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# STRUCTURAL STUDIES OF THE *Haemophilus influenzae* TYPE e CAPSULAR POLYSACCHARIDE

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## ABSTRACT

The structure of the *Haemophilus influenzae* type e capsular polysaccharide has been determined by a combination of chemical and spectroscopic methods. The structure of the repeating unit of the polymer was found to be →3)-β-D-GlcNAc-(1→4)-β-D-ManANAc-(1→ ; both sugars were present in the pyranoid form.

## INTRODUCTION

Of the six *Haemophilus influenzae* serotypes, designated a through f, only one, type b, is a serious pathogen in humans, with meningitis being the most frequent disease encountered. Children, aged six months through three years, are the most susceptible group[1,2]. The polysaccharide capsule of the type b organism confers virulence on that organism; the remaining five capsular serotypes are only rarely associated with human disease[3]. The molecular basis for the virulence of type b organisms, as opposed to the non-virulence of the other serotype organisms, is not understood[4]. This study was, therefore, undertaken to gain such understanding through structural comparison of type b with other, non-virulent, capsular types. The structures of the type b (ref. 5), type a (ref. 6), type c (ref. 7), and type f (ref. 8) capsules have been reported. We present herein our findings with *H. influenzae* type e.

## RESULTS AND DISCUSSION

The capsular polysaccharide from *H. influenzae* type e, subsequent to drying over phosphorus pentaoxide *in vacuo*, had $[\alpha]_D^{22}$ −40° (*c* 0.05, $H_2O$). Following carbodiimide-mediated, carboxyl group reduction, and acid-catalyzed hydrolysis, only two monosaccharides were detectable in appreciable amounts, namely, 2-amino-2-deoxyglucose and 2-amino-2-deoxymannose. (The reduction is required prior to acid-catalyzed hydrolysis, as the product deriving from the 2-acetamido-2-deoxy-mannosyluronic residue in the polymer is unstable to the reaction conditions; *vide infra*). The two amino sugars were identified by their retention times in an automated, amino acid analyzer; the addition of samples of the authentic amino sugars (2-

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amino-2-deoxyglucose and 2-amino-2-deoxymannose) to the hydrolyzate did not result in the production of new or broadened peaks. The ratio of 2-amino-2-deoxyglucose to 2-amino-2-deoxymannose was ~1.0:0.8 after hydrolysis for 16 h.

Ninhydrin oxidation of the products in the hydrolyzate resulted in formation of the anticipated product, arabinose, identified as the alditol acetate by gas–liquid chromatography (g.l.c.). This arabinose possessed the D configuration, as determined by g.l.c. comparison with the set of diastereoisomers formed by glycosidation of D- and L-arabinose with *d*-2-octanol. Nitrous acid deamination of the hydrolyzate resulted in the appearance of D-glucose, identified by its retention time in an automated, neutral-sugar analyzer, and its disappearance (in the same analyzer) on treatment with D-glucose oxidase. It should, however, be noted that glucose was formed in

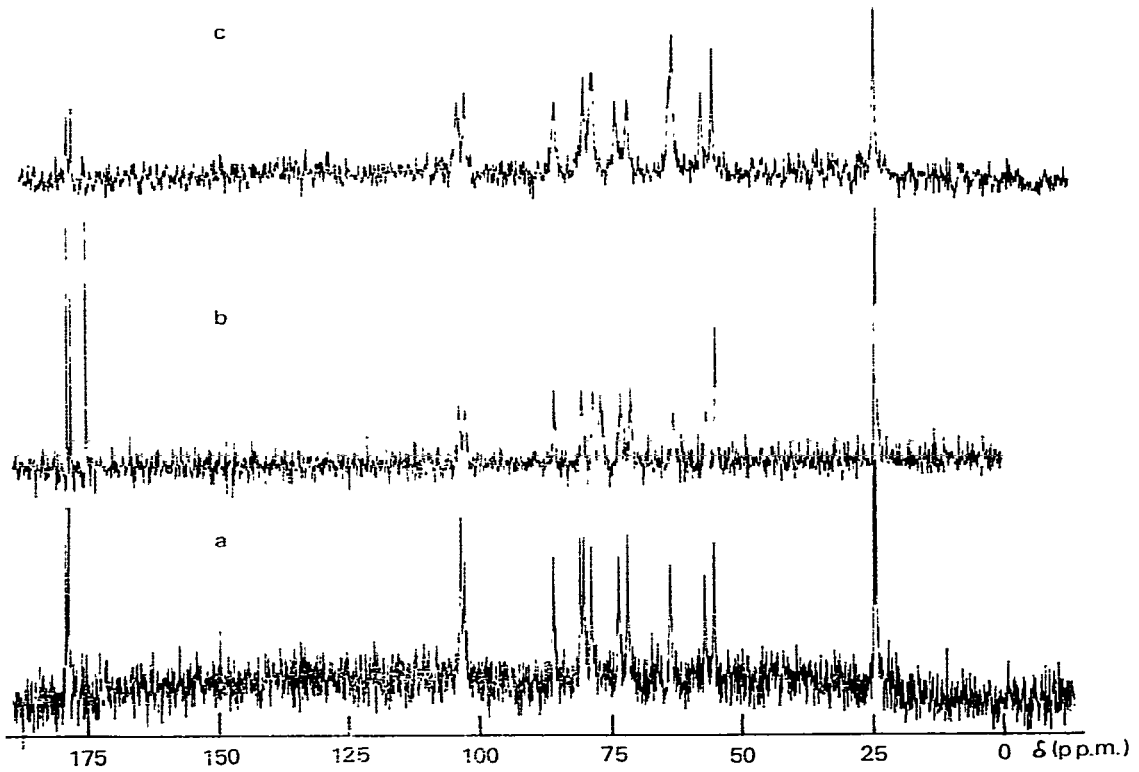



Fig. 1. $^{13}$C-N.m.r. spectra (25 MHz) of (a) *H. influenzae* type e polysaccharide at pH 7.0; (b) *H. influenzae* type e polysaccharide at pH 1.0; (c) CMCT–$NaBH_4$-reduced *H. influenzae* type e polysaccharide. [Samples (~30 mg) were dissolved in 1:19 $D_2O$–$H_2O$ (~0.4 mL) containing 0.01M EDTA and placed in 5-mm n.m.r. tubes. Spectra were recorded at 70°, in order to improve resolution. Approximately 30,000 transients were collected for each spectrum; spectra were obtained by employing 90° pulses (12 μs), 8192 data points, a 5-kHz spectral window, a 5-kHz filter, and a pulse repetition time of 2.0 s. Prior to Fourier transformation, the free-induction-decay signal was zero-filled with 8192 data points, and exponentially multiplied, resulting in an additional 1.0-Hz line-broadening in the frequency-domain spectrum. In a separate experiment, sodium 3-(trimethylsilyl)propanoate-*2,2,3,3-d*$_4$ (TSP) was added as an internal standard.]

only 17% of the theoretical yield, and, moreover, a peak that could be attributed to 2,5 anhydromannose, the anticipated product from deamination[9] of 2-amino-2-deoxyglucose, was not observed.

The native, *H. influenzae* type e polysaccharide is negatively charged at neutral pH, as evidenced by its anodic migration during immunoelectrophoresis, and by titration. Following acid-catalyzed hydrolysis, only 2-amino-2-deoxyglucose and a labile amino sugar (having a retention time shorter than that of 2-amino-2-deoxyglucose) were detected with the amino acid analyzer, and neutral sugars were not observed. After hydrolysis for 6 h, the ratio of 2-amino-2-deoxyglucose to labile amino sugar was 2.6:1.0; after 24 h, the ratio had changed to 12.7:1.0, with the absolute amount of 2-amino-2-deoxyglucose being virtually unchanged. The labile sugar was not identified. A 24-h hydrolyzate was subjected to analysis by g.l.c.; 2-amino-2-deoxyglucose was identified (as the trimethylsilyl derivative) by comparison with an authentic sample. Ninhydrin oxidation of the hydrolyzate resulted in arabinose having the D configuration, ascertained by the method described for the reduced polymer.

Based on these analyses, the *H. influenzae* type e polymer is a copolymer of 2-amino-2-deoxyglucose and 2-amino-2-deoxymannuronic acid, both sugars having the D configuration. The structural details of the polysaccharide were established by $^{1}H$- and $^{13}C$-nuclear magnetic resonance (n.m.r.) spectroscopy (the application of n.m.r. to structure elucidation in polysaccharides is reviewed in, for example, ref. 10).

The $^{13}C$-n.m.r. spectrum of the type e polysaccharide, at pH values of 7.0 and 1.0, is displayed in Fig. 1; the number and position of the resonances in the 50–110-p.p.m. region of the spectrum were indicative of a disaccharide repeating-unit of the composition already presented. Moreover, it may immediately be seen that both amino sugars are acetylated; two $-NC(O)CH_3$ resonances were observed in the ~24–25-p.p.m. region, and the corresponding $-NC(O)CH_3$ resonances in the ~177–178-p.p.m. region. The C-2 resonances of the amino sugars occur in their characteristic regions, at 55.6 and 57.2 p.p.m. (at pH 7.0). At each pH value, only a single, primary hydroxyl group resonance is observed, at 63.6 p.p.m. at pH 7.0 and at 62.7 p.p.m. at pH 1.0; the presence of only a single hydroxymethyl group is in accord with chemical analysis, which showed the presence of a C-6-containing carboxylic acid group. The carboxylic acid resonance was observed at 177.1 p.p.m. at pH 7.0, and, as anticipated, this shifted to higher field on lowering the pH (174.8 p.p.m. at pH 1.0). Concomitant with the titration of the carboxylic acid resonance, a second resonance, in the 75–80-p.p.m. region of the spectrum, was observed to shift position: it is assignable to C-5 of the 2-acetamido-2-deoxymannosyluronic acid residue of the repeating unit: the resonance occurred at 80.0 p.p.m. at pH 7.0, and shifted to 76.7 p.p.m. at pH 1.0. For the remaining resonances, changes in chemical shift as a function of pH were $<1$ p.p.m. $^{13}C$-N.m.r. spectral data for the type e polysaccharide, as well as for model compounds, are collected in Table I.

The $^{13}C$-n.m.r. spectrum of the reduced polysaccharide is shown in Fig. 1c (chemical shifts are tabulated in Table I). As expected for the reduced polymer, the carboxylic acid resonance is not present; a new hydroxymethyl group is, however,

TABLE I

$^{13}$C-N.M.R.-SPECTRAL DATA FOR THE *H. influenzae* TYPE e POLYSACCHARIDE, 2-ACETAMIDO-2-DEOXY-GLUCOSE, AND 2-ACETAMIDO-2-DEOXYMANNOSE[a]

| *Carbon atom* | *α-GlcNAc* | *β-GlcNAc* | *α-ManNAc* | *β-ManNAc* | H. influenzae *type e, pH 7.0*[b] | H. influenzae *type e, reduced* |
|---|---|---|---|---|---|---|
| C-1 | 93.7 | 97.2 | | | 103.1 (103.8) | 103.8 |
| C-2 | 56.9 | 59.6 | | | 57.2 (56.5) | 57.5 |
| C-3 | 73.6 | 76.8 | | | 85.8 (85.8) | 85.3 |
| C-4 | 73.0 | 72.8 | | | 71.7 (70.9) | 71.6 |
| C-5 | 74.2 | 78.8 | | | 78.5 (78.2) | 78.1 |
| C-6 | 63.5 | 63.6 | | | 63.6 (62.7) | 63.8 |
| -NC(O)*C*H$_3$ | 24.9 | 25.1 | | | 25.3 (24.4) | 24.9 |
| -NC(O)CH$_3$ | 177.3 | 177.5 | | | 177.1 (177.4) | 176.8 |
| C-1′ | | | 95.9 | 95.9 | 102.6 (102.8) | 102.4 |
| C-2′ | | | 56.0 | 56.9 | 55.6 (54.6) | 55.6 |
| C-3′ | | | 71.7 | 74.6 | 73.5 (72.9) | 73.9 |
| C-4′ | | | 69.6 | 69.4 | 80.7 (80.4) | 79.8 |
| C-5′ | | | 74.8 | 79.1 | 80.0 (76.7) | 78.4 |
| C-6′ | | | 63.3 | 63.3 | 177.1 (174.8) | 63.2 |
| -NC(O)*C*H$_3$ | | | 24.8 | 24.8 | 24.9 (23.6) | 24.9 |
| -NC(O)CH$_3$ | | | 177.5 | 178.4 | 177.9 (178.1) | 177.7 |

[a]Primed carbon atoms refer to the 2-acetamido-2-deoxymannosyluronic (or 2-acetamido-2-deoxymannosyl) residue of the polymer repeating-unit. [b]Values in parentheses refer to chemical shifts at pH 1.0.

observed. By comparison with the spectrum of the unreduced polysaccharide, as well as those of model compounds, the resonance at 78.4 p.p.m. is assigned to C-5 of the 2-acetamido-2-deoxymannosyl residue.

At pH 7.0, anomeric-carbon resonances for the intact type e polysaccharide are observed at 103.1 and 102.6 p.p.m. By comparison with those of model compounds, these shifts demonstrate that the C-1 atom in each sugar is glycosidated[10]. Moreover, the resonance position of C-1 of the 2-acetamido-2-deoxyglucosyl residue (103.1 p.p.m.) is indicative of the β-anomeric configuration. The chemical shift for C-1 of the 2-acetamido-2-deoxymannosyluronic acid residue (or the reduced material) is not indicative of anomeric stereochemistry; for this residue, however, the position of the C-5 resonance is indicative of anomeric stereochemistry, occurring at ~79 p.p.m. for the β-anomeric configuration, and at 75 p.p.m. for the α configuration. The C-5 resonance of the 2-acetamido-2-deoxymannosyluronic residue occurred at 80.0 p.p.m. at pH 7.0, and at 76.7 p.p.m. at pH 1.0; for the CMCT*–$NaBH_4$ reduced polymer, the C-5 resonance occurred at 78.4 p.p.m. These shifts indicated the β-anomeric stereochemistry at C-1 for this residue.

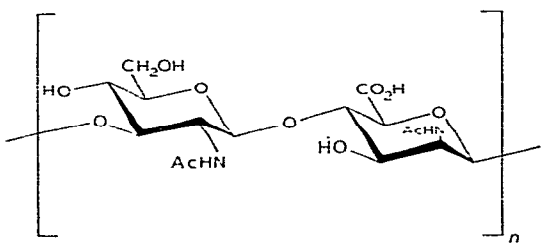
The chemical shift of C-6 for the 2-acetamido-2-deoxyglucosyl residue is

*Sodium 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate.

characteristic of an unsubstituted hydroxymethyl group. By default, this residue must be linked to the mannosyluronic residue at either C-3 or C-4. Ancillary evidence for this conclusion derives from the observation that the polymer does not consume periodate, even under forcing conditions; hence, it must be substituted at either C-3 or C-4 in the 2-acetamido-2-deoxyglucosyl residue. The resonance at 85.5 p.p.m. is highly characteristic of alkoxylation at C-3 of the *β*-anomeric form of glucose and, accordingly, it was concluded that the 2-acetamido-2-deoxyglucosyl residue is linked at C-3, as well as at C-1.

The 2-acetamido-2-deoxymannosyluronic residue, in addition to a linkage site at C-1, must, by default, be linked at either C-3 or C-4. On the basis of comparison of chemical shifts of model compounds with those found for the polymer, it was concluded that the linkage site is C-4. If the 2-acetamido-2-deoxymannosyluronic residue were linked at C-3, a resonance due to C-4 would be expected to occur at ~69 p.p.m., but a resonance at 69 p.p.m. was not observed in the $^{13}C$ spectrum of the polymer (the only resonance in that region was due to C-4 of glucose, at ~71.5 p.p.m.). After hydrazine-induced *N*-deacetylation, the CMCT–$NaBH_4$-reduced polysaccharide consumed periodate, oxidizing the 2-acetamido-2-deoxymannosyl residue; this is consistent with a C-4 linkage (after *N*-deacetylation, periodate oxidation, and acid-catalyzed hydrolysis, 2-amino-2-deoxyglucose was observable, whereas 2-amino-2-deoxymannose was not). In addition, erythrose, as the alditol acetate, was detected by g.l.c. analysis of the hydrolyzed, periodate-oxidized material. Observation of the proton signals for the *N*-methylacetamido groups in the reduced polymer, before and after treatment with hydrazine, indicated that only one acetyl group was removed by hydrazine and, from the foregoing evidence, this must be the acetyl group of 2-acetamido-2-deoxymannose. That the 2-acetamido-2-deoxyglucosyl residue is resistant to *N*-deacetylation is consistent with a linkage site[11] at C-3.

On the basis of the chemical and spectroscopic data, it is concluded that the *H. influenzae* type e capsular polysaccharide has structure **1**.




**1**

The type e capsular polysaccharide bears some similarities to type a (ref. 6), type b (ref. 5), type c (ref. 7), and type f (ref. 8). All are acidic, and contain disaccharide repeating-units. However, type e and type d (ref. 12) differ from the other *H. influenzae* capsular polysaccharides in that they do not possess phosphoric diester linkages; the acidity of the type e and type d (ref. 12) polysaccharides derives from a carboxylic acid group, namely, that in 2-acetamido-2-deoxymannuronic acid. Al-

TABLE II

ANALYTICAL RESULTS OBTAINED WITH *H. influenzae* TYPE e POLYSACCHARIDE

| *Parameter* | *Value* |
|---|---|
| $K_d$ (Sepharose 4B-CL) | 0.05 |
| Moisture (%, w/w) | 11 |
| Protein (%, w/w) | 2.29 |
| Endotoxin (%, w/w) | 0.01 |
| Inorganic residue (%, w/w) | 5.21 |
| 2-Amino-2-deoxyglucose (μmol/mg)[a] | 2.74 |
| 2-Amino-2-deoxymannose (μmol/mg)[a] | 2.11 |

[a]Determined using the type e polysaccharide twice reduced with CMCT–$NaBH_4$ (see Experimental).

though previously reported as a component of bacterial capsular-polysaccharides, as, for example, in the *Escherichia coli* K7 capsule[13], 2-acetamido-2-deoxymannuronic acid is not a common, capsular constituent. It is, therefore, of probable phylogenetic significance that this uronic acid residue occurs in both the *H. influenzae* type e and type d capsular polysaccharides.

## EXPERIMENTAL

*Isolation of polysaccharides.* — The capsular polysaccharide from *H. influenzae* type e was isolated as described[14] for Bureau of Biologics strains "Hernandez" (courtesy of Dr. David Hodes, Mount Sinai Hospital, New York) and "801" (Dr. Margaret Pittman, Bureau of Biologics). Strain Hernandez was used for the structural-determination studies: $^{13}$C-n.m.r. spectroscopy showed that Hernandez and 801 polysaccharides were structurally identical. Determinations of protein, endotoxin, moisture, and molecular weight were conducted by described methods[7b], and are presented in Table II, together with chemical analyses.

*Materials.* — 2-Acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-mannose, and D- and L-arabinose were obtained from Calbiochem (San Diego, CA). D-Glucose oxidase was obtained from Worthington Biochemical Corp. (Freehold, NJ), and Sepharose and Sephadex for gel-permeation chromatography, from Pharmacia (Piscataway, NJ). All other analytical reagents were of the highest purity available.

*Polysaccharide sugar analysis.* — Hydrolysis of the native, capsular polysaccharide and chemically treated derivatives was conducted as described, using methanesulfonic acid[7b,15]. Amino sugars were analyzed with a Beckman 120-B amino acid analyzer. Neutral sugars were analyzed either underivatized, in an automated sugar-analyzer[15], or derivatized (as described[7b]), in a Varian Associates 3700 gas chromatograph equipped with a WCOT, OV-225 capillary column (25 m × 0.9 mm). Identification of sugars was achieved by comparison with authentic samples.

*Reduction of the type e polysaccharide with sodium borohydride.* — The polysaccharide was reduced according to the method of Taylor and Conrad[16]. 3-(3-Dimethylaminopropyl)-1-ethylcarbodiimide · HCl) and CMCT were used interchangeably. The polysaccharide was reduced twice by this method (the reduction was somewhat sluggish, necessitating recycling).

*Oxidation with ninhydrin.* — An aqueous solution of the reduced polysaccharide (6 mg) was hydrolyzed with 4M HCl in a closed, nitrogen-purged, screw-capped vial for 4 h at 100°, concentrated with a stream of nitrogen to ~100 μL, washed with water (2 × 250 μL), centrifuge-evaporated, and the product dried over $P_2O_5$ and NaOH pellets in a vacuum desiccator. The slightly yellow residue was treated with 24:1 2% aqueous ninhydrin–pyridine (2 mL) in a nitrogen-purged, screw-capped hydrolysis tube, and heated for 30 min at 100°. The dark-purple solution resulting was desalted in a column of Dowex-50 X-8 ($H^+$) ion-exchange resin followed by an identical column of Dowex-1 X-8 ($HCO_3^-$) resin. The fractions containing a pentose (Bial modified orcinol test[17]) were pooled, and lyophilized. The lyophilizate was analyzed as the alditol acetate by g.l.c. and, as the underivatized sugar, by an automated sugar-analyzer, and identified as arabinose. Glycosidation with *d*-2-octanol, followed by peracetylation, gave a mixture of diastereoisomers that were identical to those obtained from authentic D-arabinose by similar treatment, and that differed from the set of diastereoisomers obtained by similar treatment of L-arabinose: analysis was performed by g.l.c., as described in ref. 8a.

N-*Deacetylation, and periodate oxidation, of the reduced polysaccharide.* — *N*-Deacetylation was accomplished as described[18], the course of the reaction being monitored by $^1$H-n.m.r. spectroscopy. A sample of the twice-reduced, type e polysaccharide (500 μg) was treated with anhydrous hydrazine (50 μL) and hydrazine sulfate (1 mg), sealed *in vacuo* in a pyrolysis tube, and heated for 8 h at 100°. The tube was cooled and opened, and water (200 μL) was added. The hydrazine-treated sample (100 μL) was added to each of two Pyrex test-tubes, and vacuum-dried over $P_2O_5$ and NaOH pellets. To one tube was added 0.03M $NaIO_4$ solution (1.8 mL); the other tube was treated identically, with the exception that the periodate solution was first quenched with ethylene glycol (50 μL). The tubes were covered with silicone-rubber stoppers, and heated overnight at 37°. The first sample was then treated with ethylene glycol (50 μL), whereupon both samples were lyophilized, dissolved in water (200 μL), transferred to separate hydrolysis tubes, dried with a centrifuge evaporator, and hydrolyzed with methanesulfonic acid for 16 h at 115°. Both samples were then subjected to amino acid analysis as already described.

*Deamination of the hydrolyzate of the reduced, type e polysaccharide*[19]. — The twice-reduced, type e polysaccharide (100 μg) was hydrolyzed with 12M HCl as already described. After being dried over $P_2O_5$ and NaOH, the hydrolyzate was cooled to 0°, and treated with acetic acid (100 μL), water (20 μL), and sodium nitrite (1.5 mg). After the initial, vigorous bubbling had subsided (~30 min), the sample was kept overnight at 4°. It was then desalted in a column of Dowex-50 X-8 ($H^+$) followed by a column of Bio-Rad AG-3 X-4 ($HCO_3^-$), and lyophilized. The

residue was dissolved in water (400 μL); half of the solution was treated with D-glucose oxidase, and half remained untreated. The samples were concentrated, and analyzed in an automated sugar-analyzer.

*Attempted periodate oxidation of the native, type e polysaccharide.* — The type e polysaccharide (5 mg) was dissolved in 0.03M $NaIO_4$ (5 mL) in a clean test-tube protected from light by aluminum foil. The sample was heated at 37°, and the consumption of periodate was monitored by u.v. spectroscopy (absorbance at 223 nm) according to the procedure of Aspinall and Ferrier[20]: a control sample (without polysaccharide) was run simultaneously.

*Additional analytical instrumentation and procedures.* — $^{13}$C-N.m.r. spectra were recorded with a JEOL FX-100 spectrometer, and $^{1}$H-n.m.r. spectra, with a Varian HA-220 n.m.r. spectrometer, as described[7b]. Specific rotations were derived from measurements made with a Cary 60 recording spectropolarimeter.

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