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STRUCTURAL STUDIES OF THE *Haemophilus influenzae* TYPE f CAPSULAR POLYSACCHARIDE

WILLIAM EGAN*, FAI-PO TSUI, AND RACHEL SCHNEERSON

Division of Bacterial Products, Bureau of Biologics, Food and Drug Administration, 8800 Rockville Pike, Bethesda, Maryland 20205 (U.S.A.)

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ABSTRACT

The structure of the *Haemophilus influenzae* type f capsular polysaccharide was studied by chemical and nuclear magnetic resonance spectroscopic techniques. The repeating unit of the polysaccharide was found to be →3)-β-D-GalNAc-(1→4)-α-D-GalNAc-(1→OP(OH)→.

2 ||
↑ O
OAc

INTRODUCTION

Capsules are necessary for causation of severe invasive disease[1] by *Haemophilus influenzae* (as well as, *inter alia*, *Neisseria meningitidis* and *Streptococcus pneumoniae*). Of the six known, capsular types of *H. influenzae*, designated by the letters a through f, only one, namely, type b, is commonly associated with disease in humans[2]. The other five types, although possessing polysaccharide capsules of overall characteristics similar to those of type b, are rarely the etiologic factor of serious, human disease. In order to increase our understanding of the virulence conferred to type b through its capsule, the structures of the normally nonvirulent serotypes have been studied for comparison. The structure of the type b capsule has been reported[3]; our studies with the type f polysaccharide are herein presented.

EXPERIMENTAL

Polysaccharide isolation. — The capsular polysaccharide from *H. influenzae* type f (NIH strain 644, ATCC No. 9833, and State Laboratories Institute, Boston, MA, No. 686, kindly supplied by Leslie Wetterlow) was isolated as described[4]. Determinations of protein, phosphorus, nucleic acid, endotoxin, moisture, *O*-acetyl, and molecular size were conducted as described[5]; these results are summarized in Table I.

*To whom correspondence should be addressed.

TABLE I

ANALYTICAL RESULTS OBTAINED FOR *Haemophilus influenzae* TYPE f CAPSULAR POLYSACCHARIDE[a]

Parameter	*Value*
K_d (on Sepharose 4B)	0.4
Moisture (%, w/w)	9
Nucleic acid (%, w/w)	0.05
Protein (%, w/w)	0.98
Endotoxin (%, w/w)	0.01
O-Acetyl (μmol/mg)	1.81
Phosphorus (μmol/mg)	1.9
2-Amino-2-deoxygalactose (μmol/mg)	3.71

[a]The values for 2-amino-2-deoxygalactose, phosphorus, *O*-acetyl, endotoxin, protein, and nucleic acid are uncorrected for moisture. Endotoxin was determined by the limulus lysate assay; 2-amino-2-deoxygalactose was determined with an amino acid analyzer.

Sugar analysis of the polysaccharide. — Hydrolysis of the polysaccharide and sugar analysis were conducted by methods described for the *H. influenzae* type c polysaccharide[6,7]. The D or L assignment of 2-amino-2-deoxygalactose was made through comparison of the specific rotation (determined with a Cary 60 recording spectropolarimeter) of the hydrochloride salt with that of the authentic compound (Calbiochem, San Diego, CA). The D or L assignment was also made by g.l.c., as follows. 2-Amino-2-deoxygalactose was oxidized with ninhydrin[8], to yield lyxose, which was then glycosidated with *d*-2-octanol [Aldrich Chemical Co., Inc.; $[\alpha]_D^{17}$ +9° (neat)]; the mixture of products (the anomers of the furanosides and pyranosides of lyxose) was peracetylated, and the esters were subjected to g.l.c. analysis. The retention times of the set of glycosides thus formed were identical to those of those obtained from the condensation of D-lyxose and *d*-2-octanol, and distinct from those of those obtained from L-lyxose and *d*-2-octanol (see ref. 9). Authentic D- and L-lyxose were obtained from ICN Pharmaceuticals, Cleveland, OH.

Phosphate hydrolysis of the polysaccharide and O-deacetylation. — Partial and total hydrolysis of the phosphate of the polysaccharide, and *O*-deacetylation of the polysaccharide, were accomplished by methods described[6]; as before, the reactions were respectively monitored by ^{31}P- and ^{1}H-n.m.r. spectroscopy.

Isolation of 2-amino-2-deoxygalactose. — The type f polysaccharide was completely dephosphorylated (and simultaneously *O*-deacetylated), and purified by ion-exchange chromatography on Dowex 50 (H^+), followed by Dowex 1 (HCO_3^-). The product was hydrolyzed for 6 h at 100° with methanesulfonic acid as described[6], and the material was dried over P_2O_5 and NaOH. The material in the hydrolyzate was chromatographed on a column of Dowex 50 (H^+) resin according to established procedure[10].

Nuclear magnetic resonance (n.m.r.) spectroscopy. — N.m.r. spectra (^{13}C and ^{31}P) were recorded with a JEOL FX-100 spectrometer as described[6]. ^{1}H-N.m.r.

spectra were recorded in the continuous-wave mode with a Varian HR-220 n.m.r. spectrometer.

RESULTS AND DISCUSSION

Acid hydrolysis of the type f polysaccharide liberates 2-amino-2-deoxygalactose and inorganic phosphate in the molar ratio of 2:1. The amino sugar was identified by chromatographic comparison with authentic material; confirmation was achieved through ninhydrin oxidation to lyxose, identified by chromatographic comparison with authentic lyxose. 2-Amino-2-deoxygalactose was the sole sugar detectable in the acid hydrolyzate, and its hydrochloride was isolated and purified; it had $[\alpha]_D^{20}$ +94° (*c* 0.5, water), thereby establishing it as the D enantiomer {lit.[11] $[\alpha]_D^{20}$ +93° (water)}. The D configurational assignment was confirmed by g.l.c. by a method, employing *d*-2-octanol, developed by Lindberg and co-workers[9].

The acidic nature of the polysaccharide was initially evidenced by its anodic migration during immunoelectrophoresis. The diester nature of the polysaccharide was established by (*1*) titration, and (*2*) its hydrolytic behavior. The ^{31}P-n.m.r. spectrum of the intact polysaccharide showed no change in chemical shift as a function of pH over the range 2 to 11. As phosphoric monoesters show[12] marked changes (~4 p.p.m.) in chemical shift in the vicinity of their second pK_a (~6), the titration data precluded the possibility of a phosphoric monoester. The intact polysaccharide did not react with alkaline phosphatase, an enzyme specific for phosphoric monoesters; however, after treatment with 0.5M HCl during 20 h at 20°, the polysaccharide was susceptible to hydrolysis catalyzed by alkaline phosphatase. This combination of mild hydrolysis with acid and that catalyzed by alkaline phosphatase yielded a neutral, reducing sugar having a low molecular weight, as evidenced by its K_d value

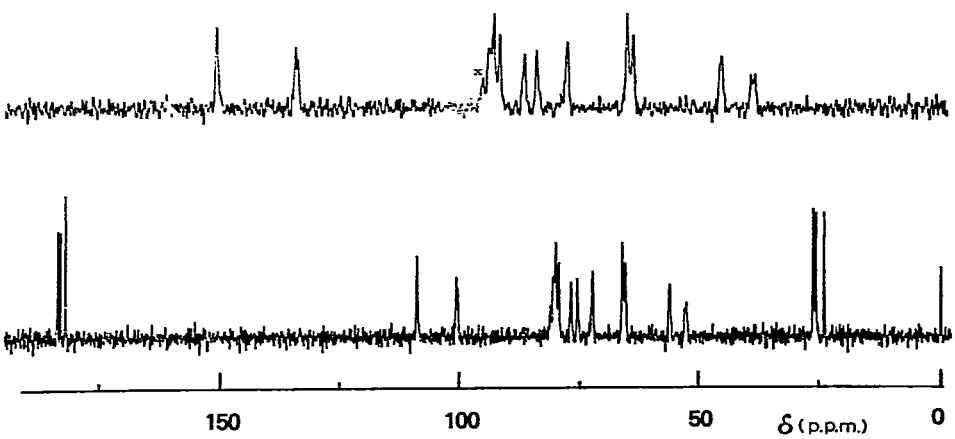


Fig. 1. ^{13}C-N.m.r. spectrum of type f *Haemophilus influenzae* capsular polysaccharide. [The chemical shifts are given on the δ scale and are relative to internal sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$ (TSP). The upper tracing is an expansion of the region between ~50 and 85 p.p.m. An impurity (unidentified) is marked "x" in the expansion.]

TABLE II

^{13}C-N.M.R.-SPECTRAL DATA FOR THE *Haemophilus influenzae* TYPE f POLYSACCHARIDE AND 2-ACETAMIDO-2-DEOXYGALACTOSE[a]

Carbon atom	*β-GalNAc*	*α-GalNAc*	H. influenzae *type f*
C-1	98.1		104.9
C-2	56.5		54.2 (5.0)[b]
C-3	73.8		77.5 (6.5)
C-4	70.6		69.6
C-5	77.8		76.3
C-6	63.8		63.3
-NC(O)CH$_3$	177.7		177.0
-NC(O)*C*H$_3$	25.0		25.2
C-1		93.8	96.9 (6.1)
C-2		53.0	50.9 (6.8)
C-3		70.4	72.8
C-4		71.3	77.0
C-5		73.2	74.0
C-6		64.0	63.6
-NC(O)CH$_3$		177.0	176.5
-NC(O)*C*H$_3$		24.8	24.7
-OC(O)CH$_3$			175.6
-OC(O)*C*H$_3$			23.0

[a]Data are taken from ref. 14 and are converted from a tetramethylsilane reference to a sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 (TSP) reference by adding 1.6 p.p.m., the shift difference between Me_4Si and TSP in an acetone solution containing both of these standards. [b]^{31}P, scalar coupling-constants are given in parentheses.

(0.55) in gel-permeation chromatography on Sephadex G-25. The reducing nature of the sugar was determined both chemically and spectroscopically. Inorganic phosphate was also liberated during the treatment with alkaline phosphatase.

The ^{13}C-n.m.r. spectrum of the type f polysaccharide is shown in Fig. 1. Twelve resonances are observed in the region 50–110 p.p.m.; additionally, three lie in the region 22–25 p.p.m., and three in the region 175–177 p.p.m. The ^{13}C-n.m.r. data are assembled in Table II. With the exception of those resonances mentioned in the text, the assignments given in Table II should be regarded as tentative. The six resonances in the ranges 22–25 and 175–177 p.p.m. are characteristic of two *N*-acetyl and one *O*-acetyl group. In concert with compositional data, the ^{13}C-n.m.r. spectrum provides evidence for a repeating unit in which two 2-acetamido-2-deoxygalactosyl groups are linked by a phosphoric diester and that bears one specifically located, *O*-acetyl group.

The position of the anomeric resonances at 105 and 97 p.p.m. indicate the presence of both the β and α configurations. The presence of the two anomers is further reflected in the resonance positions of the respective C-2 atoms, especially subsequent to removal of the *O*-acetyl groups (see later). The resonances at 63.1 and 63.3 p.p.m. are characteristic of the carbon atom of a hydroxymethyl group, and

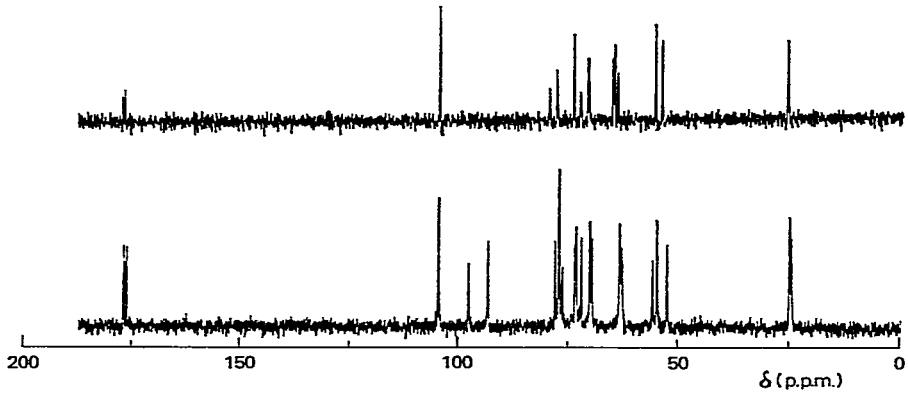


Fig. 2. ^{13}C-N.m.r. spectra of the type f polysaccharide following *O*-dephosphorylation (mild hydrolysis with acid followed by alkaline phosphatase-catalyzed hydrolysis) (bottom tracing) and subsequent sodium borohydride reduction (top tracing). (Both scales are in p.p.m. and are relative to internal TSP.)

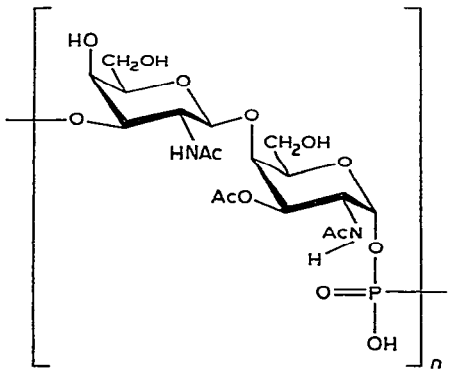
demonstrate that O-6 of each 2-amino-2-deoxygalactosyl residue is unsubstituted; that at least one such residue contains an unsubstituted 6-hydroxyl group is shown by the failure of the polymer to consume periodate, a reagent specific for vicinal hydroxyl groups.

To ascertain the site of *O*-acetylation, the polysaccharide was *O*-deacetylated with ammonium hydroxide. Removal of the acetyl group was seen to cause, *inter alia*, an ~2-p.p.m., downfield shift of the C-2 resonance of the 2-acetamido-2-deoxy-α-galactosyl residue (50.9 to 52.7 p.p.m.); such a shift is characteristically observed following hydrolysis of an acetate (or phosphate) group at a vicinal carbon atom[13]. The *O*-deacetylation thus established the site of *O*-acetylation as O-3 of the 2-acetamido-2-deoxy-α-galactosyl residue.

The presence of ^{31}P–^{13}C, scalar coupling (couplings were confirmed by recording ^{13}C-n.m.r. spectra at two field-strengths, namely, 2.3 and 6.2 T) to *both* C-1 and C-2 of the 2-acetamido-2-deoxy-α-galactosyl residue indicates attachment of the phosphate at C-1. As expected, mild *O*-dephosphorylation with acid, followed by treatment with alkaline phosphatase, resulted in the loss of all ^{31}P–^{13}C, scalar couplings. Simultaneously, a number of new resonances appeared, the most telling of which were those appearing at 98.3 and 93.8 p.p.m., attributable to the free (*i.e.*, reducing) C-1 atom of the anomeric 2-acetamido-2-deoxygalactosyl residues. The 105-p.p.m. resonance in the starting polymer was not shifted by this treatment. Reaction of the hydrolyzed material with $NaBH_4$ caused the loss of the two resonances at 98.3 and 93.8 p.p.m. (confirming their assignment to reducing carbon atoms) and left the 105-p.p.m. resonance unaltered in shift. The product obtained by treating the sugar with sodium borohydride showed a simplified ^{13}C spectrum (as regards the number of resonances). The ^{13}C-n.m.r. spectra for the *O*-dephosphorylated and $NaBH_4$-treated materials are shown in Fig. 2.

The structure of the type f polysaccharide follows from the foregoing ^{13}C-

n.m.r. observations. Commencing with the 2-acetamido-2-deoxy-α-galactosyl residue, it was concluded that phosphate is attached to C-1, O-3 is acetylated, and O-6 is unsubstituted. By elimination, therefore, O-4 must be the site of linkage to the second 2-acetamido-2-deoxygalactosyl residue of the repeating unit. The terminus of this linkage is C-1 of the 2-acetamido-2-deoxy-β-galactosyl residue, as the C-1-β resonance is unperturbed by *O*-dephosphorylation. Additionally, as O-6 of the 2-acetamido-2-deoxy-β-galactosyl residue is unsubstituted, the phosphate group must be attached to either C-3 or C-4. The observation of a ^{31}P, scalar coupling (J 5.0 Hz) to C-2-β evidences attachment at C-3. Such a large coupling-constant through four bonds (as would be the case for attachment at C-4) would be unexpected, whereas it is expected for a three-bond coupling[14]. Attachment of the phosphate at C-3 is substantiated by the observation of an ~1.2-p.p.m., downfield shift of the C-2 resonance on *O*-dephosphorylation[13]. The structure of the type f polysaccharide is therefore as depicted. Branefors-Helander *et al.*[15] have independently determined the same structure for the type f polysaccharide.



The type f polysaccharide was originally studied by Rosenberg *et al.*[16], who concluded that the polysaccharide contains two, non-equivalent 2-amino-2-deoxy-galactosyl residues linked 1→1, and that it possesses a 3→4 phosphate linkage; moreover, they did not detect the *O*-acetyl group. The structure originally proposed is at variance with that now derived. Rosenberg *et al.*[16] argued against attachment of phosphate at C-1, on the basis of the rate constant of hydrolysis of the phosphoric diester; the present work, which unambiguously establishes attachment of phosphate at C-1, thus emphasizes the hazard of employing arguments based on hydrolysis rate-constants in the absence of suitable, model compounds. In accord with the ^{13}C-n.m.r. data, the *O*-dephosphorylated sugar gave a positive Park–Johnson[17] reducing-sugar test.

The type f capsule is structurally similar to the capsules obtained from type b (ref. 3), type c (refs. 6 and 18), and type a (ref. 19) insofar as all have phosphoric diester-linked, disaccharide repeating-units. Types d and e are also acidic polymers of disaccharide repeating-units; however, they do not contain phosphoric diester

linkages[20]. The apparent chemical distinction between type b and the remaining serotype polysaccharides is that type b is composed of two pentoses whereas the rest, including[20] d and e, contain at least one hexosyl residue in the repeating unit.

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