Laurell, C. B., Pierce, J., Persson, U., & Thulin, E. (1975) *Eur. J. Biochem. 57*, 107–113.
Lienhard, G. E., Crabb, H. H., & Ransome, K. J. (1984) *Biochim. Biophys. Acta 769*, 404–410.
Manjunath, P., & Sairam, M. R. (1982) *J. Biol. Chem. 257*, 7109–7115.
Mega, T., Jujan, E., & Yoshida, A. (1980a) *J. Biol. Chem. 255*, 4053–4056.
Mega, T., Jujan, E., & Yoshida, A. (1980b) *J. Biol. Chem. 255*, 4057–4061.
Mort, A. J., & Lamport, D. T. A. (1977) *Anal. Biochem. 82*, 289–309.
Ogata, S.-I., & Lloyd, K. O. (1982) *Anal. Biochem. 119*, 351–359.
Olden, K., Parent, J. B., & White, S. L. (1982) *Biochim. Biophys. Acta 650*, 209–232.
Plummer, T. H., Jr., & Tarentino, A. L. (1981) *J. Biol. Chem. 256*, 10243–10246.
Plummer, T. H., Jr., Elder, J. H., Alexander, S., Phelan, A. W., & Tarentino, A. L. (1984) *J. Biol. Chem. 259*, 10700–10704.
Sairam, M. R. (1980) *Arch. Biochem. Biophys. 204*, 199–206.
Saklatvala, J., Wood, G. C., & White, D. D. (1976) *Biochem. J. 157*, 339–351.
Shifrin, S., Consiglio, E., & Kohn, L. D. (1983) *J. Biol. Chem. 258*, 3780–3786.
Taga, E. M., Waheed, A., & van Etten, R. L. (1984) *Biochemistry 23*, 815–822.
Takahashi, O., & Nishibe, H. (1981) *Biochim. Biophys. Acta 657*, 457–467.
Takahashi, N., Toda, H., Nishibe, H., & Yamamoto, K. (1982) *Biochim. Biophys. Acta 707*, 235–242.
Takasaki, S., Mizuochi, R., & Kobata, A. (1982) *Methods Enzymol. 83*, 263–268.
Tarentino, A. L., & Maley, F. (1974) *J. Biol. Chem. 249*, 811–817.
Tarentino, A. L., & Maley, F. (1975) *Biochem. Biophys. Res. Commun. 67*, 455–462.
Tarentino, A. L., & Plummer, T. H., Jr. (1982) *J. Biol. Chem. 257*, 10776–10780.
Tarentino, A. L., Plummer, T. H., Jr., & Maley, F. (1974) *J. Biol. Chem. 249*, 818–824.
Trimble, R. B., & Maley, F. (1977a) *Biochem. Biophys. Res. Commun. 78*, 935–944.
Trimble, R. B., & Maley, F. (1977b) *J. Biol. Chem. 252*, 4409–4412.
Trimble, R. B., Maley, F., & Watorek, W. (1981) *J. Biol. Chem. 256*, 10037–10043.

Structural Determination of the Capsular Polysaccharide of *Neisseria meningitidis* Group I: A Two-Dimensional NMR Analysis†

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ABSTRACT: The capsular polysaccharide antigen of *Neisseria meningitidis* group I was isolated by Cetavlon precipitation and purified by ion-exchange chromatography. The structure of the I polysaccharide was determined largely by comprehensive proton and carbon-13 nuclear magnetic resonance studies in which both one-dimensional and two-dimensional experiments were carried out directly on the I polysaccharide. The I polysaccharide is composed of the repeating unit →4)α-L-GulpNAcA(1→3)[4-OAc]β-D-Man*p*NAcA(→ in which the former residue adopts the $_4C^1$ (L) conformation and the latter residue adopts the $^4C_1$ (D) conformation. The one-bond coupling between the anomeric carbon and proton ($^1J_{^{13}C,H}$) of the 2-acetamido-2-deoxy-β-D-mannuronopyranosyl residue is not consistent with its β-D configuration. This anomalous value of $^1J_{^{13}C,H}$ for this residue is due to through-space anisotropy effects on its anomeric proton, generated by the proximity of the carboxyl group of the neighboring 2-acetamido-2-deoxy-α-L-gulurono-pyranosyl residue. The *O*-acetyl substituents of the I polysaccharide are essential for its antigenicity to group I polysaccharide-specific antibodies.

Neisseria meningitidis is a Gram-negative organism that has been classified serologically into groups A, B, C, D, 29E, L, W-135, X, Y, and Z (Gotschlich et al., 1969; Jennings, 1983; Ashton et al., 1983). Except for group D, each group produces a unique capsular polysaccharide that is the group-specific antigen, and the structures of all the polysaccharides have been determined (Jennings, 1983; Jennings et al., 1983). Ding et al. (1981) described three new meningococcal groups and designated them H, I, and K. The structure of the H polysaccharide has also been recently reported (Michon et al., 1984; Van der Kaaden et al., 1984). As a continuation of our structural studies on the meningococcal polysaccharides, we now report the structure of the I polysaccharide. The structure of the K polysaccharide has been reported elsewhere (Michon et al., 1985).

Nuclear magnetic resonance spectroscopy has played an increasingly important role in the structural elucidation of the meningococcal polysaccharides (Jennings, 1983; Jennings et al., 1983; Michon et al., 1984; Van der Kaaden et al., 1984), and the utility of this technique has been further demonstrated in the structural elucidation of the K (Michon et al., 1985) and I polysaccharides, where a number of one-dimensional and

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two-dimensional, including nuclear Overhauser enhancement (NOE), techniques were employed. Although two-dimensional techniques (Patt, 1984) have been used frequently on oligosaccharides since they were first reported in 1982 (Bernstein & Hall, 1982; Prestegard et al., 1982; Bruch & Bruch, 1982), examples of the direct application of these techniques to polysaccharides are few (Barrow et al., 1984).

Experimental Procedures

Materials. Strain 1486 (I) of *Neisseria meningitidis* was obtained from Dr. Shao-quing Ding, National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China, and was grown in a chemically defined medium (Bundle et al., 1974). The group I capsular polysaccharide was isolated and partially purified as previously described (Bundle et al., 1974) using an initial Cetavlon precipitaton to obtain the crude polysaccharide. Purification of the I polysaccharide was accomplished by the application of the polysaccharide, in 0.01 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer at pH 7.4, to a column of DEAE-Sephadex A-25 (Cl^- form, Pharmacia Fine Chemicals) and the development of the column with a linear gradient of NaCl to 0.5 M. The polysaccharide was detected by Ouchterlony immunodiffusion using antisera to the I polysaccharide made by injecting meningococcal group I organisms in rabbits as previously described (Ashton et al., 1979). By use of this assay, the I polysaccharide eluted as a single peak with approximately 0.3 M NaCl. However, by monitoring the eluate of the ion-exchange column with phenol–sulfuric reagent (Dubois et al., 1956), an additional minor polysaccharide component was detected which was easily separated from the I polysaccharide by virtue of its prior elution from the column. This polysaccharide contaminant was serologically inactive and was not investigated further. The I polysaccharide had $[\alpha]_D$ −112° (*c* 0.24, water). D-Gulosamine was obtained from Dr. M. B. Perry of this laboratory.

The de-O-acetylated I polysaccharide was obtained by treating the I polysaccharide with 0.1 M NaOH at 25 °C for 16 h, with subsequent neutralization, dialysis, and lyophilization of the solution. Carboxyl-reduced I polysaccharide was obtained according to the method Taylor & Conrad (1972) in which sodium borohydride was added to the carbodiimide complex of the I polysaccharide at pH 7.0. Two treatments were necessary to achieve complete reduction of all the carboxyl groups.

Instrumental Methods. Solutions were concentrated in a rotary evaporator under reduced pressure below 40 °C. Optical rotations were determined on a Perkin-Elmer 243 instrument with 1-dm semimicro cells at 23 ± 1 °C.

Gas–liquid chromatography (GLC) was performed on a Hewlett-Packard 5710A instrument equipped with a flame ionization detector and a Model 3380A electronic integrator using the following columns: (i) a glass column (180 × 0.15 cm) containing SP2340 on Supelcoport (80–100 mesh) operated at 180 °C (delay 2 min) to 240 °C at 4 °C/min; (ii) a fused silica capillary column (0.3 mm × 25 m) containing 3% OV17 operated at 180–230 °C at 2 °C/min and held for 10 min at 230 °C. Combined gas–liquid chromatography–mass spectrometry (GLC–MS) was performed on a Hewlett-Packard 5985B instrument using the above columns and an ionization potential of 70 eV.

^{13}C and ^1H NMR spectra were recorded on a Bruker AM500 spectrometer. Acetone was used as an internal chemical shift reference for ^1H NMR (2.225 ppm), and dioxane was used as the external chemical shift reference for ^{13}C NMR (67.4 ppm). Polysaccharides were exchanged twice with D_2O and then run in 0.4 mL of D_2O (5-mm tubes) at a concentration of 60 mg/mL.

Proton homonuclear shift correlated two-dimensional (2-D) NMR experiments (H,H-COSY) and proton dipolar correlated 2-D NMR experiments (NOESY) were performed by using the standard 90°–t_1–90° and 90°–t_1–90°–τ–90° pulse sequences, respectively. A mixing time of 100 ms was used for the NOESY experiment. Quadrature detection in both dimensions was employed. All phase cyclings were according to the standard software provided by Bruker (DISN MRP 840301.1). The initial (t_1, t_2) matrices were 64 × 512 data points with 4 Hz per point digital resolution in the second domain. Data points in both dimensions were weighted by unshifted sine bell functions prior to Fourier transformation. The final (f_1, f_2) matrix was zero filled to 512 × 512 points. Magnitude spectra which are symmetrized about the diagonal were used to represent the data.

^{13}C–H shift correlation with proton decoupling in the F_1 domain was done according to Bax (1983). The initial (t_1, t_2) matrix of 64 × 1024 points was Fourier transformed to a final matrix of 256 × 1024 points corresponding to a digital resolution of 23 Hz per point in the F2 domain and 15 Hz per point in the F1 domain.

Analytical Methods. Paper chromatography was carried out by the descending method using ethyl acetate–pyridine–water (5:2:5 v/v, top layer) and 1-butanol–pyridine–water (6:4:3 v/v) as eluants. Thin-layer chromatography (TLC) was performed on precoated cellulose TLC plates using ethyl acetate–pyridine–acetic acid–water (5:5:1:3 v/v) as ascending eluants. Compounds were visualized after the plates were sprayed with ninhydrin (amino sugars) or alkaline silver nitrate and *p*-anisidine reagents (pentoses).

Hexosamines were detected in the hydrolysates of the carboxyl-reduced I polysaccharide either directly by using a Technicon autoanalyzer or indirectly by the identification of the products of their deamination (Dmitriev et al., 1975) and ninhydrin degradation (Stoffyn & Jeanloz, 1954). For the purposes of deamination, the I polysaccharide was first carboxyl reduced using sodium borodeuteride before being hydrolyzed (2 N HCl for 90 min at 100 °C) until complete de-N-acetylation had occurred. Following deamination and conversion to alditol acetates (Sawardeker et al., 1965), the products were identified by GC–MS using column i described under Instrumental Methods. In preparation for ninhydrin degradation, the carboxyl-reduced I polysaccharide was first depolymerized with anhydrous HF (Sanger & Lamport, 1983) for 16 h at 25 °C, and the free hexosamines were generated by treating the depolymerized products with 1 M HCl at 100 °C for 3 h. Following ninhydrin degradation, the derived pentoses (arabinose and xylose) were identified by paper chromatography and by GLC–MS of their pentitol acetates using column i.

Hydrolysis of the Carboxyl-Reduced I Polysaccharide. The 2-amino-2-deoxy-L-guluronic and 2-amino-2-deoxy-D-mannuronic acid components of the I polysaccharide were identified by using the procedure of Torii et al. (1973). Following carboxyl reduction (Taylor & Conrad, 1972), the I polysaccharide was hydrolyzed with 4 M HCl for 2 h at 100 °C, and the products of hydrolysis, 2-amino-2-deoxygulose and 2-amino-2-deoxymannose, were separated on a Dowex 50X-8 (H^+) ion-exchange column using 0.3 M HCl as eluant. Gulosamine hydrochloride had $[\alpha]_D$ +17° (*c* 0.35, water), consistent with it having the L configuration (Heyns et al., 1957). Because of the low specific rotations of D- and L-mannosamine, it was difficult to assign the configuration of mannosamine

Table I: Carbon-13 Chemical Shifts[a] of Polysaccharides and Component Saccharides

polysaccharides and component saccharides	residue	C1	C2	C3	C4	C5	C6	CH_3, *N*-acetyl	CH_3, *O*-acetyl
native I polysaccharide	A	95.15[b]	46.38	69.46[c]	77.62	67.47		22.91[c]	
	B	99.51[d]	50.17	74.26	69.26[c]	75.50		22.84[c]	21.33
de-O-acetylated I polysaccharide[e]	A	95.85	46.68	69.70	77.79	67.82		23.03	
	B	100.10	50.53	77.36	68.57	78.24		23.03	
carboxyl-reduced I polysaccharide[e]	A	96.12[f]	46.88	68.60	76.99	66.17	61.55	23.01	
	B	100.56[g]	50.68	77.36	66.84	78.50	61.40	23.01	
4-*O*-(2-acetamido-2-deoxy-β-D-mannopyranosyl)-2-acetamido-2-deoxy-L-gulitol[e,h]	B	100.4[i]	54.4	73.1	67.8	77.3	61.6	23.1[c]	
	A	61.4	53.5	73.0[c]	78.8	69.6[c]	63.1	22.9[c]	
2-acetamido-2-deoxy-α-D-gulopyranose		92.4	47.5	70.4	69.6	67.1	62.2	23.2	
2-acetamido-2-deoxy-β-D-gulopyranose		93.9	52.4	71.2	69.6	75.1	62.2	23.2	

[a] In ppm from external dioxane. [b] $^1J_{^{13}C,H}$ = 171.2 Hz. [c] Tentative assignments. [d] $^1J_{^{13}C,H}$ = 167.9 Hz. [e] Assignments confirmed by 2-D ^{13}C,H-COSY. [f] $^1J_{^{13}C,H}$ = 170.0 Hz. [g] $^1J_{^{13}C,H}$ = 160.0 Hz. [h] Correspond to residues B and A, respectively, of the carboxyl-reduced I polysaccharide. [i] $^1J_{^{13}C,H}$ = 163.0 Hz.

hydrochloride using this criterion. Therefore, it was degraded to arabinose as previously described, which had $[\alpha]_D$ −84° (*c* 0.3, water), a value consistent with its D configuration.

Partial Hydrolysis of the I Polysaccharide. The de-O-acetylated I polysaccharide (60 mg) was treated with anhydrous HF (5 mL) (Sanger & Lamport, 1983) for 2 h at 25 °C. The HF was removed under reduced pressure, and an aqueous solution of the residue was neutralized with 0.02 M NH_4OH. The products were fractionated on a Bio-Gel P-2 column (1.5 cm × 90 cm) using 0.1 M ammonium acetate as eluant. The eluted products were monitored by using a Waters R403 differential refractometer, and the fraction corresponding to a disaccharide component was collected and lyophilized. To label the anomeric position of the reducing sugar residue, the disaccharide fraction was reduced with sodium borodeuteride, and the disaccharide–alditol was then carboxyl reduced according to the method of Taylor & Conrad (1972). The reduced product was deionized by passage through a Bio-Rad AG50-X8 ion-exchange column and refractionated on a Bio-Gel P2 column. The disaccharide fraction yielded only one disaccharide–alditol, the structure of which was elucidated by methylation (GLC) and NMR analyses.

Methylation Analysis. The carboxyl-reduced I polysaccharide and oligosaccharide were methylated with methyl iodide in the presence of methylsulfinyl anion according to the procedure of Hakomori (1964). The products were then purified on a small Sephadex LH-20 column (20 cm × 0.5 cm) using chloroform as eluant. The fractions were monitored by spotting the eluant on silica gel TLC plates, spraying the plates with 10% H_2SO_4 in ethanol, and charring the plates in an oven for 5 min at 130 °C. Fractions shown to contain permethylated polysaccharide were pooled and evaporated to dryness, and the residue was then hydrolyzed with 1 M trifluoroacetic acid for 16 h at 105 °C. Following evaporation of the acid, the partially methylated monomers were reduced with $NaBH_4$ and acetylated, and the products were analyzed by GLC–MS (Lindberg, 1972) using column ii. Methylated oligosaccharides were analyzed directly by GLC–MS also using column ii.

Quantitative Microprecipitin Experiments. Quantitative microprecipitins were carried out by the method of Kabat & Mayer (1961) using 0.1 mL of a rabbit antiserum to the I polysaccharide (diluted 5 times in PBS at pH 7.0) and 0.1 mL of antigen solution containing from 2 to 40 μg of the I polysaccharide. Precipitates were dissolved in 0.1 mL of sodium hydroxide (0.1 M), and the protein content was determined by the method of Lowry (1951).

Results and Discussion

Characterization of the Components of the I Polysaccharide. The presence of 2-acetamido-2-deoxy-L-guluronic acid and 2-acetamido-2-deoxy-D-mannuronic acid as constituents of the I polysaccharide was indicated when two components having the same elution volumes (amino acid analyzer) as 2-amino-2-deoxygulose and 2-amino-2-deoxymannose were identified in the hydrolysate of the carboxyl-reduced I polysaccharide. That the amino sugars were N-acetylated in the native I polysaccharide was ascertained from NMR spectroscopic data (vide infra). The presence of the above amino sugars was also confirmed by the identification of xylose and arabinose (paper chromatography and GLC analysis), the anticipated products of the ninhydrin degradation (Stoffyn & Jeanloz, 1954) of gulosamine and mannosamine, respectively, in the ninhydrin-treated hydrolysate of the carboxyl-reduced I polysaccharide. Also, 2,5-anhydroiditol and glucose were identified (GLC–MS analysis) as the major products of the deamination of the de-N-acetylated (hydrolyzed), carboxyl-reduced I polysaccharide. These products are known to be derived from the deamination of gulosamine and mannosamine, respectively (Williams, 1975). Determination of the configuration of the aminouronic acids in the I polysaccharide was achieved by assigning the configuration of their carboxyl-reduced analogues (gulosamine and mannosamine) obtained by hydrolysis of the carboxyl-reduced I polysaccharide. The specific rotations of gulosamine hydrochloride and the ninhydrin-degraded product (arabinose) of mannosamine were consistent with the amino sugars having the L and D configurations, respectively.

Partial Hydrolysis of the I Polysaccharide. Controlled depolymerization of the de-O-acetylated I polysaccharide using anhydrous hydrogen fluoride yielded, in addition to monomers and larger oligomers, one disaccharide shown to be 4-*O*-(2-acetamido-2-deoxy-β-D-mannuronopyranosyl)-2-acetamido-2-deoxy-L-guluronic acid. The disaccharide was reduced with sodium borodeuteride in order to label the reducing residue and then carboxyl reduced by the method of Taylor & Conrad (1972). It was then methylated and subjected to GLC–MS analysis, and in this analysis, the fragmentation pattern was only consistent with the 4-linked structure shown in Figure 1. The fact that 2-acetamido-2-deoxy-β-D-mannopyranose was the nonreducing residue was deduced from the ^{13}C NMR spectrum of the reduced disaccharide. The ^{13}C signals of the two residues of the reduced disaccharide are listed in Table I, and, except for C1, those associated with the nonreducing residue are almost identical with those previously assigned to 2-acetamido-2-deoxy-β-D-mannopyranose (Bundle et al., 1973). Only one disaccharide was detected in all the above analyses, indicating that this method of depolymerization had resulted in a highly selective cleavage of the 1 → 3 linkages of the I polysaccharide.

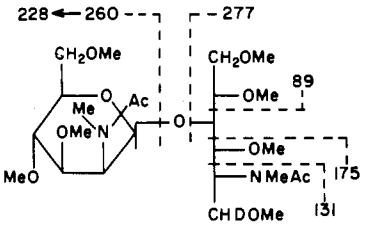
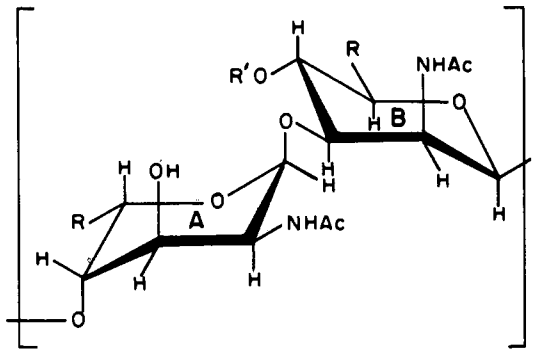


FIGURE 1: Methylated reduced oligosaccharide obtained from the I polysaccharide with some primary fragments.



a) $R = COO^-$, $R' = COCH_3$
b) $R = COO^-$, $R' = H$
c) $R = CH_2OH$, $R' = H$

FIGURE 2: Repeating units of the native, de-O-acetylated, and carboxyl-reduced I polysaccharide.

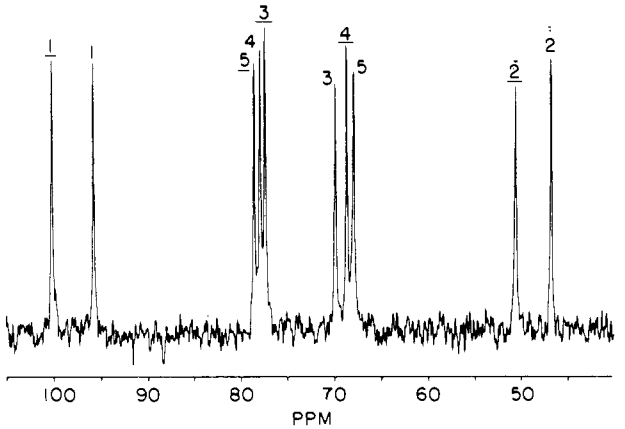


FIGURE 3: Fourier-transformed ^{13}C NMR spectrum of the de-O-acetylated I polysaccharide.

Structure of the I Polysaccharide. A preliminary analysis of the ^{13}C NMR spectrum of the I polysaccharide is consistent with it being composed of a mono-O-acetylated disaccharide repeating unit containing only *N*-acetylhexuronic acid residues, previously identified as 2-acetamido-2-deoxy-L-guluronic and 2-acetamido-2-deoxy-D-mannuronic acids. The structures of the repeating units of the native, de-O-acetylated, and carboxyl-reduced I polysaccharide are shown in modifications a, b, and c, respectively, of Figure 2. The ^{13}C NMR spectrum of the de-O-acetylated I polysaccharide is shown in Figure 3, and the chemical shifts of the carbons of all three repeating units (Figure 2a–c) are listed in Table I. Evidence strongly indicative of a disaccharide repeating unit was provided by the observation of two distinct anomeric carbon (95.15 and 99.51 ppm) and anomeric proton (4.78 and 5.06 ppm) signals in the respective ^{13}C and ^{1}H (Figure 4) NMR spectra of the I polysaccharide. The presence of two *N*-acetyl groups was deduced by the observation of two signals in the above ^{13}C

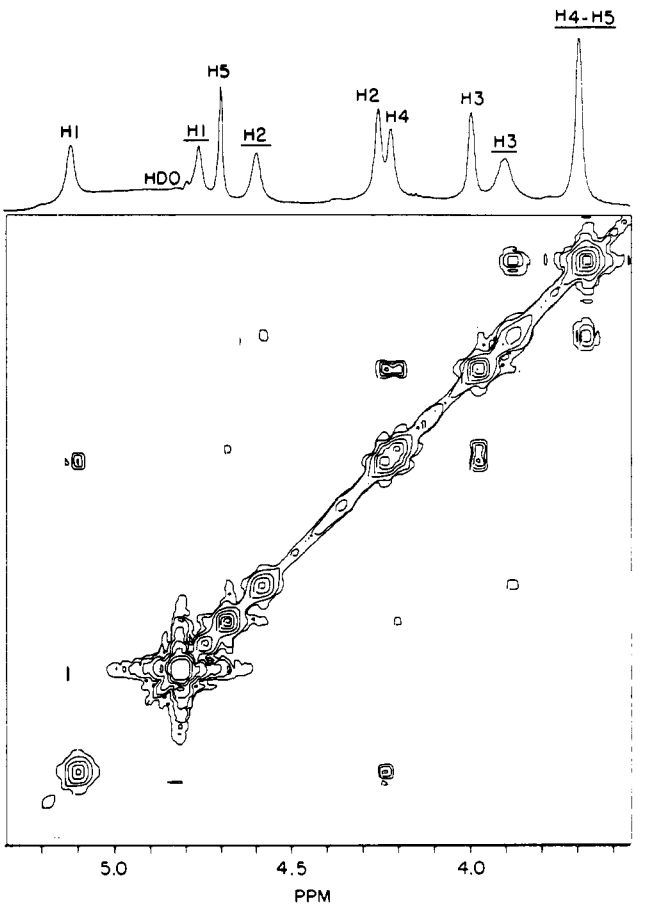


FIGURE 4: Homonuclear 2-D shift correlated H,H-COSY spectrum of the de-O-acetylated I polysaccharide (Figure 2b) in D_2O (310 K) with the 1-D spectrum above. The corresponding assignments for residue A and residue B (underlined) are shown.

NMR spectrum characteristic of the *N*-acetyl groups at 50.17 and 46.38 ppm (C–N) and at 22.91 and 22.81 ppm (acetamido methyl groups). The spectrum also exhibited five carbonyl signals at 176.4, 175.9, 175.3, 174.8, and 174.1 ppm consistent with the presence of two acetamido, two carboxyl, and one *O*-acetyl group in the repeating unit (Figure 2a) of the I polysaccharide. One of the above signals and the signal at 21.60 ppm disappeared following base treatment of the I polysaccharide which meant that these signals could be readily assigned to the *O*-acetyl carbonyl and methyl carbons of the above repeating unit (Figure 2a). No signals were observed in the hydroxymethyl region of the ^{13}C NMR spectrum of the I polysaccharide, but, as expected, two signals (61.55 and 61.40 ppm) appeared in this region of the carboxyl-reduced I polysaccharide (Figure 2c).

Although monomeric *N*-acetylguluronic and *N*-acetylmannuronic acids were not available as models to assist in the assignment of the signals in the ^{13}C NMR spectrum of the de-O-acetylated I polysaccharide (Figure 3), it was possible to make an assignment of the carboxyl-reduced I polysaccharide by using the previously assigned signals of 2-acetamido-2-deoxy-α-L-gulose and 2-acetamido-2-deoxy-β-L-gulose (Table I) and 2-acetamido-2-deoxy-α-D-mannose and 2-acetamido-2-deoxy-β-D-mannose (Bundle et al., 1973) for reference. This type of analysis is purely empirical and because of the relative complexity of the polysaccharide involves a considerable risk of making incorrect assignments. Because these assignments were crucial to the structural elucidation of the I polysaccharide, they were made unambiguously without model compounds by using a number of two-dimen-

Table II: Chemical Shifts[a] and Coupling Constants[b] of Signals in ^{1}H NMR Spectra of I Polysaccharides and a Monomeric Unit

	residue A			residue B			2-acetamido-2-deoxy-D-gulopyranose	
proton	native I	de-O-acetylated I[c]	carboxyl-reduced I[c]	native I	de-O-acetylated I[c]	carboxyl-reduced I[c]	α anomer	β anomer
H1	5.06 (<3)	5.08 (<3)	4.89	4.78 (<3)	4.75 (<3)	4.72	5.17 (3.7)	4.93 (8.4)
H2	na[d]	4.25	4.24	4.40	4.58	4.52	4.22 (3.6)	4.00
H3	na	3.98	4.01	na	3.89	3.71	4.95 (3.3)	4.00
H4	na	4.21	3.75	5.06	3.69	3.51	3.86 (<1)	3.75
H5	4.63	4.70	4.18	na	3.69	3.29	4.31	4.00
CH_3 (*N*-acetyl)	1.97	2.10	2.09	1.97	2.07	2.07	2.07	2.05
CH_3 (*O*-acetyl)	1.99			1.99				

[a] Expressed relative to internal acetone in ppm. [b] In parentheses (in hertz). [c] Chemical shifts assigned by 2-D H,H-COSY. [d] na, not assigned.

sional NMR techniques. This type of analysis is now routinely used for the assignment of oligosaccharide NMR signals, but only one report of its direct application to polysaccharides has so far been described (Barrow et al., 1984). The method chosen was to correlate the carbon spectrum of the de-O-acetylated I polysaccharide with its assigned proton spectrum, thus assigning the carbon spectrum indirectly.

The ^{1}H NMR spectrum of the de-O-acetylated I polysaccharide shown in Figure 4 was difficult to assign directly due to its complexity. Therefore, a proton homonuclear shift correlated 2-D NMR (H,H-COSY) experiment was performed, and the spectrum is also shown in Figure 4. As a result of this experiment, it was possible to assign all the protons of both the 2-acetamido-2-deoxyguluronopyranosyl (A) and 2-acetamido-2-deoxymannuronopyranosyl (B) residues of the repeating unit of the de-O-acetylated I polysaccharide (Figure 2b). These assignments and coupling constants together with those of the monomer 2-acetamido-2-deoxy-D-gulopyranose are listed in Table II. With these data, a heteronuclear shift correlated 2-D NMR (^{13}C,H-COSY) experiment was performed which enabled the unambiguous assignment of all the carbons in the repeating unit of the de-O-acetylated I polysaccharide (Figure 2b) to be made. These assignments are listed in Table I. Because of the importance of ^{13}C chemical shift data for making linkage and configurational assignments (Jennings & Smith, 1978; Gorin, 1981) on the I polysaccharide, and the lack of suitable monomer models, the above two-dimensional experiments were also performed on the carboxyl-reduced I polysaccharide. This enabled 2-acetamido-2-deoxy-α-D-gulopyranose and 2-acetamido-2-deoxy-β-D-gulopyranose and 2-acetmido-2-deoxy-α-D-mannopyranose and 2-acetamido-2-deoxy-β-D-mannopyranose (Bundle et al., 1973) to be used as satisfactory models for these assignments. The chemical shift data from the two-dimensional experiments are listed in Tables I and II.

By studying patterns of chemical shift differences between the above model compounds and residues A and B of the repeating unit of the carboxyl-reduced I polysaccharide (Figure 2c), it can be seen that regardless of anomeric configuration, although residues A and B were later assigned the α-L and β-D configurations, respectively (vide infra), A is linked at O4 and B at O3. This is consistent with characteristic large downfield displacements on C4 of A (7.39 ppm) and C3 of B (7.26 ppm) plus an upfield displacement on C2 of B (3.72 ppm) (β effect) in comparison with the chemical shifts of 2-acetamido-2-deoxy-α-D-gulopyranose (Table I) and 2-acetamido-2-deoxy-β-D-mannopyranose (Bundle et al., 1973).

The above linkage assignment was consistent with the methylation of the reduced disaccharide (Figure 1) obtained from the I polysaccharide, which indicated that the *N*-acetylgulosamine residue was linked at O4. In addition, both 3,6- and 4,6-di-*O*-methyl-2-(*N*-methylacetamido)-2-deoxyhexoses were detected in the methylation analysis of the carboxyl-reduced I polysaccharide, from which it could be deduced that the *N*-acetylmannosamine residue of the I polysaccharide was linked at O3.

By comparing chemical shifts, it was also possible to make assignments of the α-L configuration to residue A and the β-D configuration to residue B of the carboxyl-reduced I polysaccharide (Figure 2c). This was achieved by comparing the chemical shifts of the anomerically sensitive C5 signals of residues A and B with those of the equivalent C5 signals of the model compounds 2-acetamido-2-deoxy-α-D-gulopyranose and 2-acetamido-2-deoxy-β-D-gulopyranose and 2-acetamido-2-deoxy-D-mannopyranose, respectively (Table I). The chemical shifts of C5 of A (66.17 ppm) and C5 of B (78.50 ppm) were more similar to the chemical shifts of C5 of their respective α-D and β-D model compounds. However, in the case of residue B, the above configurational assignment was not definitive because of conflicting evidence obtained by using ^{1}H NMR spectroscopy.

In order to make configurational assignments on hexopyranosyl derivatives, it is often convenient to use the anomeric one-bond ^{13}C–^{1}H coupling constants ($^1J_{^{13}C,H}$) for this purpose. The magnitude of $^1J_{^{13}C,H}$ has been demonstrated to be sensitive to change in the anomeric configuration (Perlin et al., 1970; Schwartz & Perlin, 1972; Bock & Pederson, 1974). For example, in the case of D-glucopyranose [$^4C_1$ (D) conformation], the α-D anomer (equatorial H1) has $^1J_{^{13}C,H}$ = 169 Hz while the β-D anomer (axial H1) has $^1J_{^{13}C,H}$ = 161 Hz (Schwartz & Perlin, 1972). These coupling constants and the 8–10-Hz difference between them have been found consistently in a large number of anomeric pairs of hexopyranoses (Perlin et al., 1970; Schwartz & Perlin, 1972; Bock & Pederson, 1974) and hexopyranosyl residues in oligosaccharides and polysaccharides (Hamer & Perlin, 1976). The values of $^1J_{^{13}C,H}$ for residues A and B of the I polysaccharide were 171.2 and 167.9 Hz, respectively, from which one could deduce that both residues had the α configuration. Of the values of $^1J_{^{13}C,H}$ for A (170.0 Hz) and B (160.0 Hz) of the carboxyl-reduced I polysaccharide, the former is consistent with the above assignment; however, the latter provides contradictory evidence that residue B has the β-D configuration. This latter assignment is consistent with the ^{13}C chemical shift data and was later confirmed by nuclear Overhauser enhancement experiments (vide infra).

Two possible explanations for the anomalous value of $^1J_{^{13}C,H}$ for residue B of the I polysaccharide are either that it has an equatorial anomeric proton due to it adopting the $_4C^1$ (D) or twist boat conformation or that it is associated with structural features peculiar to the I polysaccharide. In fact, the latter explanation proved to be the relevant one because unambiguous assignment of the anomeric configuration (β-D) and conformation [$^4C_1$ (D)] of residue B of the I polysaccharide (Figure 2a) was obtained by 2-D nuclear Overhauser experiments carried out directly on the de-O-acetylated I polysaccharide

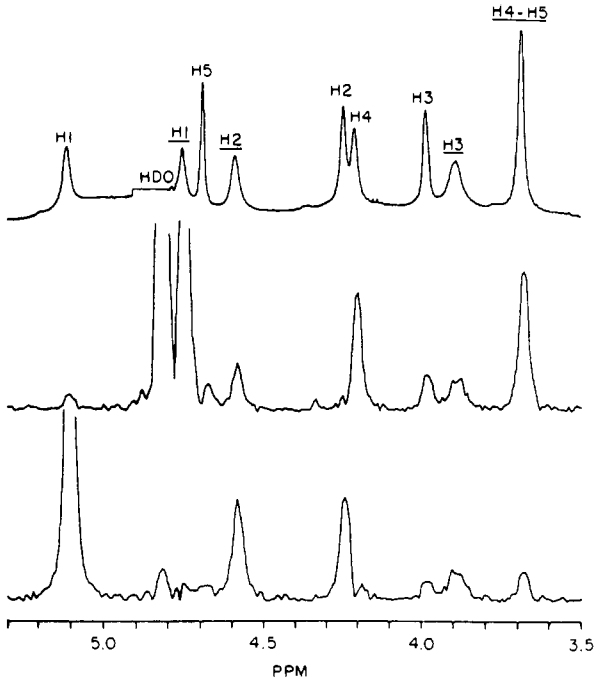


FIGURE 5: ^{1}H NMR spectrum (top) of the de-O-acetylated I polysaccharide (Figure 2b) in D_2O (310 K) with the assignments for residue A and residue B (underlined) (top). Cross-sections of the 2-D NOESY contour map showing the NOE's for H1 of residue B (middle) and H1 of residue A (bottom).

(Figure 5). In a similar experiment, the α-L configuration and $_4C^1$ (L) conformation of residue A were also confirmed (Figure 5). A number of nuclear Overhauser enhancements were observed on the resonance of protons in close proximity to the anomeric protons of residues A and B (Figure 5). Critical to the above assignments are the intraresidue enhancements observed between H1–H3 and H1–H5 of ring B which indicated that H3 and H5 were both cis and 1→3-diaxially disposed to H1. For residue A, the only strong intraresidue enhancement observed was between H1 and H2 which is consistent in having the configuration (α-L) and conformation [$_4C^1$ (L)] depicted in Figure 2b.

Interresidue enhancements between H1 and H2 and between H1 and H3 are a consequence of the α-L-(1→3) linkage between residues A and B, and those between H1 and H4 and between H1 and H3 are a consequence of the β-D-(1→4) linkage between residues B and A (Figure 2b). Since interresidue NOE's are dependent on the linkage conformation, the magnitude of these enhancements reflects the relative orientation of these residues with respect to each other.

The most probable explanation of the anomalous value of $^1J_{^{13}C,H}$ for residue B in the I polysaccharide is that it is due to electronic effects. That the carboxylate groups are involved in this effect can be deduced from the more conventional values of $^1J_{^{13}C,H}$ obtained for residue B in the carboxyl-reduced I polysaccharide (Table I). Although this type of effect has been observed in hexopyranosyl derivatives having electronegative substituents at the anomeric center (Bock & Pederson, 1974), it has not been observed in hexuronopyranoses where the carboxyl group is more remote from the anomeric center. The following evidence indicates strongly that in the case of the I polysaccharide the anomalous $^1J_{^{13}C,H}$ value of residue B is due to an interresidue through-space anisotropy effect associated with the carboxyl group of the neighboring glycosidically linked residue A (Figure 2b). Residue B is also a constituent of the de-O-acetylated meningococcal K polysaccharide which has the repeating unit →4)β-D-Man*p*NAcA(1→3)β-D-Man*p*NAcA(1→ (Michon et al., 1985). In this environment, residue B and the 4-linked β-D-mannuronopyranosyl residue both have glycosidically linked hexuronopyranosyl residues, and both exhibit similar and anomalous $^1J_{^{13}C,H}$ values (171.0 and 168.8 Hz, respectively).

In contrast, residue B is also a constituent of the type e *Haemophilus influenzae* capsular polysaccharide, in which structure it alternates with uncharged 3-linked 2-acetamido-2-deoxy-β-D-glucopyranosyl residues (Branefors-Helander et al., 1981). In this environment, it has $^1J_{^{13}C,H}$ = 164 Hz, a value which, although still not free from ambiguity, is more compatible with its β-D configuration than the larger values of $^1J_{^{13}C,H}$ of residue B in the native I and K polysaccharides. There remains now the question as to why the carboxylate group of residue B does not conform to the above hypothesis and similarly modify $^1J_{^{13}C,H}$ of residue A in the I polysaccharide. One plausible explanation is that this interresidue effect is dependent on the distance of the carboxyl group from the anomeric proton. Therefore, the distance between the carboxylate group of residue A and the anomeric proton of residue B must be greater than the equivalent distance between the same groups on residues B and A, respectively. This distance is dependent on the orientation of these glycosidic linkages, and as such, the orientation of the glycosidic linkage between residues B and A of the I polysaccharide differs substantially from that between residues A and B of the same polysaccharide, and also from the orientation of both glycosidic linkages between the two 2-acetamido-2-deoxy-β-D-mannuronopyranosyl residues of the K polysaccharide (Michon et al., 1985).

The simple pattern of chemical shift displacement observed by comparing the ^{13}C NMR signals of the de-O-acetylated (Figure 2b) with the native I (Figure 2a) polysaccharide (Table I) indicated that the repeating unit of the latter is specifically monosubstituted by the *O*-acetyl at O4 of residue B (Figure 2a). Consistent with the above assignment, only three significant displacements were observed in the signals of the I polysaccharide: a rather small downfield displacement of 0.69 ppm on C4 of B and fairly large but characteristic displacements on the vicinal C3 and C5 signals of the same residue (3.10 and 2.74 ppm, respectively). This assignment was also confirmed by comparing the ^{1}H NMR spectra of the de-O-acetylated (Figure 2b) and native I (Figure 2a) polysaccharide, where the only proton signal of the I polysaccharide to undergo a substantial downfield displacement (1.37 ppm) indicative of *O*-acetyl substitution was H4 of residue B (Table II).

Immunological Properties of the I Polysaccharide. Quantitative serological precipitation experiments using the native (Figure 2a) and de-O-acetylated (Figure 2b) I polysaccharide with a meningococcal group I specific antiserum are shown in Figure 6. These experiments demonstrate that the *O*-acetyl group is essential to the formation of the group-specific determinant. While the native I polysaccharide precipitated close to 80 μg of antibody from 1 mL of antiserum, the de-O-acetylated I polysaccharide precipitated less than 10 μg. Probably due to their common 3-linked 4-*O*-acetyl-2-acetamido-2-deoxy-β-D-mannuronopyranosyl residues, the meningococcal K polysaccharide cross-reacts with the I polysaccharide specific antiserum and, interestingly, cross-reacts more strongly than the homologous de-O-acetylated I polysaccharide.

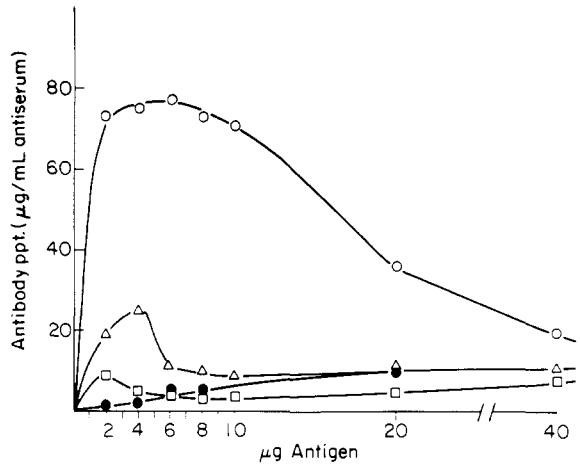


FIGURE 6: Quantitative precipitin curves of the native (○), de-O-acetylated (□), and carboxyl-reduced (●) I polysaccharide and the native K polysaccharide (△).

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REFERENCES

Ashton, F. E., Ryan, A., Diena, B. B., & Frasch, C. E. (1979) *Can. J. Microbiol. 25*, 784–787.

Ashton, F. E., Ryan, A., Diena, B., & Jennings, H. J. (1983) *J. Clin. Microbiol. 17*, 722–727.

Barrow, K. D., Collins, J. G., Rogers, P. L., & Smith, G. M. (1984) *Eur. J. Biochem. 145*, 173–179.

Bax, A., & Freeman, R. (1981) *J. Magn. Reson. 44*, 542–561.

Bernstein, M. A., & Hall, L. D. (1982) *J. Am. Chem. Soc. 104*, 5553–5555.

Bock, K., & Pedersen, C. (1974) *J. Chem. Soc., Perkin Trans. 2*, 293–297.

Branefors-Helander, P., Kenne, L., Lindberg, B., Peterson, K., & Unger, P. (1981) *Carbohydr. Res. 88*, 77–84.

Bruch, R. C., & Bruch, M. D. (1982) *J. Biol. Chem. 257*, 3409–3413.

Bundle, D. R., Jennings, H. J., & Smith, I. C. P. (1973) *Can. J. Chem. 51*, 3812–3819.

Bundle, D. R., Jennings, H. J., & Kenney, C. P. (1974) *J. Biol. Chem. 249*, 4797–4801.

Ding, S.-Q., Ye, R.-B., & Zang, H.-C. (1981) *J. Biol. Stand. 9*, 307–315.

Dmitriev, B. A., Backinowsky, L. V., Lvov, V. L., Kotchetkov, M. K., & Hofman, I. L. (1975) *Eur. J. Biochem. 50*, 539–547.

Dubois, M., Gilles, K. A., Hamilton, P. A., Rebers, P. A., & Smith, F. (1956) *Anal. Chem. 28*, 350–356.

Gorin, P. A. J. (1981) *Adv. Carbohydr. Chem. Biochem. 38*, 13–104.

Gotschlich, E. C., Lui, T.-Y., & Artenstein, M. S. (1969) *J. Exp. Med. 129*, 1349–1365.

Hakomori, S. (1964) *J. Biochem. (Tokyo) 55*, 205–208.

Hamer, G. K., & Perlin, A. S. (1976) *Carbohydr. Res. 49*, 37–48.

Heyns, K., Paulsen, H., Eichstedt, R., & Rolle, M. (1957) *Chem. Ber. 90*, 2039–2049.

Jennings, H. J. (1983) *Adv. Carbohydr. Chem. Biochem. 41*, 155–208.

Jennings, H. J., & Smith, I. C. P. (1978) *Methods Enzymol. 50*, 39–50.

Jennings, H. J., Lugowski, C. W., Ashton, F. E., & Ryan, J. A. (1983) *Carbohydr. Res. 112*, 105–111.

Kabat, E. A., & Mayer, M. M. (1961) in *Experimental Immunochemistry*, pp 241–267, Charles C. Thomas, Springfield, IL.

Lindberg, B. (1972) *Methods Enzymol. 28*, 178–195.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randale, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Michon, F., Roy, R., Jennings, H. J., & Ashton, F. E. (1984) *Can. J. Chem. 62*, 1519–1524.

Michon, F., Brisson, J. R., Roy, R., Ashton, F. E., & Jennings, H. J. (1985) *Can. J. Chem.* (in press).

Patt, S. L. (1984) *J. Carbohydr. Chem. 3*, 493–512.

Perlin, A. S., Casu, B., & Koch, H. J. (1970) *Can. J. Chem. 48*, 2596–2606.

Prestegard, J. H., Koerner, T. A. W., Demon, P. C., & Yu, R. K. (1982) *J. Am. Chem. Soc. 104*, 4993–4995.

Sanger, M. P., & Lamport, D. T. A. (1983) *Anal. Biochem. 128*, 66–70.

Sawardeker, J. S., Sloneker, J. H., & Jeanes, A. R. (1965) *Anal. Chem. 37*, 1602–1604.

Schwartz, J. A., & Perlin, A. S. (1972) *Can. J. Chem. 50*, 3667–3676.

Stoffyn, P. J., & Jeanloz, R. W. (1954) *Arch. Biochem. Biophys. 52*, 373–379.

Taylor, R. L., & Conrad, H. E. (1972) *Biochemistry 11*, 1383–1388.

Torii, M., Sakakihara, K., & Kuroda, K. (1973) *Eur. J. Biochem. 37*, 401–405.

Van der Kaaden, A., Van Doorn-Van Wakeren, J. I. M., Kamerling, J. P., Vliegenthart, J. F. G., & Tiesjema, R. H. (1984) *Eur. J. Biochem. 141*, 513–519.

Williams, J. M. (1975) *Adv. Carbohydr. Chem. Biochem. 31*, 9–79.