Carbohydrate Research, 80 (1980) 305–316

STRUCTURAL AND IMMUNOLOGICAL STUDIES OF THE *Haemophilus influenzae* TYPE c CAPSULAR POLYSACCHARIDE

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(Received July 16th, 1979; accepted for publication, September 19th, 1979)

ABSTRACT

The structure of the *Haemophilus influenzae* type c capsular polysaccharide was studied by chemical and spectroscopic techniques. The repeating unit was shown to be →4)-β-D-Glc*p*NAc-(1→3)-α-D-Gal*p*(1→OP(OH)→; ~80% of the 2-acetamido-2-deoxy-D-glucosyl residues are acetylated at O-3. Competitive inhibition of antigen–antibody precipitation shows that the *O*-acetyl group confers an antigenic specificity on the type c capsular polysaccharide.

INTRODUCTION

Haemophilus influenzae is a frequent cause of serious human diseases, including acute bacterial meningitis, epiglotitis, and osteomyelitis[1]. *H. influenzae* virulence is directly related to its polysaccharide capsule, and serum antibodies specific for the capsule confer resistance to invasive disease caused by this organism[2]. Of the six known *H. influenzae* capsule serotypes, designated a through f, virtually all serious disease is caused by type b.

The relationship between *H. influenzae* type b virulence and capsule structure is as yet unknown and so this study was undertaken in order to gain such understanding by structural comparison of type b with other, nonvirulent, capsular types. The structure of the type a (ref. 3) and type b (refs. 4 and 5) capsules have been reported. We herein present our findings with *H. influenzae* type c.

EXPERIMENTAL

Materials. — D-Galactose oxidase (Chromogen for Galactostat) was obtained from Worthington Biochemical Corporation (Freehold, NJ). Authentic sugars were obtained from Sigma Chemical Company (St. Louis, MO). Sepharose and

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TABLE I

ANALYTICAL RESULTS OBTAINED WITH *Haemophilus influenzae* TYPE c CAPSULAR POLYSACCHARIDE

Parameter	*Value*
K_d (on Sepharose 4B)[a]	0.41
Moisture (%, w/w)	14
Protein (%, w/w)	0.09
Endotoxin (%, w/w)	0.06
Phosphorus (μmol/mg)	1.63
Galactose[b] (μmol/mg)	1.65
2-Acetamido-2-deoxyglucose[b] (μmol/mg)	1.50
O-Acetyl (μmol/mg)	1.35

[a]K_d defined in ref. 7. [b]Determined by ion-exchange chromatography (see Experimental section).

Sephadex for gel-permeation chromatography were obtained from Pharmacia (Piscataway, NJ). All analytical reagents were of the highest purity available.

Polysaccharide isolation. — The capsular polysaccharide from *Haemophilus influenzae* type c was isolated as described[6]. Strain 624, originally isolated by Dr. Chapman (1/13/42) (ATCC No. 9007, from the NIH/BoB culture collection) was utilized. Determinations of protein, phosphorus, nucleic acid, endotoxin, and moisture, and molecular-size characterization were conducted by methods described[7]. The results are summarized in Table I.

Polysaccharide sugar analysis. — The purified polysaccharide (0.5 mg) was dissolved in de-ionized water (480 μL) and digested for 16 h at 95° with 2M methanesulfonic acid (20 μL) and Dowex 50-X8 (200–400 mesh) ion-exchange resin[8] (10 mg). An aliquot (10 μL) of the hydrolyzate was diluted with ethanol (90 μL), and loaded onto a column (0.6 × 20 cm) of Dowex 50-X8 (10 ±1 μm particle size) ion-exchange resin equilibrated at 80° under nitrogen at a pressure of ~275 lb.in.$^{-2}$. The sample was continuously eluted with 90% (v/v) ethanol; reducing sugars were detected photometrically as their Tetrazolium Blue reaction-product[8]. L-Fucose or L-rhamnose was added to a duplicate run as an internal standard for determination of retention time and concentration.

Amino sugar was analyzed with a Beckman 120-B amino acid analyzer; the chromatography was performed with an accelerated system according to the procedure of Spackman[9]. The hydrolysis was conducted as follows. The polysaccharide (10 μg) was hydrolyzed with M methanesulfonic acid (50 μL) in a sealed, evacuated tube at 115° (ref. 10), and samples were removed at 2, 4, 6, 8, 10, and 24 h. The hydrolyzate was made neutral with 2M NaOH (40 μL), and diluted with 0.2M sodium citrate buffer[10] (pH 2.2).

Sugar composition was also determined by gas–liquid chromatography in a Varian Model 3700 Gas Chromatograph equipped with a WCOT OV-225 capillary column. Identification of the sugars was made by comparison with authentic samples.

Neutral sugars were identified as (*1*) the alditol acetate, and (*2*) the peracetylated derivative of the reducing form; a programmed column-temperature was used (rising at 1°.min^{-1} from 170 to 220°) after an isothermal hold for 1 min. Amino sugar was determined as the trimethylsilyl derivative of the reducing form thereof; the temperature of the column was programmed to rise at 1°.min^{-1} after an isothermal hold of 1 min at 120°. The flow rate was 1 mL.min^{-1}.

Nuclear magnetic resonance (n.m.r.) spectroscopy. — Natural abundance, ^{13}C-n.m.r. spectra were routinely recorded at 25.04 MHz with a JEOL FX-100 spectrometer for solutions (~50 mg/mL) at pH 7.0 and 20°, with sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 (TSP) as the internal, chemical-shift standard; shifts are reported on the δ scale. Spectral parameters (5-mm, n.m.r. tube) were as follows: 12-μs, $\pi/2$ pulse; 2.5-s, pulse-repetition rate; 5-kHz, spectral window; 8192 data points. Prior to Fourier transformation, the free-induction decay (f.i.d.) signal was zero-filled with 8192 data points, and exponentially multiplied so as to result in an additional line-broadening of 0.68 Hz in the frequency-domain spectrum. For quantitative analysis, ^{1}H decoupling was gated off, except during the acquisition of the f.i.d.; for determining the number of directly bonded hydrogen atoms, the ^{1}H decoupler was gated off during the acquisition of the f.i.d. For routine spectra, the ^{1}H decoupling field was on continuously.

^{31}P-N.m.r. spectra were recorded at 40.25 MHz with a JEOL FX-100 spectrometer (10-mm tube) as follows: a 26-μs, $\pi/2$ pulse; 5-kHz spectral window; 2.5-s pulse-repetition rate; 8192 data points. Prior to Fourier transformation, the f.i.d. was zero-filled with 8192 data points, and exponentially multiplied so as to result in an additional, 1.0-Hz line-broadening in the frequency-domain spectrum. Broad-band, ^{1}H decoupling was employed, but was gated off, except during data acquisition. Chemical shifts are in p.p.m. relative to 25% H_3PO_4 contained in an internal capillary tube.

^{1}H-N.m.r. spectra were recorded in the continuous-wave mode with either a Varian HR-220 or a Varian XL-100/15 spectrometer at respective probe temperatures of 20 and 30°. Chemical shifts are in p.p.m. relative to internal TSP.

Polysaccharide phosphate hydrolysis. — The polysaccharide (20 mg) was dissolved in ^2H_2O (1 mL); 0.5M HCl (1 mL) was added, and the ^{31}P-n.m.r. spectrum was immediately recorded. A single resonance at −2.7 p.p.m. was observed. With time, this resonance decreased in intensity as two new resonances, at −1.75 and −1.30 p.p.m., appeared. When no further change in the spectrum was noted, the pH was adjusted to 11.5; a redistribution in the site of phosphate attachment was observed (see Fig. 1). After 30 min at pH 11.5, the pH was lowered to 10.0, and alkaline phosphatase [Sigma, type VII (calf intestine); 5 mg] was added. After 10 h, the monophosphate peaks disappeared and a new resonance, at 1.1 p.p.m., attributable to inorganic phosphate, appeared. The time course of the alkaline phosphatase-catalyzed hydrolysis is shown in Fig. 1.

Polysaccharide O-deacetylation. — The polysaccharide (50 mg) was dissolved in ^2H_2O (0.5 mL), the pH was adjusted to 10.5 with deuterated ammonium hydroxide

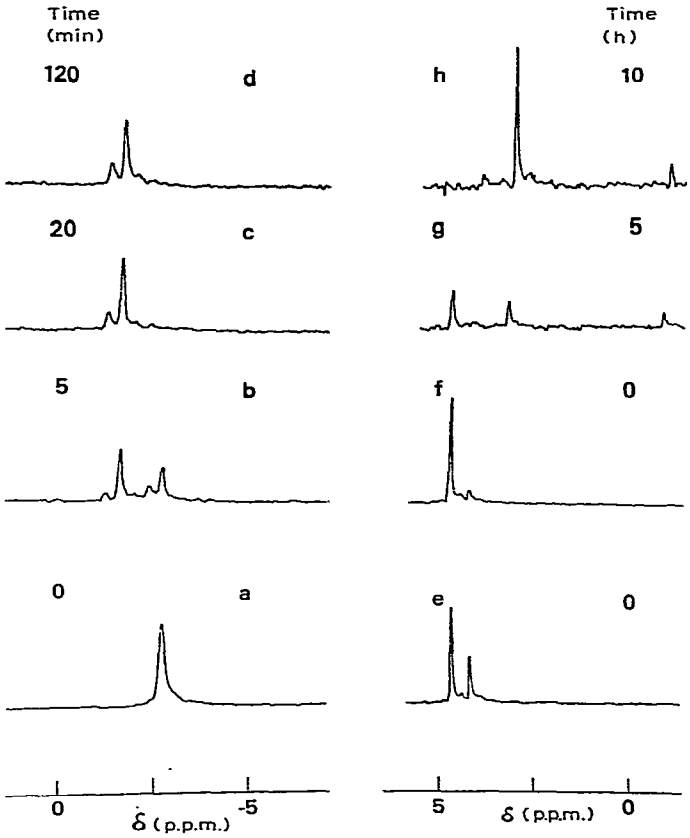


Fig. 1. ^{31}P-N.m.r. spectrum of the time-course of the acid-catalyzed hydrolysis of the *H. influenzae* type c polysaccharide with 0.25M HCl at 20° (40 mg/2.0 mL). [a–d, Time course of the acid-catalyzed hydrolysis; e–h, time course of the alkaline phosphatase-catalyzed dephosphorylation. The redistribution of monophosphates (compare d and e) is due to a base-catalyzed rearrangement (see Experimental section).]

(Aldrich Chemical Company), and the mixture was kept at 5° until the *O*-deacetylation was complete (as indicated by ^{1}H-n.m.r. spectroscopy).

Antigen–antibody, competitive-inhibition studies. — Sheep No. 841 was immunized with formalin-treated *H. influenzae* type c (Strain ATCC No. 9007) as described[11]. The serum was decanted from the clotted blood, centrifuged under sterile conditions, and the supernatant liquor stored at −20° without a preservative.

The antigen concentration to precipitate maximum antibody from sheep anti-*H influenzae* type c antiserum was determined by a precipitin curve; 0.5 mL of antiserum was incubated with 0.5 mL of a 0.15M NaCl solution of *H. influenzae* type c polysaccharide for 1 h at 37°, and for 3 days at 4°. The tubes were occasionally agitated gently. The precipitates were collected by centrifugation, washed three times with chilled, 0.15M NaCl, and then dissolved in 0.8% sodium lauryl sulfate (2.0 mL). The absorbance of the dissolved antigen–antibody complex was measured at 280 nm with a Gilford spectrophotometer. 50 μg (maximum precipitation) and 25 μg (anti-

body excess) of type c antigen were chosen for a study of the inhibitory effect of the *O*-deacetylated, type c polysaccharide upon the precipitation between the untreated type c polysaccharide and the sheep serum. Aliquots (0.5 mL) of the *O*-deacetylated preparation containing 5, 25, and 2500 μg were added to 0.5 mL of antiserum, and incubated for 1 h at 37°. 25 or 50 μg of *H. influenzae* type c polysaccharide was then added, mixed, and incubated for 1 h at 37° and for 3 days at 4°. *H. influenzae* type b polysaccharide[12], used as a control, displayed no effect upon the precipitation-reaction of type c with its homologous antiserum. No precipitation occurred between the type c antiserum and the *O*-deacetylated polysaccharide alone at any concentration (5, 50, or 2500 μg).

RESULTS

The capsular polysaccharide from *H. influenzae* type c had $[\alpha]_D^{20}$ +47° (*c* 0.13, H_2O). Following acid hydrolysis, ion-exchange chromatography revealed galactose as the sole, reducing, neutral sugar, and 2-amino-2-deoxyglucose as the sole amino sugar. Their identities were confirmed by g.l.c., in which no additional sugars were observed.

Treatment of the hydrolyzate with D-galactose oxidase resulted in the quantitative elimination of D-galactose, whereas the 2-amino-2-deoxyglucose was unaffected. The enzymically transformed galactose had the same retention time (Dowex 50 ion-exchange resin) as material derived from the identical treatment of authentic D-galactose.

2-Amino-2-deoxyglucose was isolated from the hydrolysis mixture by ion-exchange chromatography. The specific rotation of the isolated sugar (as the hydro-

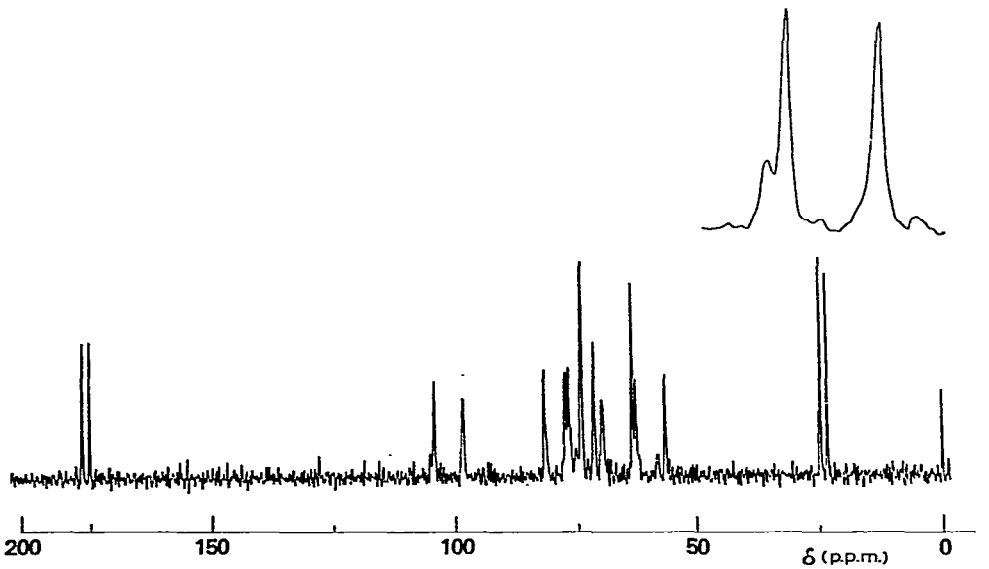


Fig. 2. ^{13}C-N.m.r. spectrum of the *H. influenzae* type c polysaccharide. (An expanded-scale display of the *N*- and *O*-acetyl methyl region is shown.)

TABLE II

^{13}C-N.M.R.-SPECTRAL DATA FOR THE *Haemophilus infiuenzae* TYPE c CAPSULAR POLYSACCHARIDE

Carbon[a]	*δ*[b] *(p.p.m.)*	*^{31}P coupling (Hz)*	*Number of directly bonded hydrogen atoms*
$OCOCH_3$	23.44		3
$NCOCH_3$	24.78		3
C-2′	56.83		1
C-6′[c]	62.91		2
C-6[c]	63.56		2
C-2	69.7	8.5	1
C-4	71.51		1
C-4′, C-5	74.07	7.0	1
C-3′	76.8		1
C-5′	77.4	4.9	1
C-3	81.82		1
C-1	98.6	6.7	1
C-1′	104.58		1
$OCOCH_3$	177.67		0
$NCOCH_3$	177.12		0

[a]Primed carbons refer to 2-acetamido-2-deoxyglucose, and unprimed carbons to galactose. [b]Chemical shifts referred to internal TSP. [c]Assignments may be reversed.

chloride salt) was $[\alpha]_D^{20}$ +74° (*c* 0.2, H_2O); an authentic sample of the hydrochloride salt had $[\alpha]_D^{20}$ +73° (*c* 0.2, H_2O). Oxidation of the isolated amino sugar with ninhydrin[13] gave a neutral sugar whose retention time by ion-exchange chromatography was identical to that of the anticipated oxidation-product, arabinose.

Consistent with the presence of phosphate, the polysaccharide was acidic, as revealed by anodic migration during immunoelectrophoresis at pH 9.5. The ^{31}P-n.m.r. signal was monitored as a function of pH, but no change was observed over the pH range 2–11; this is indicative of a phosphoric diester linkage[14]. Analysis for *O*-acetyl by the Hestrin method revealed 1.35 μmol.mg^{-1} (see Table I).

The natural-abundance, ^{13}C-n.m.r. spectrum of the type c polysaccharide is shown in Fig. 2. Twelve major resonances, four of which exhibited ^{31}P–^{13}C, scalar couplings, were distinguishable in the 50–110-p.p.m. region. Additionally, the spectrum showed resonances characteristic of *N*-acetyl and *O*-acetyl groups. The spectrum is thus in accord with the compositional analysis presented in Table I. Spectral data are collected in Table II; the resonance assignments presented therein derive from the presence of scalar couplings to phosphorus, and comparison of resonance positions of the type c polysaccharide with its reaction products and model compounds. (A number of resonance assignments are tentative; however, these do not affect structural conclusions.) Several resonance-assignments may be readily made, and their structural consequences derived. The resonances at 24.8 and 177.1 p.p.m. correspond to the $C(O)CH_3$ and $C(O)CH_3$ carbon atoms of the *N*-acetyl group; the resonances at

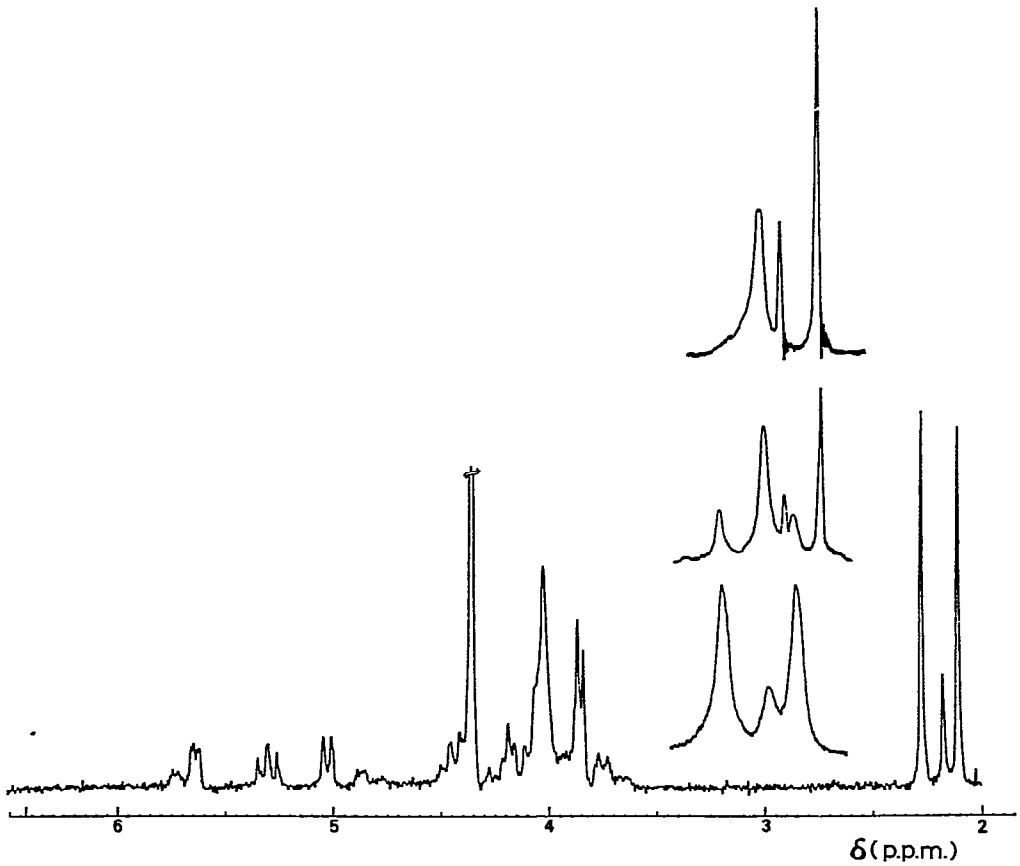


Fig. 3. ^{1}H-N.m.r. spectra (220 MHz) of the *H. influenzae* type c polysaccharide prior to treatment with ammonium hydroxide (pH 10.5). (The expanded, inset spectra of the acetyl methyl region shows the time course of the hydrolysis with NH_4OH.)

23.49 and 175.7 p.p.m., to the C(O)CH_3 and C(O)CH_3 carbon atoms of the *O*-acetyl group; and that at 56.83 p.p.m. corresponds to C-2 of 2-acetamido-2-deoxyglucose. The resonances at 63.56 and 62.91 p.p.m. are characteristic of primary carbon atoms, and correspond to C-6 of 2-acetamido-2-deoxyglucose and galactose. The resonances at 104.58 and 98.6 p.p.m. correspond to the anomeric carbon atoms of 2-acetamido-2-deoxyglucose and galactose.

A number of minor resonances are also discernible in the ^{13}C-n.m.r. spectrum, the most significant of which is a shoulder, at 25.10 p.p.m., on the *N*-acetyl -CH_3 resonance. It is likely that these minor resonances derive from repeating units that are not *O*-acetylated, consistent with the chemically derived stoichiometry (see Table I). Comparative integration of the ^{13}C resonances at 25.10 and 24.78 p.p.m. *versus* that at 23.44 p.p.m. showed that ~80% of the repeating units were *O*-acetylated. To establish firmly that the minor resonances derive from non-*O*-acetylated units, the polymer was totally *O*-deacetylated by treatment with ammonium hydroxide.

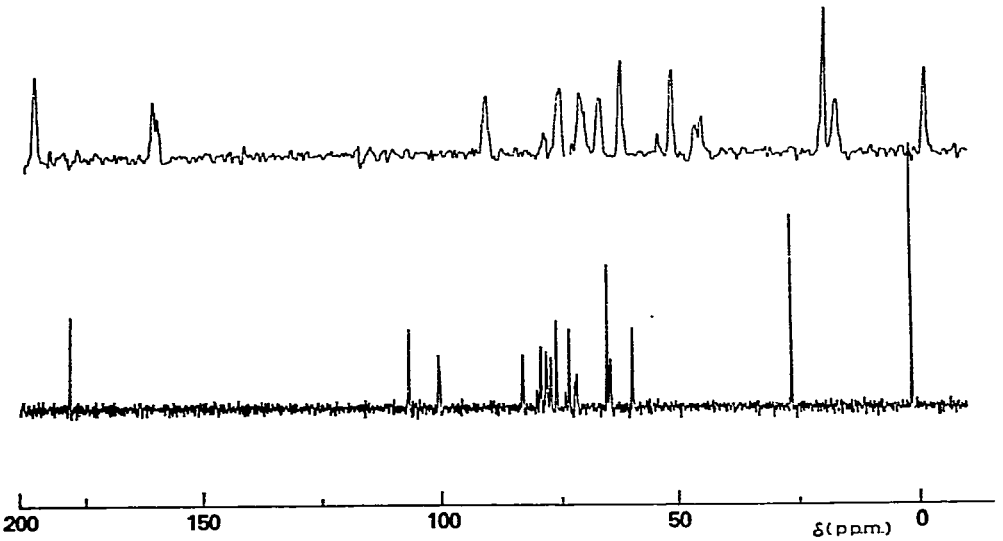


Fig. 4. ^{13}C-N.m.r. spectrum of the *H. influenzae* type c polysaccharide following *O*-deacetylation with ammonium hydroxide (the polymer was dialyzed to remove acetamide and acetate). The top tracing is an expansion of the ~50–110-p.p.m. region.

The time course of the *O*-deacetylation was monitored by ^{1}H-n.m.r. spectroscopy (see Fig. 3). The signals at 2.10 and 2.28 p.p.m. are major, *N*-acetyl and *O*-acetyl resonances, and the less-intense signal at 2.17 p.p.m. is the *N*-acetyl signal from non-*O*-acetylated units. Comparative integration of these signals indicated that ~80% of the residues are *O*-acetylated, in agreement with the ^{13}C-n.m.r. results. Subsequent to addition of ammonium hydroxide, the two major resonances decreased in intensity, while the minor resonance increased in intensity. Concomitantly, two new (and narrower) resonances arose that are attributable to acetate and acetamide. At completion, integration of the *N*-acetyl to (acetate + acetamide) methyl resonances demonstrated that 80% of the residues were *O*-acetylated in the original sample, confirming the above sets of data.

The ^{13}C-n.m.r. spectrum of the *O*-deacetylated polymer is shown in Fig. 4. In addition to the absence of *O*-acetyl-group resonances and of the previously observed, minor resonances, one further feature of the spectrum is noteworthy. The resonance for C-2 of 2-acetamido-2-deoxyglucose had shifted downfield by 1.6 p.p.m.; this change in chemical shift establishes the 3-hydroxyl group of the 2-acetamido-2-deoxyglucosyl residue as the site of *O*-acetylation in the starting polysaccharide[15].

Mild hydrolysis of the starting polysaccharide with acid, followed by treatment with alkaline phosphatase, resulted in a fragment having a low molecular weight, presumably a disaccharide (as judged by gel-permeation chromatography on Sephadex G-25). Examination of the ^{13}C-n.m.r. spectrum of this material (see Fig. 5) showed that the linkage of C-1 of 2-acetamido-2-deoxyglucose to galactose remained intact, whereas the C-1 linkage of galactose was disrupted. The resonance at 104.6 p.p.m.

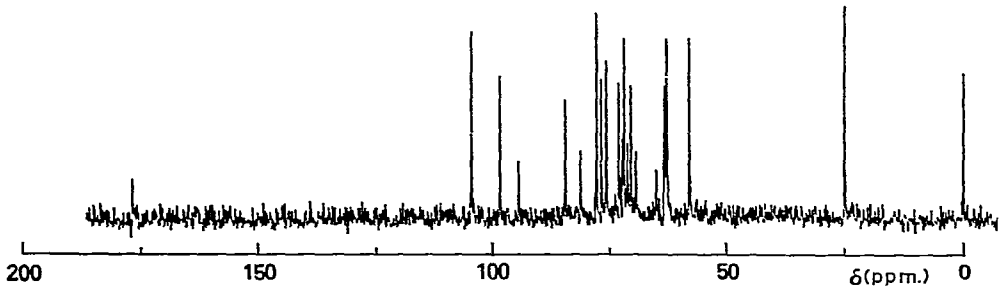


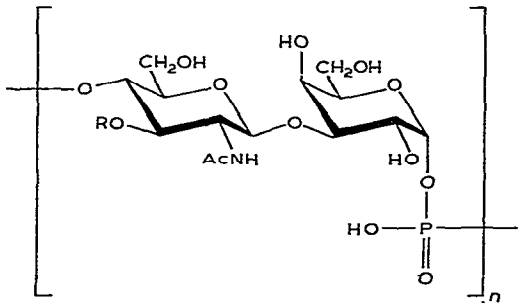
Fig. 5. ^{13}C-N.m.r. spectrum of the *H. influenzae* type c polysaccharide following mild treatment with acid (0.25M HCl) and with alkaline phosphatase.

remained unaltered, and that at 98.6 p.p.m., along with its scalar coupling to phosphorus, disappeared. Two new resonances, at 99.0 and 94.93 p.p.m., arose. (The C-1 resonance for the β and α anomers of galactose appear at 99.16 and 95.0 p.p.m., respectively; the corresponding resonances for 2-acetamido-2-deoxyglucose occur at 97.7 and 93.61 p.p.m., respectively.) Upon treatment of the hydrolyzed material with $NaBH_4$, the resonances at 99.0 and 94.93 p.p.m. disappeared. Sugar analysis of the borohydride-reduced material revealed the presence of 2-acetamido-2-deoxyglucose but no galactose. Phosphate is thus attached to C-1 of galactose.

The type c polysaccharide did not consume periodate, even after prolonged treatment at room temperature. Accordingly, it contains no vicinal hydroxyl groups. With this information, the structure of the polysaccharide may be derived. The anomeric carbon atom of 2-acetamido-2-deoxyglucose is directly linked to galactose, O-3 is acetylated, and O-6 is unsubstituted (see earlier). Therefore, the site of attachment of galactose to 2-acetamido-2-deoxyglucose is O-4; galactose is attached to O-4 of 2-acetamido-2-deoxyglucose *via* a phosphoric diester linkage. The diester nature of the phosphate linkage is established by (*a*) the acidity of the polysaccharide, (*b*) the hydrolytic stability of the phosphate bond, and (*c*) the titration behavior of the phosphate[12].

The galactosyl terminus of the phosphoric diester linkage is C-1. As the 6-hydroxyl group of galactose is unsubstituted, and the polymer does not consume periodate, C-1 of 2-acetamido-2-deoxyglucose is linked to O-3 of the galactosyl residue.

From the observed values of the H-1–H-2, scalar coupling-constants of galactose and 2-acetamido-2-deoxyglucose, in conjunction with the Karplus relationship[16], the α or β chirality of C-1 can be determined. An 8.5-Hz, H-1–H-2 coupling-constant was measured for the dephosphorylated *O*-(2-acetamido-2-deoxyglucosyl)galactitol; accordingly, the 2-acetamido-2-deoxyglucosyl group possesses the β configuration. The anomeric configuration of the galactose was established by utilizing the *O*-deacetylated polymer. To distinguish ^{31}P from ^{1}H-2 coupling to H-1, the ^{31}P resonance was irradiated. With ^{31}P decoupling, the doublet for H-1 (J 6 Hz) collapsed to a singlet having a line width at half-height of ~2 Hz. Consequently, the coupling



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R = $-C(O)CH_3$ (0.8)

R = $-H$ (0.2)

between H-1 and H-2 of the galactose must be small, and, accordingly, the galactose must possess the α configuration at C-1. The structure of the *H. influenzae* type c polysaccharide is, therefore, that depicted in formula **1**.

From Fig. 6a, it may be seen that the *H. influenzae* type c polysaccharide produces a typical, precipitin curve with the sheep antiserum. Maximum precipitation (equivalence zone) occurred with 50 μg of type c polysaccharide per 0.5 mL of antiserum. Accordingly, this and a lower antigen content (25 μg in 0.5 mL; antibody excess) were chosen in a study of the effect of the *O*-deacetylated, type c polysaccharide

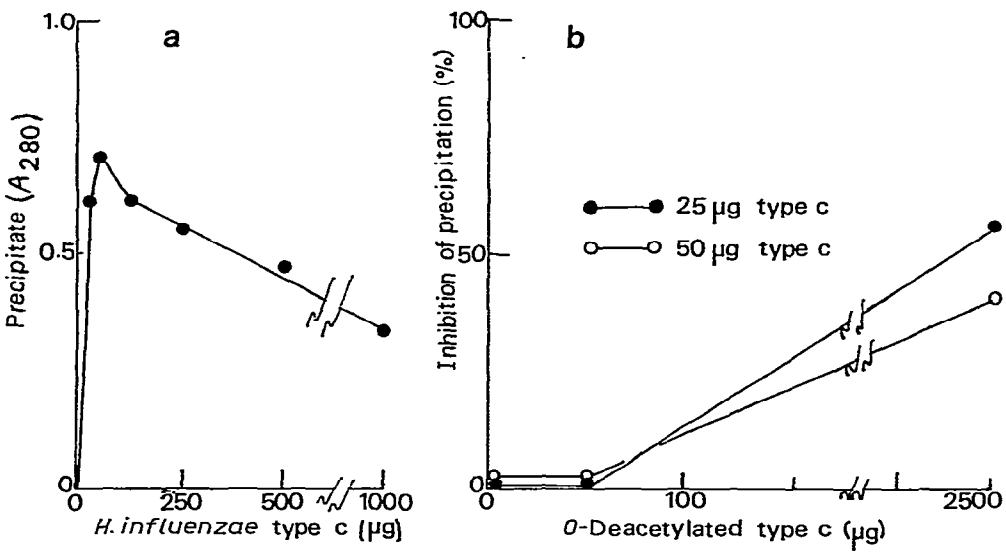

Fig. 6. (a) Precipitin curve between *H. influenzae* type c polysaccharide and homologous, sheep antiserum. (b) The inhibitory effect of *O*-deacetylated, type c polysaccharide on the precipitation reaction between type c polysaccharide and homologous, type c antiserum at equivalence (50 μg of polysaccharide) and at antibody excess (25 μg of polysaccharide).

upon the homologous, type c antibody precipitation. Fig. 6b shows the inhibitory effect induced by the *O*-deacetylated preparation upon the homologous, type c antibody precipitation. At comparable concentrations, the *O*-deacetylated preparation had no inhibitory effect upon the precipitation induced by 25 or 50 μg of type c polysaccharide. At fifty times the concentration of type c, the *O*-deacetylated preparation showed an inhibitory effect that was greater upon the 25-μg (57% inhibition) than upon the 50-μg level (43% inhibition) of the type c polysaccharide. No precipitation was observed with the *O*-deacetylated preparation or with *H. influenzae* type b polysaccharide.

DISCUSSION

The type c polysaccharide is a phosphoric diester-linked polymer of galactose and 2-acetamido-2-deoxyglucose. Phosphoric diester-linked disaccharide residues in capsular polysaccharides have been reported for the *H. influenzae* types a, b, and f organisms (refs. 3, 4, 5, and 17); although the structures of the *H. influenzae* types d and e capsules have not yet been completely elucidated, their repeating units do not contain phosphoric diester linkages[17,18]. Both the d and the e capsular polysaccharides contain residues of 2-acetamido-2-deoxyglucose and of an additional (acidic) sugar in their repeating units[17,18]. Only the type b organism has a capsule composed of units of two pentose residues and phosphate. The remaining types have units containing one or more hexose residues.

The type c polysaccharide cross-reacts with the *Streptococcus pneumonia* type 11 polysaccharide[11]. Although the structure of the polysaccharide of the type 11 capsule has not yet been fully elucidated, it contains 2-acetamido-2-deoxyglucose and galactose residues in its repeating unit[19].

A loss of antigenic activity has been shown[20,21] to accompany the *O*-deacetylation of pneumococcal types 11A and 18C. The presence of *O*-acetyl groups also affects both the immunogenicity and antigenicity of the pneumococcal type 1 polysaccharide[22], the *Escherichia coli* K1 polysaccharide[23], and the *Neisseria meningitidis* Group C polysaccharide[24]. The antigenic significance of the *O*-acetyl group was probed in the present study of the *H. influenzae* type c polysaccharide by studying the inhibitory effect of the *O*-deacetylated type c polysaccharide on the precipitation reaction between the untreated, type c polysaccharide and its homologous antiserum. The *O*-deacetylated preparation did not precipitate with the type c antiserum, and showed a low inhibitory activity in the homologous, precipitation reaction. Removal of the *O*-acetyl group was accompanied by a decrease in molecular size, as the K_d value (defined in ref. 7) fell from 0.4 to 0.8 on a column of Sepharose 4B. Based on our laboratory experience, such an alteration in K_d value is unlikely to account for the loss in precipitability of the *O*-deacetylated polysaccharide with the type c antiserum. By extension, it may be concluded that the *O*-acetyl group confers an antigenic specificity to the type c polysaccharide.

ACKNOWLEDGMENTS

We thank Dr. Robert Highet (National Institutes of Health, Bethesda, Maryland) for making a Varian XL-100/15 spectrometer available to us for the ^{31}P-decoupling experiment, Mr. Robert Boykins (Bureau of Biologics) for his expert help with sugar analyses, and Drs. John B. Robbins and Teh-Yung Liu (Bureau of Biologics) for critical discussions and helpful advice.

NOTE ADDED

The structure of the type c *Haemophilus influenzae* capsular polysaccharide has recently been elucidated[25]; the structure presented herein is in agreement with that determined by these workers.

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