Structural Determination of the Sialic Acid Polysaccharide Antigens of Neisseria meningitidis Serogroups B and C with Carbon 13 Nuclear Magnetic Resonance*

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The application of 13C nuclear magnetic resonance to the analysis of some sialic acid containing meningococcal polysaccharide antigens is described. Complete assignments of the spectra of both the native serogroup B and the de-O-acetylated serogroup C polysaccharides have been made. These assignments were based on the corresponding data for some related monomers (sialic acid and its α- and β-methylglycosides) and on supportive chemical evidence. The data indicate that the serogroup B polysaccharide is a 2→8-α-linked homopolymer of sialic acid, identical in structure with colominic acid from Escherichia coli, whereas the de-O-acetylated serogroup C polysaccharide is a 2→9-α-linked homopolymer. The native serogroup C polysaccharide is O-acetylated (1.16 mol of O-acetyl per sialic acid residue), all the O-acetyl substituents being located only at C-7 and C-8 of the sialic acid residues, and in addition contains unacetylated residues (24%). The polysaccharide contains di-O-acetylated residues (O-acetyl on C-7 and C-8), and at least one of the possible mono-O-acetylated residues at C-7 or C-8.

Both the meningococcal serogroup B and C polysaccharides are homopolymers of sialic acid, the latter being partially O-acetylated (1) and exhibiting protective properties in man (2, 3). Due to the relative instability of these polysaccharides to chemical treatment it was advantageous to elucidate their structures by a nondestructive technique. In addition, as the structure of these polysaccharide immunogens is intimately linked to their effectiveness as vaccines, the development of a technique for rapid structural determination would be invaluable. We have previously demonstrated that 13C nuclear magnetic resonance is an extremely powerful tool in such an investigation (4). We have now applied it to the sialic acid polymers. Together with complementary chemical evidence we have confirmed the basic structure of the serogroup B polysaccharide as a 2→8-α-linked homopolymer of sialic acid identical in structure with colominic acid (5). The serogroup C polysaccharide is a 2→9-α-linked homopolymer of sialic acid containing 1.16 mol of O-acetyl per sialic acid residue, all the O-acetyl substituents being located on C-7 or C-8 of the individual residues. The polysaccharide contains unacetylated residues (24%), di-O-acetylated residues (C-7 and C-8), and at least 1 mono-O-acetylated residue at C-7 or C-8.

EXPERIMENTAL PROCEDURE

Materials—The cells of serogroups B and C (strains 608 and 2241, respectively) were obtained from the culture collection of the Laboratory for Disease Control, Ottawa) and were grown as previously described (6). The polysaccharides were isolated and purified as previously described with Cetavlon to precipitate the crude polysaccharides (7). Colominic acid (Escherichia coli) was obtained from Koch-Light Laboratories, Colnbrook, England, and N-acetylneuraminic acid (sialic acid) was obtained from Nutritional Biochemicals Ltd., Cleveland, Ohio. Neuraminidase (Vibrio cholerae) and neuraminidase (Clostridium perfringens) were obtained from Calbiochem, San Diego, Calif., and Worthington Biochemical Corp., Freehold, N. J., respectively. The methyl α- and β-glycosides of sialic acid were prepared according to the methods of Yu and Ledeen (8) and Kuhn et al. (9), respectively. The tetra-O-acetyl derivative of the methyl α-glycoside was prepared by conventional procedures using pyridine and acetic anhydride.

De-O-acetylation of Serogroup C Polysaccharide—Initially the serogroup C polysaccharide was de-O-acetylated using 0.02 M NaOH as described by Gotschlich et al. (1). In our hands this method yielded only a partially de-O-acetylated product as determined by 13C NMR (Fig. 1). For complete de-O-acetylation the polysaccharide (150 mg) was dissolved in 0.1 M NaOH (50 ml) and incubated at 37°C for 4 hours. The solution was neutralized with acetic acid (pH 7.0), dialyzed, and lyophilized to yield the completely de-O-acetylated product (110 mg) as determined by 13C NMR (Fig. 1).

Periodate Oxidation of Polysaccharides—Periodate oxidations were carried out on 5 to 10 mg of the polysaccharides in 0.02 M sodium metaperiodate (5 ml). The periodate uptake was estimated spectrophotometrically (10). One larger scale oxidation was carried out on 25 mg of the serogroup C polysaccharide in 0.1 M sodium periodate (5 ml). The oxidation was terminated by the addition of a few drops of ethylene glycol after 0.3 mol of periodate per mol of sialic acid had been consumed. The resultant solution was concentrated to 2 ml and used in a gel filtration experiment (Fig. 2).

Gel Filtration—Gel filtration was performed on a Sephadex G-200 column (2.4 × 70 cm) with a 0.1 M phosphate buffer. The column was calibrated with blue dextran (MW 2,000,000) and the sialic acid...
FIG. 1. Fourier-transformed $\textsuperscript{13}$C NMR spectra of meningococcal polysaccharides in $\text{D}_2\text{O}$, PD 7, 100 mg per ml, 31°, taken with acquisition time 0.4 s, pulse angle 90°, exponential weighting constant 0.2 s, and spectral width 5 kHz. Chemical shifts are expressed relative to Me$_4$Si contained in a concentric tube of outside diameter 5 mm. Number of accumulated free induction decays: upper, 54,000; middle: 137,000; lower, 257,000. The inset in the upper spectrum is the transform free induction decay without experimental weighting.

RESULTS AND DISCUSSION

Assignment of Resonances in Monomer Units—In the spectrum of N-acetylneuraminic acid only 11 resonances were detected due to its predominant existence as the $\beta$ anomer in the equilibrated solution. The chemical shift assignments are shown in Table I. Some can readily be made by correlation with those for carbon atoms in similar chemical environments. Thus the high field signals at 23.2, 39.9, and 53.2 ppm are assigned to the methyl moiety of the acetamido group at C-5 (13), the deoxy carbon at C-3 (14), and C-5 (13), respectively. The low field signals at 176.0 and 174.3 ppm were assigned to the carbonyl carbon of the acetamido group at C-5 (13), and the carbonyl carbon of the carboxylic acid group (C-1) (14), respectively. The resonance at 96.4 ppm was attributed to the anomeric carbon (C-2) by comparison with the approximately equivalent chemical shifts of the anomeric resonances of the hexapyranoses (15, 16) and 2-acetamidohexapyranoses (13). Further evidence for the assignment of this signal was provided by its low intensity (approximately 50% less intense than that of the anomeric carbons of the hexapyranoses (15, 16) and the 2-acetamidohexapyranoses (13)). This is due to the unique feature of C-2 of sialic acid not having any $\alpha$ protons, and consequently obtaining less nuclear Overhauser enhancement.
Table 1

Carbon 13 chemical shifts* of polysaccharides and relevant monomers

| Polysaccharides and Monomers          | C-1 | C-2 | C-3 | C-4 | C-5 | C-6 | C-7 | C-8 | CH₃ (HCOCH₃) | CH₂OH (glycol) | OCH₃ (methyl glycolate) | CH₃ (HCOCH₃) | C=O (HCOCH₃) |
|--------------------------------------|-----|-----|-----|-----|-----|-----|-----|-----|--------------|---------------|------------------------|--------------|--------------|
| N-Acetyl Neuraminic Acid             | 174.3 | 96.4 | 39.9 | 67.8 | 53.2 | 71.3 | 69.4 | 71.5 | 64.3         | 23.2          | -                       | -            | 176.0        |
| N-Glycolyl Neuraminic Acid           | 174.3 | 96.4 | 40.0 | 67.6 | 52.9 | 71.5 | 69.3 | 71.5 | 64.3         | -             | 62.2                    | -            | 176.8        |
| Methyl(methyl α-β-N-Acetyl Neuraminidiate) | 171.6 | 100.5 | 40.5 | 67.6 | 53.0 | 71.2 | 69.4 | 71.6 | 64.6         | 23.3          | -                       | 52.3         | 176.0        |
| Methyl(methyl α-β-N-Acetyl Neuraminidiate) | 171.1 | 100.3 | 40.1 | 68.2 | 52.9 | 74.0 | 69.6 | 71.7 | 64.3         | 23.3          | -                       | 52.9         | 176.1        |
| Methyl α-β-N-Acetyl Neuraminic Acid  | 174.1 | 100.6 | 40.6 | 67.8 | 53.1 | 71.9 | 69.4 | 71.8 | 64.7         | 23.3          | -                       | 52.1         | 176.1        |
| Methyl α-β-N-Acetyl Neuraminic Acid  | 172.5 | 100.3 | 40.0 | 68.5 | 52.9 | 74.0 | 69.5 | 72.0 | 64.3         | 23.2          | -                       | 52.7         | 176.1        |
| D Polysaccharide                     | 174.4 | 102.1 | 40.9 | 69.4 | 53.6 | 74.3 | 70.4 | 78.8 | 62.4         | 23.6          | -                       | 176.1        |
| Colominic Acid                       | 174.4 | 102.2 | 42.0 | 69.4 | 53.7 | 74.4 | 70.6 | 78.6 | 62.5         | 23.7          | -                       | 176.1        |
| De-O-acetylated C Polysaccharide     | 174.9 | 101.4 | 41.8 | 69.5 | 53.5 | 73.6 | 69.5 | 71.4 | 66.3         | 23.3          | -                       | 176.1        |
| C Polysaccharide                     | 174.9 | 102.0 | 41.4 | 69.5 | 53.1 | 73.6 | 69.5 | 71.4 | 66.3         | 23.3          | -                       | 22.2         | 22.0         |
| Methyl 1,7,8,9-tetra-O-acetyl α-β-N-Acetyl Neuraminic Acid | 38.4 | 50.2 | 23.0 | -   | 53.2 | 21.7 | 21.3 | 175.5 | -            | -              | -                       | -            | -            |
| Methyl 9-O-acetyl (methyl α-β-N-Acetyl Neuraminidiate) | 171.5 | 100.3 | 40.3 | 67.5 | 52.8 | 71.5 | 69.3 | 68.7 | 67.7         | 23.2          | -                       | 52.1         | 21.4         |

*a) In parts per million from external tetramethylsilane.
*b) Assigned by specific proton decoupling experiment.
*c) Tentative assignments.
*d) Extra resonance due to the proximity of O-acetyl substituents.
*e) C=O (H-glycolate)

(17). This is also demonstrated in all the spectra of the sialic acid homopolymers (e.g. the C-2 resonance of the de-O-acetylated serogroup C polysaccharide at 101.4 ppm shown in Fig. 1). Similar low intensity signals are exhibited by the two carbonyl carbon atoms of sialic acid mentioned previously and for the sialic acid homopolymers, e.g. see Fig. 1.

The resonance at 64.3 ppm was assigned to C-9 by the appearance of a triplet of relative intensity 1:2:1 in the 'H-coupled ¹³C spectrum of sialic acid. The resonance at 67.8 ppm was assigned to C-4 by specific proton decoupling experiments. The ¹³C resonance of C-4 has an easily recognizable complex ¹³C-'H coupling pattern in the coupled spectrum due to presence of three vicinal protons. A specific decoupling experiment was carried out by irradiating at the 'H NMR frequency of the two protons attached to C-3. This simplified the complex ¹³C-'H coupling at C-4 without changing the other resonances to any significant extent. The resonance at 71.3 ppm was assigned to C-8 by correlation of the spectrum of methyl (methyl β-p-N-acetylneuraminidate) with that of its previously synthesized 9-O-acetyl derivative¹ (Table I). All the resonances of the two compounds were almost identical except

¹H. J. Jennings and A. Martin, unpublished results.
for that of C-9 (downfield displacement of 3.1 ppm), and that of C-8 (upfield displacement of 3.1 ppm). These chemical shifts are characteristic of O-acetyl substitution (13), the latter being attributed to β shielding by the O-acetyl group on the neighboring carbon. Of the remaining unassigned resonances (C-6 and C-7) the C-6 resonance was assigned by virtue of its sensitivity to change in anomeric configuration. In the hexapyranoses (15, 16) and acetamidohexapyranoses (13), the resonances affected by anomeric change are C-1, C-2, C-3, and C-5, with C-3 and C-6 being the most sensitive to this change. These latter resonances would correspond to C-4 and C-6 of the pyranose ring of sialic acid. The exocyclic C-7 should be relatively insensitive to change in anomeric configuration due to its remoteness from the anomeric center, as has been shown to be the case with exocyclic C-6 of other pyranose ring systems (13, 15, 16). In comparison with the chemical shifts of C-6 and C-7 of methyl α- and β-N-acetylneuraminic acid, the resonance at 71.2 ppm was assigned to C-6 and that at 69.4 ppm to C-7, as only the resonance at 71.2 ppm proved to be sensitive to change in anomeric configuration (Table I).

The assignment of the β configuration to the predominant form of sialic acid in solution was made by virtue of the fact that, except for the C-1, C-2, and C-3 signals (due to differing aglycones), all the other resonances in the spectrum were almost identical with those of methyl β-d-N-acetylneuraminic acid, whereas this was not the case with the methyl α anomer (Table I). The anomeric configurations of methyl α- and β-Ν-N-acetylneuraminic acid have been previously established (8, 18) and recently it has been shown that crystalline sialic acid also adopts the β configuration (19). In order to elucidate the anomeric configuration of the polysaccharides we required the effect of change in anomeric configuration on the resonances of the monomer (sialic acid). As this information was not available in the spectrum of sialic acid the comparison was made using the methyl α- and β-glycosides.

Most of the resonances of the α anomer were assigned by previously described procedures, except that in this case C-7 and C-8 were assigned by correlation with the previous assignments made for the β anomer. The remaining resonances at 68.5 and 74.0 ppm were attributed to C-4 and C-6, respectively. This assignment was preferred to the reverse assignment because changing the anomeric configuration of the methyl β-d-N-acetylneuraminic acid to α produces downfield displacements of both resonances of 0.7 ppm for C-4 and 2.8 ppm for C-6. Although these values are lower than the downfield displacements produced by the same procedure on the equivalent C-3 and C-5 position of the acetamidohexoses (13), the direction of displacement for both carbon atoms is the same. The reverse assignment for C-4 and C-6 produces a downfield displacement of 6.2 ppm for C-4 and an upfield displacement of 3.3 ppm for C-6. On this evidence alone the reverse assignment cannot be discarded, as direct comparisons of the chemical shifts of the carbon atoms in the pyranose rings of acetamidohexoses and sialic acid are not possible due to basic structural and conformational differences. However, for the purposes of this paper, even a reversal of the assignments of C-4 and C-6 of methyl α-d-N-acetylneuraminic acid does not detract from the arguments used in determining the structure and configuration of the sialic acid polymers.

Structure and Configuration of Serogroup B Polysaccharide—The B polysaccharide gave a simple 11 resonance spectrum indicating that it was a simple homopolymer of sialic acid with complete anomic homogeneity. The chemical shifts were identical with those of colimonic acid (Table I), indicating that these two polysaccharides are similar in gross structural detail. This could account for the serological cross reactions previously described between the B polysaccharide and E. coli K1 (20). Evidence based on the periodate oxidation and neuraminidase treatment of the free acid form of colimonic acid had indicated that it was 2-8-α-linked (5); similar results were obtained with the B polysaccharide (1). However, the periodate oxidations were not definitive as some periodate was consumed and this could have been interpreted as being due to mixed linkages in the polysaccharide. We also found that substantial quantities of periodate were consumed by our preparation of the B polysaccharide (Table III). In addition the neuraminidase specificity for sialic acid residues in the α configuration was based on its preferential specificity to cleave only the methyl α-glycoside of the model compounds methyl α- and β-d-N-acetylneuraminic acid (8). However the 13C NMR spectrum of the B polysaccharide provides evidence to substantiate the 2-8-α-linked structure. Most likely the uptake of periodate by the B polysaccharide was due to the presence of considerable amounts of reducing end group in the polysaccharide as the molecular weight determination (Fig. 2) indicated that it contained large quantities of low molecular weight material.

The chemical shifts of the individual carbon atoms of the B polysaccharide and colimonic acid are listed in Table I. The C-1, C-2, C-3, C-5, C-9, CH$_3$ (acetamido group), and carbonyl (acetamido group) were assigned by procedures similar to those used previously for sialic acid and its methyl α- and β-glycosides. The fact that the C-9 resonance moves upfield by 1.9 ppm in comparison with the C-9 resonance of methyl α-d-neuraminic acid (Table II) indicates that it is vicinal to the carbon involved in the linkage (C-8). Similar upfield displacements due to the shielding of β carbons by substituents have been documented for O-acetyl groups (4, 13) and for other sugar rings in oligosaccharides (16, 21). Having established the C-8 linkage the low field signal at 78.8 ppm was assigned to C-8, as such a low field signal did not appear in the spectra of either methyl α- or β-N-acetylneuraminic acid (Table I).

### Table II

| Polysaccharides – methyl glycosides of neuraminic acid | C-1 | C-2 | C-3 | C-4 | C-5 | C-6 | C-7 | C-8 | CH$_3$ (NHCOCO$_2$H) | C=O (NHCOCO$_2$H) |
|------------------------------------------------------|-----|-----|-----|-----|-----|-----|-----|-----|---------------------|-------------------|
| B – α-d-anomer                                        | +2.1| +1.8| +0.9| +0.9| +0.7| +0.3| +0.9| +6.8| –1.9                | +0.4              |
| R – β-d-anomer                                        | +0.3| +1.5| –0.3| +1.6| +0.5| +3.1| +1.0| +7.0| –2.3                | +0.3              |
| De-O-acetylated C – α-d-anomer                        | +2.6| +1.1| +1.2| +1.0| +0.1| +0.4| 0.0 | +6.0| +2.0                | +0.1              |
| De-O-acetylated C – β-d-anomer                        | +0.3| +0.8| +0.6| +1.7| –0.1| +2.4| +0.1| –0.4| +1.6                | 0.0               |

*Obtained from data in Table I; a positive difference indicates that the resonance in the polysaccharide occurs at lower magnetic field (larger chemical shift from Me$_3$Si).
Therefore, in comparison with the C-8 signal of methyl \( \alpha\)-D-neuraminic acid the C-8 signal of the B polysaccharide experiences a downfield shift of 6.8 ppm. The resonance at 70.4 ppm was assigned to C-7 as it would be expected that the chemical shift to the other carbon \( \beta \) to C-8 would also be affected. In this case the displacement is to lower field by 0.9 ppm in comparison with C-7 of methyl \( \alpha\)-D-N-acetylenuraminic acid. Similar downfield displacements for carbons \( \beta \) to the linkage point have also been documented in the oligosaccharides (16, 21). The major case for assigning the \( \alpha \) configuration to the B polysaccharide is based on the remaining resonances at 74.3 and 69.4 ppm which are more comparable to the equivalent C-6 and C-4 resonances of methyl \( \alpha\)-D-N-acetylenuraminic acid than those of the methyl \( \beta\)-D anomer. The chemical shift differences between the resonances of the B polysaccharide and those of methyl \( \alpha\) - and \( \beta\)-D-N-acetylenuraminic acid are shown in Table II. Although there are large differences in chemical shift at C-7, C-8, and C-9 in comparison with both anomers, these differences are comparable for both anomers, indicating their insensitivity to anomeric change. The differences can be attributed to the 2–8-linkage of the polysaccharide. Only small differences are apparent in the C-2, C-5, CH\( \text{acetyl} \) (acetamido group), and carbonyl (acetamido) signals, indicating that they are also insensitive to change in anomeric configuration. However, at C-4 and C-6 considerable differences exist in comparison with the methyl \( \beta \) anomer thus enabling the \( \alpha \) configuration to be assigned to the B polysaccharide. It would appear that the alternate assignment (\( \beta \) configuration) could be made if one considered only the C-1 and C-3 resonances as their chemical shift differences are significantly larger in comparison with the methyl \( \alpha \) anomer. However these differences are probably due to the comparison of sialic acid residues with differing glycosidic bonds. Both these carbons would be influenced by the differing glycosidic groups because of their location, indicating the limitations of the methyl glycosides as model compounds for the sialic acid polymers.

Structure and Configuration of De-O-acetylated Serogroup C polysaccharide—The \( ^{13} \text{C} \) NMR spectrum of the de-O-acetylated C polysaccharide (Fig. 1) shows that it is a simple anomerically homogeneous polymer of sialic acid. The chemical composition had been previously ascertained by chemical procedures (1) but no structural detail was proposed. The spectrum is only consistent with the polysaccharide having a 2–9–\( \alpha\)-linked structure. Assignment of the C-1, C-2, C-3, C-5, C-6, CH\( \text{acetyl} \) (acetamido), and carbonyl (acetamido) signals were made as previously described for the B polysaccharide. The downfield displacement of 2.0 ppm of C-9 as compared to the free C-9 of methyl \( \alpha\)-D-N-acetylenuraminic acid is independent of anomeric configuration (Table II) and indicative of C-9 being the linkage point. This value is small compared to the downfield displacements associated with the substituted primary hydroxyl group of structurally related isomaltitol (8.1 ppm) (22). This is probably due to the influence of the carboxyl group (C-1) on the anomeric center of the preceding residue. This phenomenon also occurs at C-8 of the B polysaccharide where the downfield displacement (6.8 ppm) is small compared to the substituted secondary hydroxyl group of structurally related maltitol (9.6 ppm) (22). Some evidence for this effect is also found in the methoxy signals of the methyl \( \alpha \)- and \( \beta\)-glycosides of sialic acid when compared to those of glucose (15). The latter have chemical shifts of 68.8 ppm (\( \alpha \) anomer) and 58.8 (\( \beta \) anomer), whereas the equivalent methoxy signals for the sialic acid methyl glycosides are significantly more shielded at 52.1 ppm (\( \alpha \) anomer) and 52.7 ppm (\( \beta \) anomer). The resonance at 71.4 ppm, assigned to C-8, is shielded by the adjacent linked carbon (C-9) to the extent of 0.6 ppm as compared to the equivalent C-8 position of methyl \( \alpha\)-D-N-acetylenuraminic acid. The remaining resonances at 69.5 ppm and 73.6 ppm are similar in chemical shift to the respective C-4 and C-6 resonances of the previously assigned B polysaccharide and as before in relation to the chemical shifts of the C-4 and C-6 resonances of methyl \( \alpha\) - and \( \beta\)-D-N-acetylenuraminic acid (Table II) the C polysaccharide can be assigned the \( \alpha \) configuration.

The above structural assignment is supported by the chemical evidence where the de-O-acetylated C polysaccharide consumed approximately 1 \( \mu \)mol of periodate per \( \mu \)mol of sialic acid (Table III) which is consistent with a 2–9–\( \alpha\)-linkage. In addition it was degraded by neuraminidase to the same extent and at a similar rate to the B polysaccharide (Table III), providing further evidence for its \( \alpha \) configuration. This result is in variance with a previous report by Gotschlich et al. (1) that the de-O-acetylated C polysaccharide was resistant to neuraminidase. A number of possible explanations could be postulated for this result including the possibility of strain differences (23, 24). However, we have other information that could be pertinent. Firstly, our preparation of the C polysaccharide was only partially de-O-acetylated by the procedure previously reported to yield the de-O-acetylated C polysaccharide (1) ("Experimental Procedure"). Secondly, we found that neuraminidase from our sources, in concentrations previously reported to degrade the B but not the de-O-acetylated C polysaccharide (1), did not degrade either of our preparations of these two polysaccharides and we had to resort to much higher concentrations of enzyme. However, the native C polysaccharide remained resistant to the enzyme (Table III) even at these levels. It has been reported that O-acetyl groups are responsible for the resistance of some glycoprotein sialic acid.

| Polysaccharide       | Free sialic released over periods of time | Periodate uptake: 24 hours |
|----------------------|------------------------------------------|---------------------------|
|                      | 3 hours | 21 hours | 45 hours* | 69 hours |
| Serogroup B          |         |          |          |          |
|                      | 3.6%    | 42.0     | 62.4     | 82.2     | 0.43     |
|                      | (12.6)* | (70.9)   | (79.4)   | (79.4)   |          |
| Serogroup C          |         |          |          |          |
|                      | 0.0     | 3.4      | 3.6      | 3.8      | 0.29     |
|                      | (0.0)   | (4.5)    | (4.8)    | (6.1)    |          |
| Serogroup C (partially de-O-acetylated) | N.D.     | N.D.     | N.D.     | N.D.     | 0.40     |
| Serogroup C (de-O-acetylated) | 6.5     | 36.6     | 50.7     | 72.0     | 1.16     |
|                      | (16.2)  | (75.5)   | (78.0)   | (80.4)   |          |

*Expressed as micromoles of periodate consumed per \( \mu \)mol of sialic acid.

*Additional enzyme added (50 units per mg of substrate).

*Values not bracketed. Action of V. cholerae.

*Values bracketed. Action of C. perfringens.

Dr. Gotschlich has recently kindly provided us with a sample of his C polysaccharide preparation, and a preliminary analysis of its \( ^{13} \text{C} \) NMR spectrum indicates that it does differ from our preparation, especially in the distribution of the O-acetyl groups.
acids residues to neuraminidase attack (25, 26), although evidence to the contrary has also been reported (27). Our evidence would tend to support the former view because removal of these groups using alkali did render the C polysaccharide prone to degradation. However, inter-sialic acid residue esters have also been postulated to account for neuraminidase resistance (5). An analysis of the $^{13}$C NMR spectrum of the C polysaccharide (Fig. 1) provided no evidence in favor of these linkages but the spectrum is complex and it would be impossible to dismiss the presence of a small percentage of these esters.

Location of the O-acetyl Groups in Serogroup C Polysaccharide—The $^{13}$C NMR spectrum of the native C polysaccharide is shown in Fig. 1. Due to the effect of O-acetyl groups on the chemical shifts of the sialic acid residues the spectrum is more complex than that of the de-O-acetylated polysaccharide. However, these effects can be used to advantage because the substitution of a 3-O-acetyl group in 2-acetamido-2-deoxy-b-glucose has been shown to produce large chemical displacements (2 to 3 ppm) on the carbon atoms $\alpha$ and $\beta$ to the O-acetyl group (13). Smaller displacements also occurred on the $\gamma$ and $\delta$ carbons. The larger chemical displacements ($\alpha$ and $\beta$ carbon atoms) were subsequently used to locate the O-acetyl groups in the meningococcal serogroup A polysaccharide (4).

As previously described (4) the degree of O-acetylation can be determined in homopolymers containing residues with acetamido groups by a comparison of the intensities of the CH$_3$ signals of the O-acetyl and N-acetyl groups. Calculated from its $^{13}$C NMR spectrum, the native C polysaccharide has an O-acetyl content of 1.16 mol of O-acetyl per sialic acid residue; this value was also confirmed by analysis (1.13 mol).

The number of ways of distributing the O-acetyl substituents in the C polysaccharide are numerous as there are three possible locations (C-4, C-7, and C-8) and also the possibility of unacetylated, mono-, di-, and tri-O-acetylated residues. However, the problem was considerably simplified by evidence which eliminated the possibility of O-acetyl substituents on C-4 of any of the sialic acid residues. This evidence was provided by studying the effect of 4-O-acetyl substitution in methyl $\alpha$-D-N-acetylneuraminic acid and methyl $\alpha$-D-N-acetylneuraminic acid and studying the chemical shifts on the carbon atoms $\beta$ to C-4. It was reasonably assumed on previous evidence (13) that the long range effects of the O-acetyl groups at C-7, C-8, and C-9 on C-3 and C-5 would be minimal compared to an O-acetyl group on the $\beta$ carbon (C-4). A comparison of the C-3 and C-5 chemical shifts of methyl $\alpha$-D-N-acetylneuraminic acid with those of its fully acetylated derivative (Table I) indicate substantial upfield displacements of 1.6 and 2.7 ppm for the respective C-3 and C-5 signals of the acetylated derivative. A similar comparison of the C-3 and C-5 signals of the native C polysaccharide with those of the fully acetylated derivative, produced displacements of comparable magnitude (even larger on C-3) thus confirming the absence of 4-O-acetyl groups in the native polysaccharide. A small displacement (0.6 ppm) on C-5 in the $^{13}$C NMR spectrum of the C polysaccharide was attributed to $\gamma$ carbon effects as explained below. Thus the O-acetyl substituents in the C polysaccharide are restricted to C-7 and C-8 of the sialic acid residues, indicating that there are a total of 4 possible different residues (1, 2, 3, and 4) as shown in Fig. 3. At least 3 of these 4 possible residues can be identified in the

![Fig. 3. The four different N-acetylneuraminic acid residues possible in the serogroup C polysaccharide.](http://www.jbc.org/)

$^{13}$C NMR spectrum of the native C polysaccharide.

The presence of unacetylated sialic acid residues (1) in the native polysaccharide was detected by resonances common to both the native and de-O-acetylated polysaccharide spectra (Fig. 1). These resonances are marked without primes in the spectrum of the native polysaccharide. The CH$_3$ (acetamido), C-2, and C-5 signals are readily discernible but those of C-6, C-8, and C-9 are only partially resolved. All of these resonances were enhanced and resolved in the $^{13}$C NMR spectrum of the partially de-O-acetylated polysaccharide (Fig. 1) which had an O-acetyl content as calculated from the spectrum (4) of 0.4 mol of O-acetyl per sialic acid residue. Chemical evidence in support of the presence of unacetylated residues in the native C polysaccharide is shown in Fig. 2. The elution profile of the native C polysaccharide from Sephadex G-200 indicates that it has a large molecular size (partially excluded from the column) as has been previously established (28). On treatment of the native C polysaccharide with periodate it consumed 0.29 $\mu$mol of periodate per $\mu$mol of sialic acid (Table III). When this oxidation was repeated on a larger scale and gel filtration was carried out on the oxidation product, most of the polysaccharide had been degraded to low molecular weight oligosaccharides (Fig. 2). This also indicated a scattered distribution of the unacetylated residues in the polysaccharide, i.e. no long sequences of unacetylated residues. The anomeric signal of the native polysaccharide is partially resolved into three signals C-2', C-2', and C-2 of intensities 52, 24, and 24%, respectively. Better resolution of these signals was obtained when an expanded spectrum was run (see insert in Fig. 1). This resolution in the C-2 signal is attributable to long range effects ($\epsilon$ or $\delta$ carbon effects) caused by the O-acetyl substitutions at C-7 and C-8 as this multiplicity disappeared on removal of these substituents. The anomeric signals indicate that the C polysaccharide contains at least 3 different sialic acid residues of which 24% are unacyetylated (from relative intensity of C-2 resonances). However, interpretation of the anomeric resonances must be approached with caution because of complex inter-residue effects. The anomeric carbon (C-2) of one residue could be equally affected by its own C-7 O-acetyl substituent or the C-8 O-acetyl substituent of the following residue. Both would be 3-bond (5) effects. Despite this, the estimation of the presence of 24% unacetylated residues agrees fairly well with the periodate oxidation results (Table III).

The total O-acetyl content of the C polysaccharide (1.16 mol per sialic acid residue) necessitates the presence of 7,8-di-O-acetylated residues (2) and the added presence of 24% unacetylated residues in the polysaccharide would require the presence
of substantial quantities of 2. In addition, at least 1 monoacetylated residue must be present to account for the two CH$_3$- (O-acetyl) signals at 21.7 and 21.3 ppm of relative intensities 25 and 75%, respectively. If only 7,8-di-O-acetylated residues were present in the C polysaccharide, then the relative intensities of these signals would have been equivalent. Some evidence that 7-O-acetyl residues (4) are present in the C polysaccharide can be deduced in the following way. The C-5 and C-5' signals of relative intensities 36 and 64% indicate that C-7 of the residues is more highly substituted by O-acetyl than C-8. This argument would be dependent on attributing the upfield displacement of 0.6 ppm on C-5 to the γ carbon effect (O-acetyl at C-7) rather than the more remote δ carbon effect (O-acetyl at C-8) (Fig. 3). This argument is reasonable as it is known that this effect rapidly diminishes with through bond distance from the carbons β to the carbon directly attached to the O-acetyl function (13). The upfield displacement could also be due to the 7,8-di-O-acetyl residue (2) but the relative intensity of C-5' (64%) would then require that the polysaccharide have 1.28 mol of O-acetyl per sialic acid residue in addition to a further quantity of O-acetyl from the other monoacetylated residue characterized previously. This amount of O-acetyl would be in excess of the O-acetyl content of the C polysaccharide (1.16 mol per sialic acid residue) and therefore the C-5' signal may possibly represent both the 7,8-di-O-acetyl (2) and 7-O-acetyl residues (4). No firm evidence for either the presence or the absence of 8-O-acetylated residues (3) in the C polysaccharide could be found in its $^{13}$C NMR spectrum.

One inexplicable signal (X) in the $^{13}$C NMR spectrum of the C polysaccharide (Fig. 1) at 60.8 ppm has been attributed to a so far unidentified impurity for the following reasons. The signal was not due to N-glycolyl substituents because of their significantly different chemical shift (62.2 ppm) (Table I), and because the signal disappeared on treating the C polysaccharide with base (Fig. 1). Also we have recently identified an identical signal in a number of other serogroup meningococcal polysaccharide preparations and found that it can be removed by DEAE-cellulose chromatography.3

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3 H. J. Jennings, C. P. Kenny, and A. Martin, unpublished results.

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