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# THE STRUCTURE OF THE CAPSULAR ANTIGEN FROM *Haemophilus influenzae* TYPE A

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## ABSTRACT

Structural investigation of the capsular antigen from *Haemophilus influenzae* type a has shown it to be composed of 4-*O*-β-D-glucopyranosyl-D-ribitol residues joined through phosphoric diester linkages between O-4 of D-glucose and O-5 of D-ribitol. Chemical degradations and $^{13}$C-n.m.r. spectroscopy were the main methods used.

## INTRODUCTION

The capsular antigens from different types of *Haemophilus influenzae* have been investigated[1–3], and the structure proposed for the type b antigen[3] has recently been revised[4,5]. It is a teichoic acid composed of chains of 1-*O*-β-D-ribofuranosyl-D-ribitol residues joined through phosphoric diester groups at O-3 of D-ribose and O-5 of D-ribitol. According to previous studies[1], the antigen from *H. influenzae* type a should be composed of D-glucose and phosphate. We now report structural studies on this antigen.

## RESULTS AND DISCUSSION

The capsular antigen from *H. influenzae* type a showed $[\alpha]_D$ −24°. On acid hydrolysis, it yielded D-glucose, ribitol, and 1,4-anhydroribitol (analysed as their alditol acetates[6,7]), together with inorganic phosphate. 1,4-Anhydroribitol is an artefact, formed, for example, when ribitol is treated with acid. Sugar and phosphate analyses indicated the antigen to be composed of equimolecular amounts of D-glucose, ribitol, and phosphate.

Treatment of the antigen first with alkali and then with alkaline phosphatase gave two components (*A* and *B*) that were isolated by paper chromatography. M.s. of *A*, as its *O*-trimethylsilyl derivative, demonstrated it to be a 2-*O*-hexopyranosyl-pentitol[8]. It showed $[\alpha]_D$ −22°, and m.p. 135–138°, and gave an acetate that melted

at 99–100°, with rearrangement of its crystal structure at 82–83°. The acetate was indistinguishable from authentic 4-*O*-β-D-glucopyranosyl-D-ribitol octa-acetate[9]. Component *B*, obtained after alkaline hydrolysis and subsequent dephosphorylation, was 2,5-anhydro-4-*O*-β-D-glucopyranosyl-D-ribitol, and its structural elucidation is discussed below.

On periodate oxidation, the antigen consumed 3 mol. of periodate with the formation of 1.5 mol. of formic acid and 0.9 mol. of formaldehyde. Although these figures may not be very accurate due to the small amount of material available for the analyses, they suggest that O-1 and O-2 of D-ribitol moieties in the polymer are unsubstituted. The periodate-oxidized material was reduced with sodium borohydride, hydrolysed with acid, reduced again with sodium borodeuteride, acetylated, and investigated by g.l.c.–m.s.[6,7]. Comparable amounts of glycerol triacetate and erythritol tetra-acetate were obtained, neither of which contained a deuterium label. In order to account for the formation of erythritol from the oxidized and reduced material, phosphate must be linked either to O-4 of D-glucose or to O-3 of D-ribitol in the untreated polymer.

Application of $^{13}$C-n.m.r. spectroscopy has proved valuable in structural studies of teichoic acids and related polymers[4,10,11], and the technique was of use in this investigation. The $^{13}$C-n.m.r. spectral data for the intact antigen, and for ribitol, methyl β-D-glucopyranoside, and 4-*O*-β-D-glucopyranosyl-D-ribitol as reference compounds, are given in Table I. Signals from 4-*O*-β-D-glucopyranosyl-D-ribitol were assigned to the different carbon atoms by the close correspondence of

TABLE I

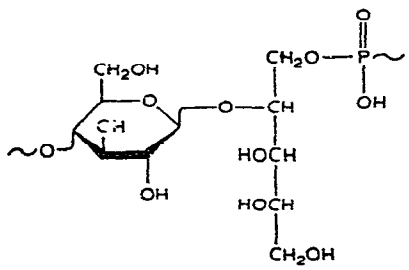
$^{13}$C-N.M.R. SHIFTS OF *H. influenzae* TYPE A ANTIGEN AND RELEVANT REFERENCE SUBSTANCES

| *Compound* | *Chemical shifts (p.p.m.)* | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *C-1* | *C-2* | *C-3* | *C-4* | *C-5* | *C-6* | *C'-1* | *C'-2* | *C'-3* | *C'-4* | *C'-5* |
| Ribitol | | | | | | | 63.2 | 72.9 | 72.9 | 72.9 | 63.5 |
| Methyl β-D-glucopyranoside | 103.9 | 73.8 | 76.5[a] | 70.4 | 76.6[a] | 61.5 | 57.9 | | | | |
| 4-*O*-β-D-Glucopyranosyl-D-ribitol | 103.1 | 74.1 | 76.4[a] | 70.4 | 76.6[c] | 61.5[b] | 63.5 | 73.7[c] | 72.2[c] | 81.7 | 61.1[b] |
| *H.i.* type a antigen | 102.9 | 74.1 | 75.6[a,d] | 74.8[e] | 75.8[a,d] | 61.4 | 63.5 | 72.6[e] | 72.4[e] | 80.1[f] | 65.7[g] |

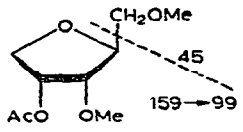
[a,b,c]The asignments may be reversed. [d]Coupled signals, coupling constants not determined. [e]$^2J^{31}P\_^{13}C = 6$ Hz. [f]$^3J^{31}P\_^{13}C = 6.5$ Hz. [g]$^2J^{31}P\_^{13}C = 5$ Hz.

their chemical shifts with those of methyl β-D-glucopyranoside and ribitol, respectively. The main differences noted were the expected downfield-shift (8.8 p.p.m.) of C-4 in ribitol[12], and the smaller upfield-shift (2.1 p.p.m.) of C-5 in the same residue. The antigen itself gave 11 signals, as expected for a polymer composed of disaccharide repeating-units. The downfield shift of C-4 in the β-D-glucopyranosyl moiety

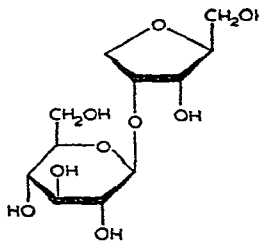
(4.4 p.p.m.), and the $^{31}P$–$^{13}C$ coupling constants observed for C-3, C-4, and C-5, demonstrated that phosphate was linked to O-4 of this unit. The downfield shift of the C-5 signal in D-ribitol (4.6 p.p.m.) and the coupling constants observed for C-4 and C-5 also demonstrated that phosphate was linked to O-5 of this moiety. On the basis of this evidence, the repeating unit **1** is proposed for the antigen from *H. influenzae* type a.




1




2




3

Component *B* formed on alkaline hydrolysis and dephosphorylation of the antigen was examined further. On acid hydrolysis, it yielded 1,4-anhydroribitol and D-glucose. The mass spectrum of its *O*-trimethylsilyl derivative was in agreement with that expected for a glucopyranosyl-(1→4)-anhydroribitol, and 2,3,4,6-tetra-*O*-methyl-D-glucose and 1,4-anhydro-3,5-di-*O*-methylribitol were the only compounds obtained on methylation analysis. Some pertinent fragments in the mass spectrum of the acetate of the latter methyl ether are indicated in formula **2**. As the D-glucopyranosyl group is linked to O-4 of D-ribitol in the antigen, compound *B* is consequently 2,5-anhydro-4-*O*-β-D-glucopyranosyl-D-ribitol (**3**). The 2,5-anhydro-D-ribitol moiety is most probably formed during treatment of the antigen with base, by nucleophilic attack of O-2, as the alkoxide, upon C-5. The possibility of such reactions occurring has been discussed[13].

From these studies, the *Haemophilus influenzae* type a antigen is therefore closely related to the ribitol teichoic acids found in bacterial cell-walls except that the ribitol residues in the latter[13] are linked together through phosphoric diester linkages to O-1 and O-5. Likewise, antigens from several pneumococci have related, but more-complex, structures[14].

## EXPERIMENTAL

*General methods.* — Solutions were concentrated under reduced pressure at bath temperatures not exceeding 40°. For g.l.c., a Perkin–Elmer 990 instrument fitted with flame-ionisation detectors was used in conjunction with a Hewlett–Packard 3370B integrator. Separations were performed on glass columns (180 × 0.15 cm) containing 3% of ECNSS-M on Gas Chrom Q, at 200° (alditol acetates) and at 170° (partially methylated alditol acetates), and 3% of OV-17 on Gas Chrom Q at 220°

(trimethylsilylated derivatives). For g.l.c.–m.s., a Varian MAT-311-SS 100, m.s.–computer system was used. Spectra were recorded at 70 eV and an ion-source temperature of 220°. For analytical and preparative paper chromatography, Whatman No. 1 and No. 3 papers were used with ethyl acetate–acetic acid–water (3:1:1) as the solvent system. Alkaline silver nitrate and ammonium molybdate were used for detection. Alkaline phosphatase (1.1 unit/mg) was obtained from Sigma Chemical Company.

*Preparation of the antigen.* — The capsular antigen from *Haemophilus influenzae* type a, strain Smith, was prepared as previously described[15]. It was purified by filtration through Sephadex G-100, followed by fractionation on a column (5 × 34 cm) of DEAE-Sephadex A-25 with a potassium acetate gradient (0.1–4M, pH 6). The antigen was eluted at 2–2.5M potassium acetate. The purified polymer showed $[\alpha]_D^{25}$ −24° (*c* 0.40, water) and contained 6.5% of P. An acid hydrolysate of the polymer (0.25M sulfuric acid, 100°, 16 h), analysed as alditol acetates[6,7], contained 1,4-anhydroribitol, ribitol, and glucose in the approximate molar proportions 0.57:0.33:1.0.

*Isolation and characterization of oligosaccharides.* — The antigen (40 mg) was treated with M aqueous sodium hydroxide, as described by Armstrong *et al.*[16]. The product was treated with alkaline phosphatase (4 mg) in a sodium hydrogen carbonate buffer of pH 10.4 (3 ml) for 5 days at 37°. The enzymic hydrolysate was passed through a mixed bed (15 ml) made from equal parts of Dowex 50($H^+$) and Dowex 1($HO^-$) resins. The eluate was concentrated to dryness and the residue (15 mg) fractionated by paper chromatography.

A component (7 mg) with $R_{GLC}$ 0.9, m.p. 135–138°, $[\alpha]_D^{25}$ −22° (*c* 0.35, water), after trimethylsilylation, gave a single peak on g.l.c. The mass spectrum displayed, *inter alia*, the following peaks: 73 (59), 74 (5), 75 (9), 103 (18), 129 (38), 147 (22), 189 (5), 191 (11), 204 (100), 205 (27), 206 (11), 217 (26), 218 (7), 243 (15), 271 (3), 272 (1), 305 (3), 306 (1), 307 (1), 319 (4), 331 (1), 333 (4), 334 (2), 361 (12), 362 (3), 363 (2), and 423 (1). The acetate, prepared by conventional treatment with acetic anhydride in pyridine, melted at 99–100° with rearrangement of the crystalline structure at 82–83°. A mixture with authentic 4-*O*-β-D-glucopyranosyl-D-ribitol octa-acetate showed the same behaviour.

A second component (5 mg), with $R_{GLC}$ 1.1, $[\alpha]_D^{25}$ −47° (*c* 0.15, water), yielded glucose and 1,4-anhydroribitol on hydrolysis. Its trimethylsilyl derivative gave a single peak on g.l.c. and, *inter alia*, the following peaks on m.s.: 73 (74), 74 (7), 75 (15), 93 (7), 103 (15), 117 (5), 129 (11), 147 (16), 171 (4), 189 (6), 191 (8), 204 (100), 205 (22), 206 (10), 217 (16), 218 (6), 261 (3), 271 (2), 305 (2), 319 (1), 361 (4), and 379 (2). Methylation analysis of the substance, with analysis of the products after borohydride reduction and acetylation[17], yielded 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol and 2-*O*-acetyl-1,4-anhydro-3,5-di-*O*-methylribitol. M.s. for the latter component showed, *inter alia*, ions at: 43 (100), 45 (51), 59 (14), 71 (39), 87 (49), 99 (38), 101 (10), and 159 (14).

*Periodate-oxidation studies.* — The polymer (12 mg) in 25mM sodium meta-

periodate (8 ml) was kept at 25°. Consumption of periodate was followed spectrophotometrically[18] at 223 nm and was complete after 48 h, when 3.0 mol. per disaccharide repeating-unit had been consumed. The formic acid (1.5 mol.) and formaldehyde (0.9 mol.) produced were determined by titration with 0.01M sodium hydroxide[18] and by reaction with chromotropic acid[19], respectively. Part of the oxidized material (1/3) was reduced with sodium borohydride (20 mg) for 14 h, neutralised with Dowex 50($H^+$) resin, filtered, codistilled with methanol (3 × 3 ml), and hydrolysed with 0.25M sulfuric acid (3 ml) for 16 h at 100°. The hydrolysate was neutralised with barium carbonate, filtered, and treated with sodium borodeuteride (10 mg) for 2 h. After neutralisation with Dowex 50($H^+$) resin and codistillation with methanol, the material was acetylated with acetic anhydride–pyridine (0.5 ml; 1:1) at 100° for 30 min. The product was analysed by g.l.c.–m.s. and gave equal amounts of glycerol and erythritol acetates, neither of which contained a deuterium label.

*$^{13}$C-N.m.r. spectroscopy.* — $^{13}$C-N.m.r. spectra were recorded with a Varian XL-100 spectrometer operating in the pulsed Fourier-transform mode at 25.2 MHz, totally proton-decoupled. Samples were dissolved in deuterium oxide and studied in 5-mm tubes at 25°. Chemical shifts were determined relative to an internal standard of methanol, and are given in p.p.m. from $Me_4Si$ ($\delta_{Me_4Si} = \delta_{MeOH} + 49.7$).

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