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

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ABSTRACT

Employing a combination of chemical and spectroscopic techniques, the structure of the *Haemophilus influenzae* type d capsular polysaccharide was found to be →4)-β-D-GlcNAc-(1→3)-β-D-ManNAcA-(1→. L-Alanine, L-serine, and L-threonine, in the molar ratios of ~1.0:1.0:0.3, were linked to C-6 of the D-mannosyluronic residue as amides; the (serine + alanine + threonine) to ManNAcA ratio was ~0.95:1.0. Removal of the amino acids by mild hydrolysis with sodium hydroxide resulted in a material that was cross-reactive with the native, type d polymer. The base-treated, type d polysaccharide was not observed to cross-react with either the *H. influenzae* type e or *Escherichia coli* K7 capsular polysaccharide, both of which are structurally similar to type d.

INTRODUCTION

Fifty years ago, Pittman[1] noted that invasive *H. influenzae* disease in humans is caused by encapsulated organisms, and that all meningitis-associated isolates were of one capsular type, namely, type b (there are six known capsular serotypes and these are designated by the letters a through f). Since then, Pittman's observations have been confirmed and extended[2,3].

The role of capsule as a virulence factor, although not yet fully elucidated, is considered to be associated with the extent of inhibition of phagocytosis by host-defense cells; in the presence of antibodies specific for the capsular polysaccharide, however, phagocytosis does occur, and protection is conferred to the host[4]. The relationship between capsular structure and type b virulence is, as yet, unknown; this study was undertaken, in part, to increase such understanding through structural comparison of type b with other, nonvirulent, capsular serotypes. The structures for the type b (ref. 5), type a (ref. 6), type c (ref. 7), type f (ref. 8), and type e (ref. 9)

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polysaccharides have been reported. We present herein our findings with *H. influenzae* type d.

RESULTS

Treatment of the *H. influenzae* type d capsular polysaccharide with 1.0M NaOH for 8 h at 25° resulted in the liberation of three amino acids, namely, threonine, serine, and alanine. The identity of these amino acids was established by chromatographic (amino acid analyzer) and spectroscopic (^{13}C- and ^{1}H-n.m.r.) comparison with authentic materials. Following hydrolysis of the type d polysaccharide with hydrochloric acid, the freed amino acids were not oxidized to their respective keto acids by the enzyme D-amino acid oxidase (EC 1.4.3.3), although authentic samples of the corresponding D-amino acids were oxidized by the enzyme, thereby establishing its activity; the amino acids associated with the type d antigen thus have the L absolute configuration. The base-treated polymer was composed of residues of 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-mannuronic acid; the identity and absolute configuration of these sugars were established by procedures described for the compositionally identical *H. influenzae* type e polysaccharide[9a]. Other sugars were not detected.

After carbodiimide-mediated reduction with sodium borohydride, acid-catalyzed hydrolysis of the NaOH-treated polysaccharide yielded 2-amino-2-deoxy-mannose and 2-amino-2-deoxyglucose in the molar ratio of ~0.9:1.0. In contrast, when the native, type d polysaccharide was hydrolyzed after similar reduction, the 2-amino-2-deoxymannose:2-amino-2-deoxyglucose ratio was ~0.05:1.0; this suggests that, in the native, type d polysaccharide, the amino acids are covalently attached to the carboxyl group of the 2-amino-2-deoxymannosyluronic acid residue (either as amides, or, in the case of serine and threonine, as esters), thereby preventing reduction of the uronic acid. (It should be noted that borohydride reduction is necessary prior to acid-catalyzed hydrolysis, as the product deriving from the 2-amino-2-deoxymannosyluronic acid residue in the polymer is unstable to the reaction conditions[9a].)

The covalently linked amino acids in the native, type d polysaccharide are resistant to oxidation by ninhydrin, and to deamination by nitrous acid. Thus, after treatment with either of these reagents, serine, threonine, and alanine are quantitatively liberated from the polysaccharide by mild, base-catalyzed hydrolysis as already described. This lack of reactivity to reagents directed to amino groups demonstrates that these groups in the three amino acids are not free, but participate in amide linkages to C-6 of mannosyluronic acid residues.

The ^{13}C-n.m.r. spectra of the base-treated, type d polysaccharide, at solution pH values of 1.5 and 7.0, are shown in Fig. 1. The spectrum at low pH displays eleven resonances, of approximately equal intensity, in the 50–110-p.p.m. region, and is in accord with the chemical composition already presented; additionally, the spectrum is characteristic of each sugar's being in the pyranoid form. Several spectral

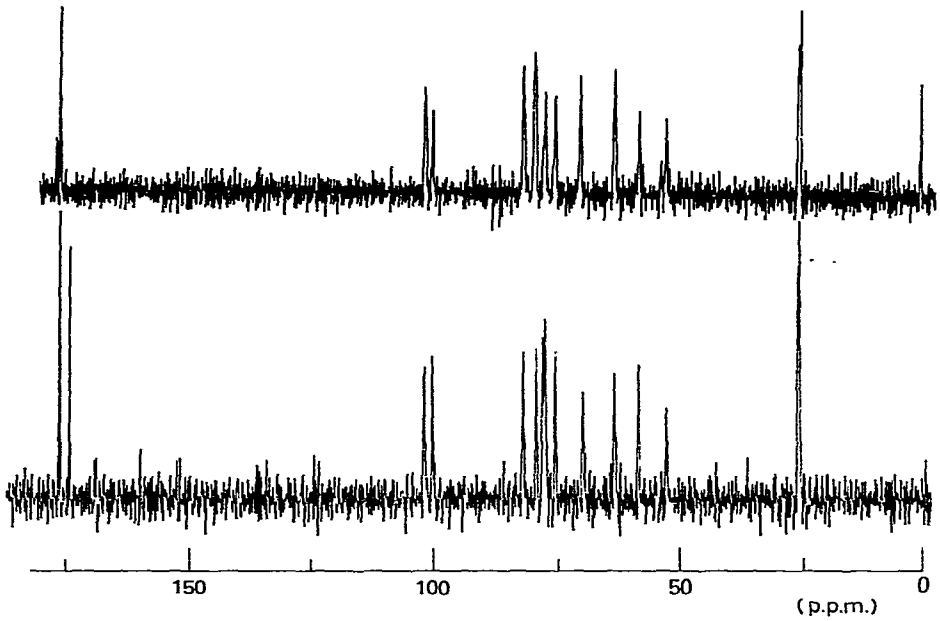


Fig. 1. ^{13}C-N.m.r. spectra (25 MHz) of the base-treated, *H. influenzae* type d polysaccharide at pH 7.0 (top trace) and pH 1.5 (bottom trace). [The polysaccharide (~30 mg) was dissolved in 1:19 D_2O–H_2O (~1.5 mL) containing 0.01M EDTA and sodium 2,2,3,3-tetradeuterio-4,4-dimethyl-4-silapentanoate (TSP) as the internal reference standard, and placed in 10-mm n.m.r. tubes. Spectra were recorded at ~70°, in order to improve the resolution. Approximately 30,000 transients were averaged for each spectrum; spectra were obtained by employing 90° pulses (18 μs), 8192 data points, a 5-kHz spectral window and matched 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 so as to result in an additional, 1-Hz line-broadening in the frequency-domain spectrum.]

assignments can be readily made*. The signals at 52.24 and 57.89 p.p.m. correspond to the C-2 atoms of the amino sugars. The 62.91-p.p.m. signal corresponds to C-6 of the 2-amino-2-deoxyglucose residue; the presence of a single hydroxymethyl group is in accord with chemical analysis, which showed the presence of a carboxylic acid group containing C-6. The six resonances in the 69–92-p.p.m. region correspond to C-3, C-4, and C-5 of the two sugars. Anomeric (C-1) carbon resonances are observed at 100.38 and 102.10 p.p.m. In addition to these eleven signals, two *N*-acetyl methyl group resonances occur at 24.85 and 25.20 p.p.m., showing that both amino sugars are *N*-acetylated. Two carbonyl-carbon resonances are seen at 175.09 and 177.08 p.p.m. ^{13}C-N.m.r. chemical-shifts for the base-treated *H. influenzae* type d polysaccharide are collected in Table I. The multiplicities of each of these carbon atoms, as observed in the proton-coupled, ^{13}C-n.m.r. spectrum[10], were in accord with the aforementioned, spectral assignments.

*The application of n.m.r. spectroscopy to structure elucidation in polysaccharides is reviewed *in*, for example, ref. 10.

TABLE I

^{13}C-N.M.R.-SPECTRAL DATA FOR THE *H. influenzae* TYPE d POLYSACCHARIDE, THE BASE-TREATED TYPE d POLYSACCHARIDE, AND THE BASE-TREATED, CARBOXYL-REDUCED POLYSACCHARIDE[a]

Carbon atom	H. influenzae *type d (native)*		H. influenzae *type d (base-treated)*		H. influenzae *type d (reduced)*
C-1	100.13	(100.8)[b]	100.48	(100.38)[b]	100.43
C-2	57.9	(57.5)	58.0	(57.89)	57.75
C-3	74.77	(74.75)	75.1	(75.1)	74.70
C-4	81.32	(81.52)	81.8	(81.77)	81.53
C-5	77.0	(77.2)	77.5	(77.58)	78.86
C-6	62.7	(63.0)	62.9	(62.91)	62.9
NC(O)CH_3	25.1[c]	25.1[c]	25.2[c]	25.2	25.1[c]
NC(O)CH_3	176.6[d]	177.4[d]	177.08[d]	(177.08)[d]	176.7[d]
C-1′	100.7[e]	(102.0)[e]	102.0[e]	(102.1)[e]	102.15[e]
C-2′	52.0	(52.7)	53.34	(2.24)	52.50
C-3′	78.7	(79.5)	79.45	(79.09)	79.49
C-4′	69.4	(69.5)	70.0	(69.34)	67.55
C-5′	77.6	(77.8)	79.45	(77.14)	77.0
C-6′	—	—	178.1	(175.09)	63.3
NC(O)CH_3	24.7[c]	24.85[c]	24.5[c]	24.85[c]	24.0[c]
NC(O)CH_3	176.7[d]	17.4[d]	177.08[d]	177.08[d]	176.7[d]
Alanine C_α	53.7	(51.0)			
C_β	20.04	(18.9)			
Serine C_α	59.69	(57.3)			
C_β	64.51	(63.5)			
Threonine C_α	—	—			
C_β	—	—			
C_γ	22.08	(22.1)			

[a]Spectral data are in p.p.m. relative to sodium 2,2,3,3-tetradeuterio-4,4-dimethyl-4-silapentanoate as the internal standard. Primed carbon atoms refer to 2-acetamido-2-deoxymannosyluronic acid, and unprimed carbon atoms, to 2-acetamido-2-deoxyglucose. [b]Values in parentheses refer to a solution pH of ~1.5; otherwise the pH was ~7.0. [c,d,e]Assignments may be reversed.

On raising the pH of the solution to 7.0, the resonance positions of two of the signals were altered by >0.1 p.p.m.; the erstwhile 77.14- and 175.09-p.p.m. signals were shifted downfield by ~2.3 and 3.0 p.p.m., respectively. Similar changes in chemical shift of a carboxyl-group carbon atom and its contiguous carbon atom characteristically accompany the ionization of a carboxylic acid[11,12]. On the basis of the pH-dependence of these signals, the 175.09-p.p.m. resonance is assigned to C-6 of the 2-acetamido-2-deoxymannosyluronic acid residue, and the 77.14-p.p.m. signal to C-5 of the same residue.

The ^{13}C-n.m.r. spectrum of the native, type d polysaccharide at pH 7.0, in the region between ~50 and 105 p.p.m., is shown in Fig. 2; ^{13}C-spectral data for this material are presented in Table I. In addition to the resonances already discussed, a number of signals, due to the amino acids, are observed. On the basis of chemical-shift comparisons with model compounds (amino acids, small peptides, and acetyl-

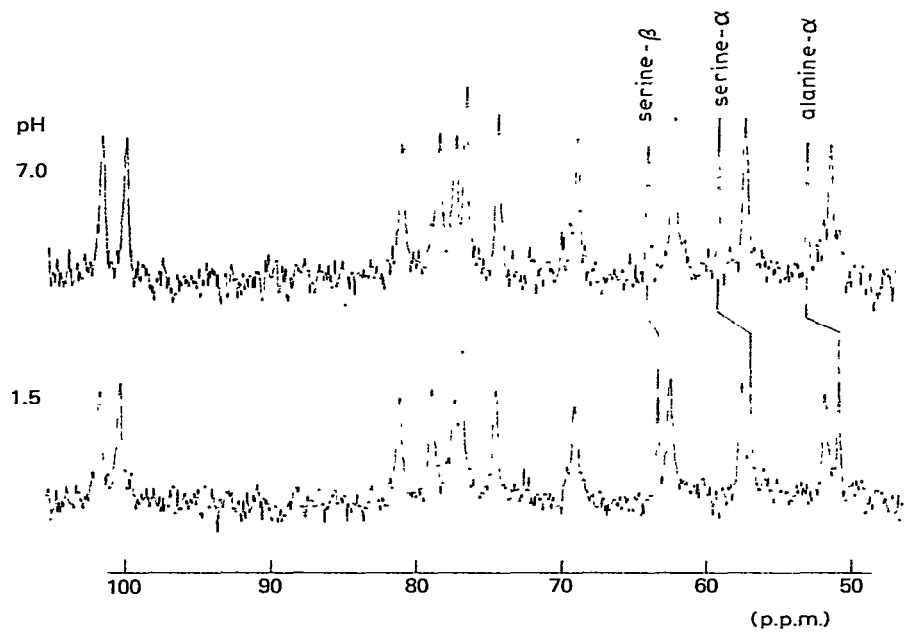


Fig. 2. ^{13}C-N.m.r. spectra (25 MHz) of the native, *H. influenzae* type d polysaccharide at pH 7.0 (top trace) and 1.5 (bottom trace); only the region between ~50 and 105 p.p.m. is displayed. Spectral conditions were as described in the legend to Fig. 1.

ated amino acids), the resonances at 20.04 and 53.7 p.p.m. are assigned to the alanine Cβ and Cα atoms, the resonances at 59.69 and 64.51 p.p.m. to the serine Cα and Cβ atoms, and the signal at 22.08 p.p.m. to the threonine Cγ atom[12]. On lowering the solution pH to 1.5, the serine and alanine Cα resonances are shifted upfield by ~2.5 p.p.m., and the alanine Cβ resonance is shifted upfield by ~1.5 p.p.m. (see Fig. 2). The signals for the 2-acetamido-2-deoxymannosyluronic acid residue were not shifted appreciably on altering the solution pH; this demonstrates the covalent attachment of the amino acids to C-6 of the mannosyluronic acid residue. Additionally, the resonance position of serine Cβ corresponds to a primary alcohol, not an ester, supporting our chemically based conclusion as to an amide linkage.

The alanine:threonine ratio, as well as the (alanine + threonine):2-acetamido-2-deoxymannosyluronic acid ratio in the type d polysaccharide was established by comparative integration of the -NC(O)CH_3 and amino acid -CHCH_3 signals in the ^{1}H-n.m.r. spectrum. The alanine:threonine ratio was ~1.0:0.3, and the (alanine+threonine):2-acetamido-2-deoxymannosyluronic acid ratio, ~0.56:1.0. ^{13}C-N.m.r. spectroscopy indicated a serine to alanine ratio of ~1:1. Following acid-catalyzed hydrolysis, amino acid analysis in an automated analyzer indicated serine:alanine:threonine ratios of ~3:3:1.

The anomeric carbon resonances for both the native and base-treated type d polysaccharides are observed at ~102.0 and 100.5 p.p.m. By comparison with those of model compounds[9a], these shifts demonstrate that C-1 in each of the sugars is

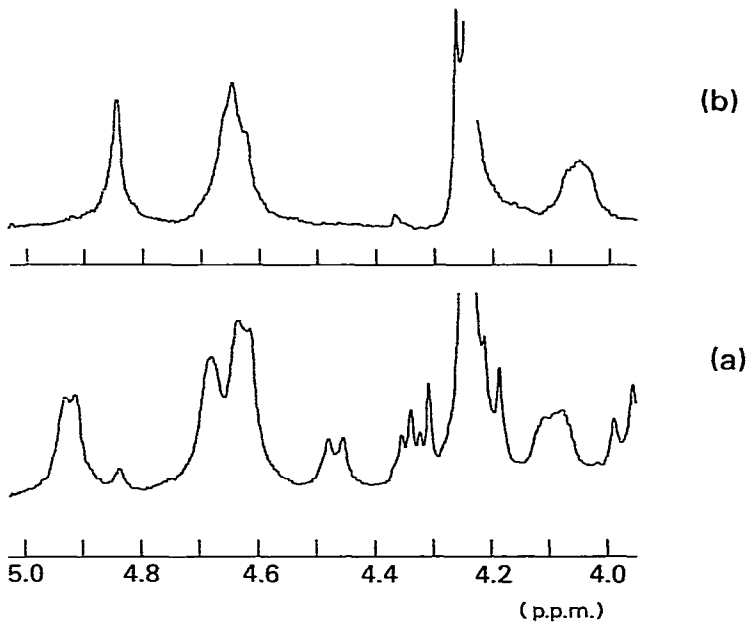


Fig. 3. ^{1}H-N.m.r. spectrum (300 MHz) of the native- (a) and base-treated (b) *H. influenzae* type d polysaccharide; only the region between ~4.0 and 5.0 p.p.m. is displayed. [The polysaccharide (~5 mg), following freeze-drying of a D_2O solution, was dissolved in D_2O (~0.5 mL) containing 0.75% of TSP as the internal reference standard, and placed in 5-mm n.m.r. tubes. Spectra were recorded at ~70°, in order to improve the resolution. Spectra were obtained by employing 90° pulses (6 μs), 8192 data points, a 3-kHz spectral window and matched filter, 32 transients, and a pulse-repetition time of 1 s. Prior to Fourier transformation, the free-induction-decay signal was zero-filled (twice), and exponentially multiplied so as to result in an additional, 0.5-Hz line-broadening in the frequency-domain spectrum.]

involved as a glycoside. In agreement with this spectroscopic conclusion, the native, and base-treated, type d polymers did not give an appreciable, positive Park–Johnson[13] test for reducing sugar. To complete the structure determination of the type d polymer, it remained to (*i*) establish the anomeric stereochemistries of the sugar residues, and (*ii*) distinguish, for each sugar, between C-3 and C-4 as the remaining linkage site. As C-6 is involved in an amide linkage in the one residue, and is in a free, hydroxymethyl group in the other, the second linkage site in each sugar must, by default, be C-3 or C-4; this conclusion is supported by the observation that neither the native nor the base-treated polymer consumes periodate, even under forcing conditions.

Several distinct, spectroscopic correlations were employed in order to determine the anomeric stereochemistry. The anomeric region of the ^{1}H-n.m.r. spectrum (300 MHz) of both the native and base-treated type d polysaccharide is shown in Fig. 3. By comparison with model sugars and polysaccharides containing 2-acetamido-2-deoxyglucose or 2-acetamido-2-deoxymannose, or both, the resonance at ~4.84 p.p.m. is assigned to H-1 of the 2-acetamido-2-deoxymannosyluronic acid residue, and it corresponds to the β configuration[14,15]. The resonance at 4.64 p.p.m.

is assigned to H-1 of the 2-acetamido-2-deoxyglucosyl residue, and it also corresponds to the β configuration[15,16]. Significantly, resonances were not observed in spectral regions that are characteristic of the α configuration of either sugar[14-16]. An additional resonance, at ~4.65 p.p.m., is observed in the anomeric region of the spectrum; on the basis of extensive, ^{1}H-decoupling studies[17] performed with the *Escherichia coli* K7 capsular polysaccharide, which contains 2-acetamido-2-deoxymannosyluronic acid and glucosyl residues in the repeating unit, the 4.65-p.p.m. signal can be assigned to H-2 of the 2-acetamido-2-deoxymannosyluronic residue. In the native, type d polymer, the *β-gluco* H-1 resonance is observed at ~4.6 p.p.m.; the mannosyluronic H-1 resonance has, however, moved downfield relative to its position for the base-treated polymer, and is, moreover, split into two resonances centered at ~4.92 p.p.m. (see Fig. 3). A small resonance is still observed at 4.84 p.p.m., and this reflects the presence of unsubstituted repeating-units (~5%).

Coupling constants for H-1–H-2 were not, in the present instance, of value in proving the anomeric stereochemistry. Both anomers of 2-acetamido-2-deoxymannosyluronic acid would, on the basis of the Karplus relationship[18], be expected to have small, and approximately equal, coupling constants. Although the anomers of glucose and its derivatives have markedly different H-1–H-2 coupling-constants, we were unable to measure them for the type d polysaccharide due to overlap with other sugar resonances, and we did not attempt "2-D" n.m.r. experiments to resolve these couplings[19]. In contrast, the directly bonded, ^{1}H-1–^{13}C-1, scalar coupling-constants were useful in assigning anomeric stereochemistry. Although the anomeric ^{13}C signals have not been assigned to specific sugar residues, both resonances exhibit coupling constants that are indicative of the β configuration, namely, 163 Hz for the 102-p.p.m. signal, and 160 Hz for the 100-p.p.m. signal[20,21].

Comparison of ^{13}C-n.m.r. chemical-shifts for 2-acetamido-2-deoxymannose and its derivatives reveals that the resonance position of C-5 is sensitive to the anomeric stereochemistry[9]. The C-5 and C-6 resonances of the 2-acetamido-2-deoxymannosyluronic acid residue of the base-treated type d polymer were assigned on the basis of titration studies (see earlier). Following reduction of the polysaccharide with borohydride, the C-5 resonance occurs at 78.86 p.p.m. From the ^{13}C chemical-shift of C-5, the β configuration may be assigned to the 2-acetamido-2-deoxymannosyluronic acid residue.

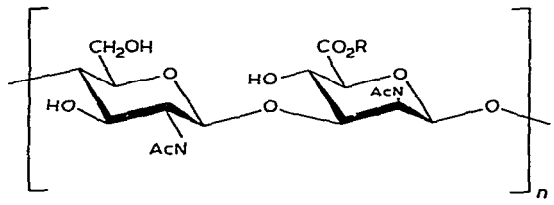
The ^{13}C-n.m.r. chemical shifts of anomeric carbon atoms are generally useful in assigning stereochemistry at this position. For example, the C-1 resonance of methyl D-glucopyranoside occurs at ~105 p.p.m. for the β, and 101 p.p.m. for the α, anomer. In the spectrum of the type d polysaccharide, however, the *gluco* shift was misleading. Regardless of which anomeric resonance was assigned to the *gluco* residue, its position indicated the α configuration. Only after considering the totality of data (^{1}H chemical-shift, all of the ^{13}C chemical-shifts, and the ^{1}H–^{13}C coupling-constant) could the β configuration be firmly assigned to the 2-acetamido-2-deoxyglucosyl residue in the type d polymer. Thus, the chemical nature of the aglycon can significantly affect the anomeric chemical-shift. We have made the same observation

with the structurally similar *E. coli* K7 capsular polysaccharide[17]. For determinations of anomeric stereochemistry, we therefore caution against placing undue emphasis on anomeric-carbon chemical-shifts.

^{13}C-N.m.r. spectroscopy showed that the *gluco* residue was substituted at C-4. In addition to the resonance positions of all carbon atoms's being consistent with C-4 substitution, signals characteristic of C-3 substitution were not observed (for the *H. influenzae* type e polysaccharide, wherein the *gluco* residue is substituted at C-3, its C-3 resonance occurs[9a] at ~85 p.p.m.).

The mannosyluronic residue is substituted at C-3. Spectroscopically, this is evidenced by (*i*) the upfield shift of the C-2 resonance, (*ii*) the presence of a C-4 resonance at its "unsubstituted" position, and (*iii*) the lack of a C-3 signal at the "unsubstituted" position. Figs. 1 and 2 show the hexosamine C-2 resonances at 52–53 and 57–58 p.p.m. (the exact value of the chemical shift depending on the pH of the solution, the presence of amino acids, and the oxidation state of the "mannosyl" C-6 atom). The ~57.75-p.p.m. resonance can be safely assigned to C-2 of the 2-acetamido-2-deoxyglucosyl residue; the 52.5-p.p.m. resonance then corresponds to C-2 of the 2-acetamido-2-deoxymannosyl residue. The *manno* C-2 resonance is shifted upfield by ~3 p.p.m., in comparison to the corresponding resonance in the spectrum of the *H. influenzae* type e polymer, wherein this residue is alkoxylated at C-4. This 3-p.p.m., upfield shift is indicative of C-3 substitution. (Alkoxylation generally causes a downfield shift of the signal of the contiguous carbon atom and a 0 to 3-p.p.m. upfield shift for those of neighboring carbon atoms[10].) Consistent with a C-3 linkage, the 67.55-p.p.m. resonance of the base-treated, borohydride-reduced polymer (see Table I) can be assigned to the unsubstituted C-4 atom; the signal is shifted ~2 p.p.m. upfield, relative to its position in the spectrum of methyl 2-acetamido-2-deoxymanno-pyranoside.

Complete *N*-deacetylation of the type d polysaccharide was not readily achieved with hydrazine–hydrazine sulfate. ^{1}H-N.m.r.-spectral analysis of the hydrazine-treated polymer revealed that one *N*-acetyl group remained, even after the mixture had been heated for 12 h at 100°. Periodate treatment of the mono-*N*-deacetylated polymer led to quantitative elimination of the 2-acetamido-2-deoxyglucosyl residue. The mono-*N*-deacetylated, periodate-oxidized polymer was dialyzed, reduced with sodium borohydride, the product hydrolyzed with 2M hydrochloric acid for 4 h at 100°, and the products peracetylated; g.l.c. analysis showed that erythritol was present in the product mixture. The quantitative elimination of glucose and the successful detection of erythritol as consequences of this reaction sequence confirm the C-4 linkage of the 2-acetamido-2-deoxyglucosyl residue. Resistance of the 2-acetamido-2-deoxymannosyluronic acid residue to hydrazine-induced *N*-deacetylation is consistent with the C-3 linkage of this residue. It is known[22] that alkoxylation adjacent to an *N*-acetyl group renders deacetylation by hydrazine difficult. The structure of the type d polymer, as deduced from the results of the chemical and spectroscopic experiments presented, is as shown.



Haemophilus influenzae Type d

R = L-serine (0.41)
L-threonine (0.41)
L-alanine (0.13)

The precipitin curve that results from admixing various amounts of the *H. influenzae* type d polysaccharide with donkey (burro)-derived antiserum (B-291) is shown in Fig. 4. Maximum precipitation (equivalence zone) occurs with ~100 μg of the type d polysaccharide per 0.5 mL of antiserum; at the equivalence zone, 1.37 mg of Hid antibodies are precipitated per mL. Preparations of native, type d polysaccharide containing low-molecular-weight antigenic species (Kd = 0.56; see Fig. 4), high-molecular-weight antigenic species (Kd = 0.0; not shown), or both (Kd = 0.03 and 0.64; see Fig. 4) gave similar results. The equivalence zone of the base-treated *H. influenzae* type d polysaccharide against the same antiserum, namely B291, was approximately the same as that found with the native, type d polysaccharide, *i.e.*,

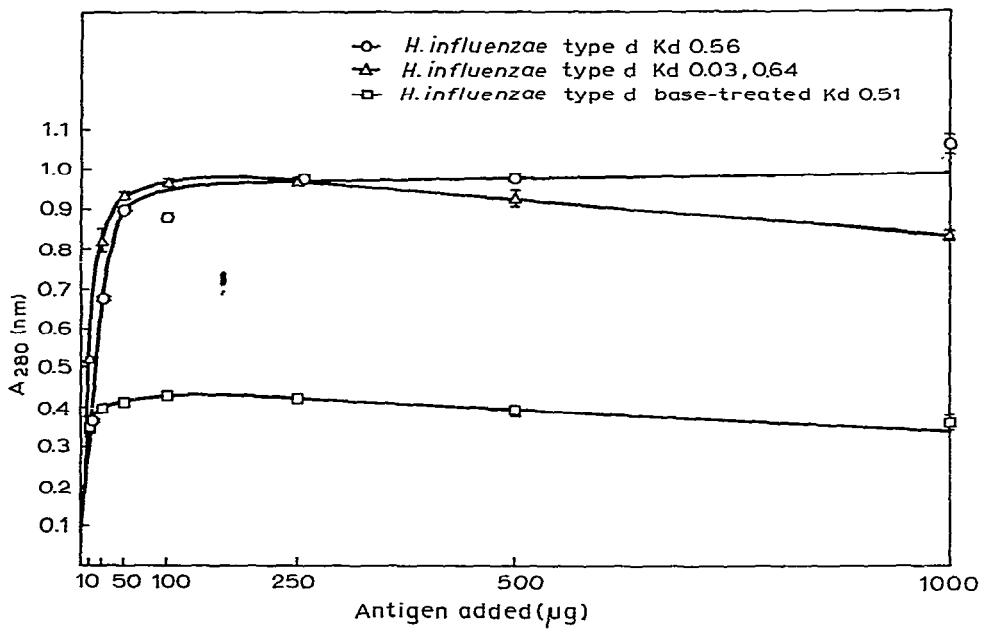


Fig. 4. Precipitin curve between the native, *H. influenzae* type d polysaccharide (circles, triangles), or the base-treated, type d polysaccharide (squares), and native, type d, burro-derived antiserum.

~100 μg of polysaccharide per 0.5 mL of antiserum; however, at the equivalence zone, only 0.61 mg of antibodies per mL was precipitated by the base-treated, type d polysaccharide. Therefore, ~55% of the combining sites of the burro-derived antibody are directed against antigenic structures composed of, or influenced by, the amino acids.

The results of these experiments were confirmed by double-immunodiffusion studies (not shown); the native, type d polysaccharide possessed antigenic determinants not present in the base-treated polysaccharide, as visibilized by a spur of the native, type d polysaccharide over the base-treated polysaccharide when reacted against B-291 antiserum. The base-treated, type d polysaccharide did not react (as ascertained by a failure to precipitate in a double-immunodiffusion experiment) with burro-derived, *H. influenzae* type e antiserum, or with rabbit-derived, *Escherichia coli* K7 antiserum. Neither the *H. influenzae* type e capsular polysaccharide nor the *E. coli* K7 capsular polysaccharide reacted (double immunodiffusion) against the B291 antiserum (the *H. influenzae* type e polysaccharide is compositionally identical[9] to the base-treated, type d polysaccharide; the *E. coli* K7 capsule is composed of equimolar amounts of 2-acetamido-2-deoxymannosyluronic acid and glucose[17]).

DISCUSSION

The *Haemophilus influenzae* type d and type e capsular polysaccharides, the structures of which have been presented here and in a preceding paper[9a], bear similarities to the other, *H. influenzae* serotype, capsular polysaccharides[5-8], in that they are polymers comprised of acidic, disaccharide repeating-units. However, they differ from the b, c, d, and f serotype polysaccharides in two major regards: first, they do not contain phosphoric diester linkages; second, each of them contains an unusual, capsular constituent, namely, 2-acetamido-2-deoxymannosyluronic acid. These distinctions are of probable phylogenetic significance. An unusual feature of the type d capsular polysaccharide is the presence of appended amino acids; such a structural feature has, to our knowledge, only been reported once[23] in bacterial, capsular polysaccharides; amino acids are, however, commonly found as constituents of bacterial, cell-wall polysaccharides.

The time course for complement-induced killing of the various *H. influenzae* serotype organisms by antibody-free, pre-colostral calf-serum has been determined[24]. On a normalized basis, the least killing was observed for type b and type a organisms, the most killing, with type d and type e organisms, and intermediate (although high) killing, with type c and type f organisms. In this regard, it is notable that the types a and b capsular polysaccharides are structurally similar, being phosphoric diester–linked polymers of ribitol–ribose and ribitol–glucose, respectively. The types d and e organisms possess the structural similarities already mentioned. The types c and f capsular polysaccharides contain *O*- and *N*-acetylated hexosamine residues.

A partial structure for the type d capsular polysaccharide was proposed by Williamson and Zamenhof[25], who reported that the type d polysaccharide is com-

posed of 2-acetamido-2-deoxyglucose and a "hexosamine-uronic acid" (2-acetamido-2-deoxyglucosyluronic acid) and contains (1→3) or (1→4) linkages. Recently, the structure of the type d capsular polysaccharide has been studied by Kenne *et al.*[26]; the results of their study are in complete accord with the structure presented herein.

The distinctions and similarities between the six *H. influenzae* serotype organisms, based on capsule structure or susceptibility to complement-induced lysis, are not mirrored[27] in their "bio-type".

EXPERIMENTAL

Materials. — 2-Acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-mannose, D- and L-arabinose, and L-serine were obtained from Calbiochem (San Diego, CA). D-Serine, D- and L-alanine, D- and L-threonine, and D-amino acid oxidase were obtained from Sigma Chemical Co. (St. Louis, MO). D-Glucose oxidase was obtained from Worthington Biochemical Corp. (Freehold, NJ). Sepharose and Sephadex for gel-permeation chromatography were obtained from Pharmacia (Piscataway, NJ). All additional, analytical reagents were of the highest purity available.

Isolation of polysaccharide. — The capsular polysaccharide from *H. influenzae* type d was isolated as described[28] for Bureau of Biologics strains "NIH-611" (Drs. Margaret Pittman and Charles Manclark, Bureau of Biologics, ATCC No. 9008) and "CDC-36" (courtesy of Dr. Robert Weaver, Center for Disease Control, Atlanta, GA; CDC Strain C8022). Compositional analysis and ^{13}C-n.m.r. spectroscopy showed that NIH-611 and CDC-36 were identical. Strain NIH-611 was used for the structure-determination studies. Determinations of protein, endotoxin, nucleic acids, moisture, and molecular size were conducted by described methods[29], and are presented in Table II.

TABLE II

ANALYTICAL RESULTS OBTAINED WITH *H. influenzae* TYPE d POLYSACCHARIDE, STRAIN 611

Parameter	*Value*
K_d (Sepharose 4B)	0.0
Moisture (%, w/w)	8
Protein (%, w/w)	1.1
Endotoxin (%, w/w)[a]	0.007
Nucleic acid (%, w/w)	0.3
Inorganic residue (%, w/w)	6.5
2-Amino-2-deoxyglucose (μmol/mg)[b]	2.75
2-Amino-2-deoxymannose (μmol/mg)[b]	2.13

[a]Determined by the limulus amebocyte lysate method. [b]Determined using the type d polysaccharide following base-hydrolysis (to remove amino acids) and carbodiimide-assisted reduction with $NaBH_4$ (see Experimental section).

Polysaccharide sugar analysis. — Hydrolysis of the native, capsular polysaccharide and chemically treated derivatives was conducted as described, using methanesulfonic acid[7b,30]. Amino sugars were analyzed with a Beckman 120-B amino acid analyzer. Neutral sugars were analyzed either underivatized, in an automated sugar-analyzer[30], or derivatized (as described[7b]), in a Varian Associates 3700 gas chromatograph equipped with a column of 3% of OV-225. Identification of sugars was accomplished by comparison with authentic samples. Absolute configurations were determined as described[9a].

Isolation and identification of polysaccharide-associated amino acids. — The type d polysaccharide (25 mg) was treated with M sodium hydroxide (2 mL) for 12 h at 25°, the base neutralized with hydrochloric acid, and the solution dialyzed under vacuum in collodion dialysis-tubing having a cut-off at mol. wt. 25,000. The dialyzate was collected, and freeze-dried; the material inside the dialysis tubing was also freeze-dried. The amino acids in the dialyzate were identified by comparison of their retention times in an automated, amino-acid analyzer with those of authentic materials, and by ^{1}H-n.m.r.-spectroscopic comparison with authentic materials.

Determination of the absolute configurations of serine, threonine, and alanine. — The type d polysaccharide (10 mg) was treated with 3M HCl (3 mL) for 2 h at 100°, to remove the amino acids. A stream of nitrogen was passed over the reaction mixture, to remove water and HCl. After being dried, the residue was dissolved in water, and the solution evaporated with a stream of nitrogen gas; this cycle was repeated three times. Solutions of authentic D-amino acids, L-amino acids, and the hydrolysis mixture were treated with D-amino acid oxidase, and aliquots of the three mixtures were analyzed at various times in an automated, amino acid analyzer. The concentration of amino acids in the solution derived from the type d polysaccharide, and in the solution of L-amino acids, did not change as a function of time; after 8 h, the concentration of D-alanine had decreased by 100%, of D-serine by 96%, and of D-threonine by 51%.

Reduction of the base-treated and native type d polysaccharide with sodium borohydride. — The polysaccharide was reduced by a modification of a method described by Taylor and Conrad[31]. Sodium 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (300 mg) was added to a stirred solution of the polysaccharide (7 mg in 2 mL of water) and the pH of the solution was adjusted to 4.75. After stirring for 2 h at room temperature, the pH was adjusted to 7.0, and 2M $NaBH_4$ solution (10 mL) was added dropwise, the pH of the mixture being maintained at 7.0 $\pm$2, and a drop of 2-octanol added to the mixture to prevent foaming. After being stirred for 2 h at room temperature, the mixture was dialyzed against water (3 × 6 L). The dialyzate was evaporated by freeze-drying, and the residue analyzed spectroscopically (^{1}H- and ^{13}C-n.m.r.) and chromatographically (automated, amino-acid analyzer). Based on the 2-amino-2-deoxyglucose and 2-amino-2-deoxymannose content (as determined in an automated, amino acid analyzer), the reduction was calculated to be 92% of the theoretical. Identical treatment of the native, type d

polysaccharide resulted in the expected amount of 2-amino-2-deoxyglucose, but only ~4% of the amount of 2-amino-2-deoxymannose.

N-*Deacetylation, and periodate oxidation, of the reduced polysaccharide.* — *N*-Deacetylation of an aliquot of the reduced polysaccharide was accomplished with hydrazine, as described[9a]. The hydrazine-treated sample 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 warmed 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, and dried with a centrifuge evaporator.

The material in one tube was then reduced with $NaBH_4$, and the product hydrolyzed with 2M HCl in a pyrolysis tube for 4 h at 100°. Water was removed by passing a stream of nitrogen over the sample. This dried hydrolyzate was then peracetylated with 1:1 (v/v) acetic anhydride–pyridine, and the acetates analyzed by g.l.c.; the material in the other tube was hydrolyzed with 2M methanesulfonic acid for 12 h at 115°, and then subjected to amino acid analysis as already described.

Nitrous acid treatment of the type d polysaccharides. — The polysaccharide (0.5 mg) was dissolved in water (0.5 mL), and 35% acetic acid (0.5 mL) and 5% sodium nitrite (0.5 mL) were added. The mixture was kept for 45 min at 5°, quenched with urea, and dialyzed against de-ionized water (3 × 1 L). Amino acid analysis of the recovered material led to no detectable change in composition, as compared with that of untreated material. Alanine, as a control, was completely deaminated under these conditions.

Quantitative precipitin analysis. — Quantitative precipitin analysis[32] was performed with Hid antiserum, raised by immunization of a burro (Bureau of Biologics, burro 291) with whole formalinized organisms (NIH stain 611). This burro antiserum was treated with the purified and the base-treated, type d polysaccharides. The reaction mixture consisted of heat-inactivated (30 min at 56°) antiserum (0.5 mL) and 0.5 mL of various concentrations of the polysaccharide antigen in saline. Following incubation for 1 h at 37° and for 5 days at 4°, the precipitates were collected by centrifugation, washed 12 times in cold, phosphate-buffered saline, and dissolved in 0.8% sodium lauryl sulfate (1 mL). The amount of antibody precipitated was calculated by assuming the optical absorbance of the immunoglobulin at 280 nm to be 14.0.

Double immunodiffusion. — Double immunodiffusion was conducted in 0.9% agarose in phosphate-buffered saline, using burro 291 type d antiserum and burro 266 type e antiserum and solutions of the native type d, base-treated type d (0.2 mg/mL), and type e polysaccharides (1.0 mg/mL). The plates were incubated for 24 h at 4°, washed for 48 h in phosphate-buffered saline, dried, and stained with 0.1% Coomassie Blue for visibilization.

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