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STRUCTURAL STUDIES OF TWO CAPSULAR POLYSACCHARIDES ELABORATED BY DIFFERENT STRAINS OF *Haemophilus influenzae* TYPE e

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ABSTRACT

The structures of two capsular polysaccharides elaborated by *Haemophilus influenzae* type e, strains NCTC 8455 and 8472, respectively, have been investigated, methylation analysis and n.m.r. spectrometry being the principal methods used. It is concluded that the polysaccharides are composed of repeating-units having the following structure:

$$\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{NAc-}(1\rightarrow 4)\text{-}\beta\text{-D-Man}p\text{NAcA-}(1\rightarrow$$
$$\begin{array}{c} 3 \\ \uparrow \\ 2 \\ \beta\text{-D-Fru}f \end{array}$$

In the polysaccharide from strain NCTC 8472, all of the repeating-units contain the β-D-fructofuranosyl group. The polysaccharide from strain NCTC 8455, however, contains only traces of D-fructose, corresponding to approximately one group per 100 repeating-units.

INTRODUCTION

Haemophilus influenzae is divided into six types (a–f) which produce different, type-specific, capsular antigens. Four of these (a, b, c, and f) are of the teichoic acid type and consist of disaccharides joined through phosphoric diester linkages. The other two, from types d[1] and e[2], are polysaccharides, according to studies by Zamenhof and co-workers.

Earlier investigators[3,4] have observed that two different antigens are produced by different strains of type e. One of these antigens, from strain NCTC 8472, is converted into the other by hydrolysis with acid under mild conditions[4]. In the light of the present investigation, the type e antigen investigated by Zamenhof and co-

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workers[2] was most probably the former. They found that it was composed of 2-amino-2-deoxy-D-glucose and an unidentified hexose (not D-glucose, D-galactose, or D-mannose). We now report structural studies of the two different *H. influenzae* type e antigens.

RESULTS AND DISCUSSION

The two capsular antigens were isolated from cultures of *H. influenzae* type e, strains NCTC 8455 and 8472, respectively, by precipitation with cetyltrimethylammonium bromide, followed by chromatography on DEAE-Sepharose.

The antigen from NCTC 8455, here called 8455, had $[\alpha]_{578}$ −30°. The ^{1}H-n.m.r. spectrum contained, *inter alia*, signals for *N*-acetyl groups at δ 1.99 (s, 6 H) and for anomeric protons at δ 4.52 (d, 1 H, J 8 Hz) and 4.78 (d, 1 H, J low). In addition to the signals for two *N*-acetyl groups, the ^{13}C-n.m.r. spectrum showed 12 distinct signals (Table I). Of these, the signals from one carboxyl carbon, two anomeric carbons, one -CH_2OH carbon, and two carbons substituted with nitrogen were readily identified.

Sugar analysis of 8455 gave 2-amino-2-deoxy-D-glucose, proved to have the D configuration as discussed below. These results therefore suggest that 8455 is composed of disaccharide repeating-units containing one 2-amino-2-deoxy-D-glucose and one aminodeoxyhexuronic acid residue. Methylation analysis yielded 2-deoxy-4,6-di-*O*-methyl-2-methylacetamido-D-glucose, demonstrating that the 2-acetamido-2-deoxy-D-glucosyl residue is pyranosidic and linked through O-3.

The polysaccharide reacted sluggishly upon carboxyl reduction by the method of Taylor *et al.*[5]. After four consecutive reductions (see Experimental), only ~65% of the carboxyl groups had been reduced. Acid hydrolysis of the product, prepared using sodium borodeuteride, yielded a mixture of 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-mannose-*6*-d_2. The latter, both as the product obtained on acetylation and as the alditol acetate, was indistinguishable from the corresponding, authentic materials in g.l.c. M.s. of the alditol acetate demonstrated the indicated deuterium-labelling. The two sugars, as the *N*-acetyl derivatives, were proved to have the D configuration by g.l.c. of their trimethylsilylated (−)-2-butyl glycosides, as devised by Gerwig *et al.*[6].

Methylation analysis of the carboxyl-reduced polysaccharide yielded 2-acetamido-2-deoxy-3,6-di-*O*-methyl-D-mannose in addition to 2-deoxy-4,6-di-*O*-methyl-2-methylacetamido-D-glucose. The 2-acetamido-2-deoxy-D-mannuronic acid residue in 8455 is consequently either pyranosidic and linked through O-4 or, less likely, furanosidic and linked through O-5.

It is evident from the chemical shifts of the anomeric protons and carbons in the ^{1}H- and ^{13}C-n.m.r. spectra, respectively, that both sugar residues in the repeating-unit of 8455 are pyranosidic and β-linked. The signals for the anomeric carbons, at 102.1 and 101.4 p.p.m., respectively, showed $^1J_{^{13}C,H}$ of 162 and 164 Hz, thus further demonstrating the β configuration of the two sugar residues[7].

TABLE I

SIGNALS IN THE ^{13}C-N.M.R. SPECTRA OF THE CAPSULAR ANTIGENS FROM *H. influenzae* TYPE c AND RELEVANT REFERENCE SUBSTANCES

Substance	*2-Acetamido-2-deoxy*-D-*glucose*						*2-Acetamido-2-deoxy*-D-*mannuronic acid*					
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
Strain NCTC 8455, pD 2	102.5	56.2	84.4	70.4	77.3	62.5	101.3	53.9	72.1	79.3	77.3	173.6
Strain NCTC 8455, pD 8	102.1	55.9	84.6	70.5	77.4	62.4	101.4	54.4	72.2	79.5	78.8	176.0[a]
Strain NCTC 8472, pD 8	101.6	55.9	84.8	71.0	78.0	62.9	101.2	55.1	75.9	79.4	79.4	176.2[a]
Strain NCTC 8472, partially hydrolysed, pD 8	102.0	55.9	84.6	70.5	77.3	62.4	101.4	54.3	72.3	79.4	78.9	175.9[a]
Me β-D-Glc*p*NAc[8]	103.2	56.7	75.2	71.2	77.1	62.0						
β-D-Man*p*NAcA[b]							94.3	54.9	72.8	69.3	76.2	—

Substance	D-*Fructose*						N-*Acetyl*		O-*Methyl*
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C=O*	*-CH₃*	*-CH₃*
Strain NCTC 8455, pD 2							175.9, 176.5	23.8, 24.1	
Strain NCTC 8455, pD 8							176.0, 176.5[a]	23.8, 24.1	
Strain NCTC 8472, pD 8	63.2	105.4	79.4	75.2	83.3	64.8	175.9[a], 176.2	23.8, 24.1	
Strain NCTC 8472, partially hydrolysed, pD 8							175.9, 176.4[a]	23.8, 24.1	
Me β-D-Glc*p*NAc[8]							176.1	23.3	58.3
β-D-Man*p*NAcA[b]							176.8	23.2	
Me β-D-Fru*f*[14]	60.0	104.7	77.7	75.9	82.1	63.6			49.8

[a]The assignments of these signals may be reversed. [b]Estimated as described in the text.

Assignments of most of the signals in the ^{13}C-n.m.r. spectrum of 8455 could be made by comparison with the spectra of the configurationally related methyl 2-acetamido-2-deoxy-β-D-glucopyranoside[8] and 2-acetamido-2-deoxy-β-D-mannopyranuronic acid. The latter spectrum was estimated from that of 2-acetamido-2-deoxy-β-D-mannose[9], assuming that the differences of the chemical shifts are the same as those between methyl β-D-glucopyranoside and methyl β-D-glucopyranosiduronic acid[10].

The signals from C-6 and C-5 of the uronic acid residue were identified from the shifts of these signals on changing the pD of the polysaccharide solution. The low-field signal at 84.6 p.p.m. is typical for C-3 of a 3-*O*-substituted glycopyranosyl residue having the *β-gluco* configuration[11]. The absence of the high-field signal (δ 69.3) for C-4 of the 2-acetamido-2-deoxy-β-D-mannopyranosyluronic acid residue and the presence of a signal at δ 79.5 strongly support the conclusion that this residue is substituted at O-4.

From the combined evidence, it is inferred that 8455 is composed of disaccharide repeating-units having the structure **1**.

→3)-β-D-Glc*p*NAc-(1→4)-β-D-Man*p*NAcA-(1→

1

The antigen from strain NCTC 8472, here called 8472, showed $[\alpha]_{578}$ −22°. The ^{1}H-n.m.r. spectrum contained, *inter alia*, signals for *N*-acetyl groups at δ 1.98 (s, 3 H) and 2.04 (s, 3 H), and for anomeric protons at δ 4.52 (d, 1 H, *J* 8 Hz) and 4.76 (d, 1 H, *J* low). The ^{13}C-n.m.r. spectrum (Table I) showed, *inter alia*, signals for two *N*-acetyl groups and three anomeric carbons.

Hydrolysis of 8472 with acid under mild conditions released a component (30%), which was identified as D-fructose by its reduction to a mixture of mannitol and glucitol, its optical rotation, $[\alpha]_{578}$ −86°, and its ^{13}C-n.m.r. spectrum. The remaining material, $[\alpha]_{578}$ −30°, gave a ^{13}C-n.m.r. spectrum indistinguishable from that given by 8455 (Table I).

Fully methylated 8472 was hydrolysed with acid under mild conditions and the sugar released was reduced ($NaBD_4$) and acetylated. G.l.c.–m.s. of the product showed it to be a 2,5-di-*O*-acetyl-1,3,4,6-tetra-*O*-methylhexitol-*2-d*. The expected glucitol and mannitol derivatives are not separated on the column used[12]. When the methylated polysaccharide was hydrolysed under stronger conditions, 2-deoxy-4,6-di-*O*-methyl-2-methylacetamido-D-glucose was released. These results therefore indicate that 8455 and 8472 contain the same linear backbone (**1**) and that, in 8472, D-fructofuranosyl groups are linked to O-3 of the uronic acid residues.

In the ^{13}C-n.m.r. spectrum of 8472 (Table I), six signals, assigned to the D-fructofuranosyl group, were sharper than the other signals. An analogous result has also been reported for the more-mobile terminal residue of a branched mannan[13]. The chemical shifts of these signals agreed much better with those reported for methyl β-D-fructofuranoside[14] (and other β-D-fructofuranosyl groups) than with the signals for the corresponding α-D-glycoside. The difference between the anomers is most

pronounced for the anomeric carbon, 109.1 p.p.m. for the α form and 104.7 p.p.m. for the β form. The only significant shift (3.6 p.p.m.) when going from 8455 to 8472 is that for C-3 in the uronic acid residue, indicating that the β-D-fructofuranosyl group is linked to that position. Substitution effects of this magnitude for β-D-fructofuranosyl groups have been observed[14,15].

Four consecutive carboxyl-reductions of 8472, using sodium borodeuteride, gave a product in which ~60% of the carboxyl groups had been reduced. Acid hydrolysis of this product yielded 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-mannose-6-d_2, which were identified as described above. On methylation analysis of the carboxyl-reduced polysaccharide, 2-deoxy-4,6-di-*O*-methyl-2-methylacetamido-D-glucose and 2-deoxy-6-*O*-methyl-2-methylacetamido-D-mannose-6-d_2 were obtained. When the carboxyl-reduced polysaccharide was treated with acid under mild conditions and then subjected to methylation analysis, the latter component was replaced by 2-acetamido-2-deoxy-3,6-di-*O*-methyl-D-mannose-6-d_2. From the combined results, it is concluded that the capsular antigen from *H. influenzae* type e, strain NCTC 8472, is composed of trisaccharide repeating-units having the structure **2**.

$$\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{NAc-}(1\rightarrow 4)\text{-}\beta\text{-D-Man}p\text{NAcA-}(1\rightarrow$$
$$\begin{array}{c} 3 \\ \uparrow \\ 2 \\ \beta\text{-D-Fru}f \end{array}$$

2

The observation that the antigen elaborated by some strains of *H. influenzae* type e, *e.g.*, NCTC 8472, is converted into the antigen elaborated by other strains, *e.g.*, NCTC 8455, on mild hydrolysis with acid is due to the facile cleavage of the β-D-fructofuranosidic linkages present in the former antigen. Immunological studies, however, revealed a slight cross-reaction between the antigens from NCTC 8455 and 8472. When the former was subjected to mild hydrolysis with acid, a small amount of D-fructose was released. The results indicate that the fructose is linked as in **2**, and although corresponding to only one β-D-fructofuranosyl group in 100 repeating-units, this should be sufficient to account for the observed cross-reaction.

Homopolysaccharides composed of β-D-fructofuranosyl residues are common in Nature, but the occurrence of D-fructose in a heteropolysaccharide is an unusual feature. It has, however, been found in the *Vibrio cholerae* lipopolysaccharide[16], but not as a component of the O-antigen, the structure of which has been determined[17]. It seems possible that D-fructose is the unknown hexose observed by the previous investigators[2] and that the 2-acetamido-2-deoxy-D-mannuronic acid was overlooked. 2-Acetamido-2-deoxy-D-mannuronic acid has been observed as a component of several bacterial polysaccharides. Thus the "common antigen" of Enterobacteriacae is composed of this acid and 2-acetamido-2-deoxy-D-glucose[18,19].

EXPERIMENTAL

General methods. — Concentrations were performed under diminished pressure at bath temperatures below 40°. G.l.c. was performed on glass columns (190 × 0.15 cm) containing (*a*) 3% of OV-225 on Gas Chrom Q (100–120 mesh) at 180° and (*b*) 3% of OV-17 on Gas Chrom Q at 190°, and on W.C.O.T. glass-capillary columns (25 m × 0.25 mm) containing (*c*) SP-1000 at 230° and (*d*) SE-30 with a temperature programme of 135→220° at 1°/min. G.l.c.–m.s. was performed with a Varian MAT 311SS 100 instrument at an ionisation potential of 70 eV. N.m.r. spectra for solutions in D_2O at 85° were recorded with a JEOL FX-100 spectrometer, using external tetramethylsilane (^{13}C) and internal sodium 1,1,2,2,3,3-hexadeuterio-4,4-dimethyl-4-silapentane-1-sulfonate (^1H) as references. The ^{13}C-n.m.r. spectra were run with complete proton-decoupling, using a pulse width of 13 μs (85° flip angle): acquisition time, 0.68 s: and pulse repetition time, 0.7 s. Optical rotations were determined with a Perkin–Elmer 241 instrument.

Preparation of the antigens. — The capsular antigens from *H. influenzae* type e. strains NCTC 8455 and 8472, were isolated from the culture filtrate by precipitation with 0.5% aqueous cetyltrimethylammonium bromide. The precipitates were dissolved in 2M aqueous sodium chloride and precipitated with ethanol (5 vol.). The antigens were dissolved in water, and recovered by dialysis against water and freeze-drying.

The 8455 antigen (540 mg) was further purified by chromatography on a column (80 × 2.6 cm) of DEAE-Sepharose CL-6B which was eluted first with water (700 ml) and then with a linear gradient of aqueous sodium chloride (1400 ml, 0→2M). The fractionation was monitored by optical rotation. The antigen was eluted as a single fraction at 0.6M sodium chloride, and the material (330 mg) was recovered by dialysis and freeze-drying. It had $[\alpha]_{578}$ −30° (*c* 0.1, water) and contained 1% of phosphorus, determined as described by Chen *et al.*[20].

The 8472 antigen (330 mg) was purified as described above, yielding 160 mg of material. It had $[\alpha]_{578}$ −22° (*c* 0.1, water) and contained 0.16% of phosphorus.

Carboxyl-reduction of the antigens. — Antigen (50 mg) was dissolved in water (7 ml) and the pH of the solution was adjusted to 4.75 with 0.1M hydrochloric acid. 1-Ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride (150 mg) was added and the pH was maintained at 4.75 by titration with 0.01M hydrochloric acid. After 1 h, no more acid was consumed. The pH was adjusted to 7 with 0.1M sodium hydroxide, and sodium borohydride (250 mg) was added. The solution was kept at 50° for 2 h. The pH was then adjusted to 4.75 and the procedure described above was repeated. The solution was left overnight at 50°, neutralised, dialysed, and freeze-dried. The extent of the reduction (~35%) was estimated from the ratio between 2-amino-2-deoxy-D-mannose and 2-amino-2-deoxy-D-glucose in the sugar analysis. The complete procedure described above was repeated. Analysis of the recovered polysaccharide (27 mg) showed that ~60% of the uronic acid residues had been reduced.

When the somewhat bulkier 1-cyclohexyl-3-(2-morpholinoethyl)carbodi-imide

metho-*p*-toluenesulfonate was used, the reduction was even less efficient. Some reductions were prepared as above, but with sodium borodeuteride.

Characterisation of sugar components. — Treatment of 8472 (25 mg) with 10% aqueous acetic acid (2 ml) at 100° for 3 h, followed by freeze-drying, and fractionation of the product on a column of Sephadex G-15, yielded a component (8.3 mg) of low molecular weight and a polymer (15.4 mg). The former had $[\alpha]_{578}$ −86° (*c* 0.2, water) and, on borohydride reduction, yielded mannitol and glucitol in the ratio 1 : 1.2, identified as their acetates by g.l.c. using columns *a* and *c*. The ^{13}C-n.m.r. spectra given by the sugar and authentic D-fructose were superposable. The polymer had $[\alpha]_{578}$ −30° (*c* 0.1, water), and its ^{13}C-n.m.r. spectrum and that of 8455 were superposable.

Hydrolysis of 8455 or 8472 (5 mg) was performed with 2M trifluoroacetic acid (1 ml) at 100° for 1 h, followed by concentration, *N*-acetylation, and repetition of the hydrolysis. The main sugar component was characterised by g.l.c.–m.s. (columns *b* and *c*) as the acetate mixture obtained with acetic anhydride–pyridine and as the alditol acetate, and was indistinguishable from the corresponding materials from authentic 2-amino-2-deoxy-D-glucose. When carboxyl-reduced 8455 or 8472 was analysed in the same way, 2-amino-2-deoxy-D-mannose was identified in addition to 2-amino-2-deoxy-D-glucose.

Determination of absolute configuration. — Using carboxyl-reduced 8455 or 8472, the absolute configurations of the sugars were determined by the method devised by *Gerwig et al.*[6].

Methylation analyses. — The method previously described[21,22] was used. Both *N*-methylacetamido and *N*-acetamido derivatives were obtained. In the text, either the major or the best-resolved peak is discussed.

Retention times for the amino sugar derivatives were not available. As the 2-amino-2-deoxy-D-mannose derivatives were only obtained after carboxyl-reduction ($NaBD_4$) and further were dideuterated on C-6, the distinction between *gluco* and *manno* derivatives caused no problems.

Complete hydrolysis of fully methylated polysaccharides was achieved by treatment with 90% formic acid followed by 2M trifluoroacetic acid at 100° for 2 h, *N*-acetylation (acetic anhydride–aqueous triethylamine), and repeated hydrolysis.

Methylation analyses of 8455, and of 8472 from which the fructofuranosyl groups had been hydrolysed, gave identical results. 2-Deoxy-4,6-di-*O*-methyl-2-methylamino-D-glucose and 2-amino-2-deoxy-3,6-di-*O*-methyl-D-mannose-*6*-d_2 were obtained, the latter only from carboxyl-reduced ($NaBD_4$) polysaccharide. In the mass spectrum of its alditol acetate, ions at *m/z* 47 and 235 (*cf.* *m/z* 45 and 233 for the corresponding non-deuterated derivative) demonstrated the indicated deuterium-labelling. The alditol acetates were well separated on column *c* (the *gluco* derivative being eluted first), but were incompletely separated on column *b*.

Fully methylated 8472 (5 mg) was dissolved in 50% aqueous acetic acid (2 ml) and kept at 100° for 1 h. The sugar released was reduced ($NaBD_4$) and acetylated. In g.l.c.–m.s., using column *a*, this derivative was indistinguishable from authentic

2,5-di-*O*-acetyl-1,3,4,6-tetra-*O*-methyl-D-glucitol, accounting for the deuterium labelling at C-2.

Methylation analysis of 8472, as described above for 8455, gave 2-deoxy-4,6-di-*O*-methyl-2-methylamino-D-glucose and 2-deoxy-6-*O*-methyl-2-methylamino-D-mannose-*6*-d_2, the latter only from carboxyl-reduced ($NaBD_4$) polysaccharide. In the mass spectrum of its alditol acetate, ions at *m/z* 47, 246, and 348 (*cf.* *m/z* 45, 244, and 346 for the corresponding non-deuterated derivative) demonstrated the indicated deuterium-labelling.

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