (1), and 259 (2)

Smith degradation of the polysaccharide. — S 14 (50 mg) in 0.1M sodium acetate buffer (pH 3.9, 75 ml) was treated with 0 2M sodium metaperiodate (25 ml) in the dark at 5° for 120 h. Excess of periodate was reduced by the addition of ethylene glycol (4 ml), the mixture was dialysed and concentrated to 100 ml, and sodium borohydride (500 mg) was added. After stirring overnight at room temperature, excess of borohydride was decomposed by adding 50% acetic acid, and the solution was dialysed and freeze-dried. Sugar analysis of part of the material (10%) showed the oxidation to have been nearly complete; D-galactose and D-glucose (ratio 12:1) and 2-acetamido-2-deoxy-D-glucose were obtained. The remaining material (90%) was methylated with methyl iodide, and the product recovered by dialysis. Part (25%) was hydrolysed, giving 2,3,6-tri-*O*-methyl-D-galactose and 2-deoxy-3-*O*-methyl-2-(*N*-methylacetamido)-D-glucose, which were detected as their alditol acetates. The remaining methylated material (75%) was treated with 90% formic acid (5 ml) for 1 h at 40°, and the solution was evaporated to dryness with repeated additions of water. The product was remethylated with trideuteriomethyl iodide, recovered by partitioning between chloroform and water, and examined by g l.c. Permethylated β-D-GlcNAc*p*-(1→3)-β-D-Gal*p*-(1→2)-erythritol (trideuteriomethylated at positions 4 and 6 of 2-acetamido-2-deoxy-D-glucose and position 3 of erythritol) was obtained. The compound had T_{LAC} 11.7 (retention time relative to permethylated lactitol on an OV-1 column at 250°), and its mass spectrum showed, *inter alia*, the following peaks: *m/e* 45 (84), 71 (100), 74 (44), 75 (25), 85 (10), 87 (20), 92 (35), 98 (16), 101 (44), 104 (18), 107 (11), 111 (20), 114 (16), 115 (52), 118 (13), 127 (41), 129 (22), 145 (14), 150 (10) [cA$_1$], 155 (4) [bA$_3$], 157 (16), 159 (38), 161 (12), 182 (8), 187 (6) [bA$_2$], 192 (9), 199 (6) [aA$_3$], 210 (7) [bcJ$_1$], 234 (43) [aA$_2$], 266 (25) [aA$_1$], 289 (6), and 322 (1).

A part of the permethylated trisaccharide alditol was hydrolysed, and the products were analysed as alditol acetates. The products were 2,4,6-tri-*O*-methyl-D-galactose, 1,3,4-tri-*O*-methyl-D-erythritol (trideuteriomethylated at O-3), and 2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-D-glucose (trideuteriomethylated at O-4 and O-6): the latter gave in its mass spectrum, *inter alia*, the following fragments: *m/e* 43 (87), 48 (25), 56 (11), 74 (43), 90 (11), 98 (38), 99 (12), 116 (100), 132 (32), 142 (36), 151 (35), 158 (40), 167 (10), 202 (15), 211 (15), 249 (1), and 250 (1).

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REFERENCES

1 M. J. How, J. S. Brimacombe, and M. Stacey, *Adv. Carbohydr. Chem.*, 19 (1964) 303–358.
2 O. Larm and B. Lindberg, *Adv. Carbohydr. Chem. Biochem.*, 33 (1976) 295–321.
3 G. W. G. Bird, *Nature (London)*, 187 (1960) 415–416.
4 G. Uhlenbruck, G. I. Pardoe, and G. W. G. Bird, *Naturwissenschaften*, 55 (1968) 347; *Z. Immunitaetsforsch.*, 137 (1969) 442–457.
5 S. Ebisu, J. Lonngren, and I. J. Goldstein, *Carbohydr. Res.*, following paper.
6 J. S. Sawardeker, J. H. Sloneker, and A. R. Jeanes, *Anal. Chem.*, 37 (1965) 1602–1604.
7 B. A. Dmitriev, L. V. Backinowsky, V. L. Lvov, N. K. Kochetkov, and I. L. Hofman, *Eur. J. Biochem.*, 50 (1975) 539–547.
8 C. Erbing, K. Granath, L. Kenne, and B. Lindberg, *Carbohydr. Res.*, 47 (1976) C5–C7.
9 H. Bjorndal, C. G. Hellerqvist, B. Lindberg, and S. Svensson, *Angew. Chem. Int. Ed. Engl.*, 9 (1970) 610–619.
10 J. Lonngren and S. Svensson, *Adv. Carbohydr. Chem. Biochem.*, 29 (1974) 41–106.
11 K. Stellner, H. Saito, and S. Hakomori, *Arch. Biochem. Biophys.*, 155 (1973) 464–472.
12 G. O. H. Schwarzmann and R. W. Jeanloz, *Carbohydr. Res.*, 34 (1974) 161–168.
13 L. Kenne, B. Lindberg, K. Petersson, and E. Romanowska, *Carbohydr. Res.*, 56 (1977) 363–370
14 G. Avigad, D. Amaral, C. Asensio, and B. L. Horecker, *J. Biol. Chem.*, 237 (1962) 2736–2743.
15 J. K. Rogers and N. S. Thompson, *Carbohydr. Res.*, 7 (1968) 66–75.
16 B. Lindberg, J. Lonngren, and J. L. Thompson, *Carbohydr. Res.*, 28 (1973) 351–357.
17 S. Hakomori, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
18 B. Lindberg, J. Lonngren, and S. Svensson, *Adv. Carbohydr. Chem. Biochem.*, 31 (1975) 185–239.
19 W. M. Watkins, in A. Gottschalk (Ed.), *Glycoproteins*, Elsevier, Amsterdam, 1972, pp. 830–891.
20 M. J. Watson and J. Baddiley, *Biochem. J.*, 137 (1974) 399–404.