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CONFORMATIONAL AND DYNAMIC DIFFERENCES BETWEEN *N. meningitidis* SEROGROUP B AND C POLYSACCHARIDES, USING N.M.R. SPECTROSCOPY AND MOLECULAR MECHANICS CALCULATIONS*

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ABSTRACT

^{1}H-N.m.r. spectroscopy has been used to determine the conformation in aqueous solution of the sialic acid residues of the *N. meningitidis* serogroup B and non-*O*-acetylated (*O*-Ac$^-$)-C polysaccharides, and of *N*-acetylneuraminic acid (NeuNAc). In all cases, the sugar adopts the $^2C_5$ conformation. The side-chain of NeuNAc adopts a conformation such that H-7 and H-8 are approximately anti-periplanar. This conformation is also found in the (*O*-Ac$^-$)-C polysaccharide, whereas H-7 and H-8 are *gauche* in the B polysaccharide. Molecular mechanics calculations have been used to probe the conformational preferences of the variously linked sialic acid residues, and the results are in general agreement with those based on the ^{1}H-n.m.r. data. The ^{13}C-n.m.r. spin–lattice relaxation-times have been interpreted in terms of the molecular dynamics of the B and (*O*-Ac$^-$)-C polysaccharides. Molecular correlation times have been calculated and details of internal rotational or segmental motion elucidated. The C polysaccharide is characterised by internal or segmental motion in the C-7 to C-9 side-chain of the sialic acid repeating-unit, whereas the B polysaccharide has little or no such movement and tumbles in solution as a rigid species with internal rotation of only the pendant C-9 group. The conformational differences suggest a substantially different three-dimensional structure in solution for these polysaccharides.

INTRODUCTION

The capsular polysaccharides of *N. meningitidis* Serogroups B and C are homopolymers of sialic acid, linked (2→8)-α and (2→9)-α, respectively[1]. Most, but not all[2], strains of the latter are *O*-acetylated at C-7 and/or C-8.

Although chemically similar, the B and C polysaccharides have quite different immunological properties. Thus, immunisation of several animal species with

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purified C polysaccharide leads to a strong immune response and protection against infection[3–5], whereas immunisation with purified B antigen does not[6,7], even though antibodies against this particular polysaccharide can be elicited by immunisation with whole bacteria or with a complex of polysaccharide with proteins[8].

Although hypotheses have been postulated to explain the poor immunogenicity of the B polysaccharide[6,9], namely, sensitivity to neuraminidases, cross-reactivity with "self" antigens[10], and intrinsic "floppiness" of the purified B polysaccharide[9], clear-cut experimental evidence is scarce. Indirect and rather circumstantial evidence suggests there could be a difference between the antibody specificity for these two polysaccharides; following conceptually the definition of antigenic determinants as envisaged by Sela *et al.*[11], one could conceive of anti-C antibodies recognising as epitope a linear oligosaccharide (*i.e.*, a sequential determinant), whereas antibodies to the B polysaccharide would recognise conformational determinants of the antigen, thus being dependent on the three-dimensional structure of the poly(sialic acid) chain.

Since there are no *a priori* reasons to equate conformational determinants in the antigen with a poor immune response, we have assumed that the three-dimensional structure is unstable, thus resulting in poor immunogenicity. A potential source of antigenic instability has been suggested[12] for the B polysaccharide, through the formation of internal esters under mild conditions, resulting in a considerable loss in antigenicity.

We now present experimental evidence on the conformation and molecular dynamics of the B and C polysaccharides by ^{1}H-n.m.r. spectroscopy, by molecular mechanics calculations, and by ^{13}C-n.m.r. spin–lattice relaxation studies. These data indicate that the B polysaccharide, in contrast to the C polysaccharide, has little internal flexibility in solution and a different three-dimensional structure. This variation is shown to explain the different capacity of the two polysaccharides to undergo internal esterification. This property, as will be demonstrated in a following paper[13], is an important factor when considering the different immunological properties of the B and C polysaccharides.

EXPERIMENTAL

N.m.r. spectroscopy. — ^{1}H-N.m.r. spectra were recorded at 360 MHz for solutions in D_2O at 23° and 70°, using a Bruker WM-360 spectrometer. At the higher temperature, no conformational changes were observed, but the resonances were sharper, enabling resolution enhancement to be performed. The spectra were acquired by using the pulse-Fourier-transform technique into 32k data points and resolution enhanced in the time domain using the Lorentzian–Gaussian transformation method[14].

^{13}C-N.m.r. spectra were recorded for 5% solutions of polysaccharides in D_2O at 27° at two field strengths, namely, 5.9T (corresponding to 250 MHz for ^{1}H) and 9.4T (corresponding to 400 MHz for ^{1}H). Assignments were based on those in

the literature[1]. For methylene and methine carbons, ^{13}C-n.m.r. spin–lattice relaxation-times were measured by using the conventional inversion–recovery pulse sequence[15]. In this method, the populations of the two n.m.r. energy levels for each nucleus are inverted by applying an r.f. pulse, a time τ is allowed for the population to re-equilibrate partially by spin–lattice relaxation, and then the degree of equilibration is ascertained by measuring the size of the n.m.r. signals. This procedure is repeated for various τ values, and the spin–lattice relaxation-time T_{1i} is obtained by plotting $\ln[M_i(\infty) - M_i(\tau)]$ against τ, where $M_i(\tau)$ is the intensity of a carbon n.m.r. peak after a delay τ. The graph yields a straight line with slope $-1/T_{1i}$.

For isotropic tumbling, the correlation time τ_R defines an autocorrelation function $G(\tau_R)$ that decays exponentially with a time constant τ_R^{-1}. This autocorrelation function can be thought of as representing the probability that, after a given time, a specific internuclear vector will be at a certain orientation. Spin–lattice relaxation can occur if there is a component of this tumbling at the appropriate n.m.r. frequency.

If isotropic reorientation is assumed, $G(\tau_R)$ is exponential and, for a C–H bond distance, r, where N is the number of hydrogens bonded to the carbon,

$$\frac{1}{NT_1} = \frac{h^2}{40\pi^2}\gamma_C^2\gamma_H^2 r^{-6}\chi,$$

where

$$\chi = \frac{\tau_R}{1 + (\omega_H - \omega_C)^2\tau_R^2} + \frac{3\tau_R}{1 + \omega_C^2\tau_R^2} + \frac{6\tau_R}{1 + (\omega_H + \omega_C)^2\tau_R^2}$$

If molecular motion is very fast, $\omega^2\tau_R^2 \ll 1$, and

$$\frac{1}{NT_1} = \frac{h^2}{4\pi^2}\gamma_H^2\gamma_C^2 r_{CH}^{-6}\tau_R,$$

and NT_1 is inversely proportional to τ_R, the so-called motional narrowing limit.

However, for slower molecular motion, the above simplification is not valid and the full equations must be used[16]. The NT_1 values for the rigid parts of the polysaccharide can be used to calculate the overall, rotational correlation times, τ_R.

For a molecule, with one degree of internal rotation, attached to a rigid backbone, Doddrell *et al.*[16] have shown that

$$J_i(\omega) = \frac{2}{r^6}\left(\frac{A\tau_R}{1 + \omega^2\tau_R^2} + \frac{B\tau_B}{1 + \omega^2\tau_B^2} + \frac{C\tau_C}{1 + \omega^2\tau_C^2}\right),$$

where

$$\frac{1}{\tau_B} = \frac{1}{\tau_R} + \frac{1}{6\tau_G},$$

$$\frac{1}{\tau_C} = \frac{1}{\tau_R} + \frac{2}{3\tau_G},$$

$A = [(3\cos^2\theta - 1)^2]/4$, $B = 3\sin^2\theta\cos^2\theta$, and $C = (3\sin^4\theta)/4$,

where θ is the angle between the C–H vector and the axis of internal rotation, and

$$\frac{1}{NT_1} = \frac{h^2}{80\pi^2}\gamma_C^2\gamma_H^2[J_0(\omega_H - \omega_C) + 3J_1(\omega_C) + 6J_2(\omega_H + \omega_C)].$$

Molecular modelling. — The molecular-modelling theoretical calculations were performed by using an in-house designed system. The hardware consisted of a DEC 11/34A computer running under RT11 vs 4 monitor, with a floating point processor and 64k words of memory. The model building and minimising software is part of a comprehensive computer chemistry and graphics system written in-house for a VT11/VR19 calligraphic terminal on the 11/34. A slow modem link between the 11/34 and a main-frame IBM VM 370 system enables molecular orbital programs to run and hence charge distributions to be estimated.

Structures are input *via* a light pen as two-dimensional pictures that are subject to a combination of Simplex and Newton–Raphson energy minimisation routines. The molecular mechanics energy calculations are based on variations in bond length, bond angle, torsional angle, van der Waals contacts, and coulombic interactions[17]. The force fields are simplified to increase general applicability, but retain the ability to produce good three-dimensional architecture. At this stage, the energy interactions are restricted to short ranges over a few neighbouring atoms for the sake of speed.

Full minimisation across the completed molecule is the next stage. For the present set of molecules, there are many rotatable bonds which have to be orientated so as to minimise the overall energy of the structure. Generally, if a full conformational space is to be investigated, an impossible number of conformations needs to be examined. To overcome this problem, an approximation (called linear search) is employed. Each of the twelve rotatable bonds is rotated through 360° by the chosen increment, say 5°, and only torsional barriers, coulombic, and van der Waals interactions are calculated. A minimum energy is found at one of the 72 positions, the bond is placed at that position, and the process is repeated for the next bond. To account for any changes in a distant torsional angle brought about by the minimisation of the current bond, the whole procedure is iterated at least

three times. A total of 72 × 12 × 3 conformations (= 2592) is examined, considerably less than the theoretically possible 72^{12}.

In order to consider coulombic interactions (H-bonds, *etc.*), the charge distributions of the molecule must be known. These are calculated theoretically by using the CNDO-molecular orbital method on the unminimised structure, which itself is a plain source of error. However, charges are not generally over-sensitive to changes of structure exemplified by these sugars, and a minimisation without charge considerations is usually acceptable as an input to charge calculations.

RESULTS

Conformation of N-*acetylneuraminic acid.* — The ^{1}H-n.m.r. spectrum of NeuNAc has been assigned in the literature[18]. Chemical shifts (δ) and spin coupling constants (J) obtained by first-order analyses in the present work, and listed in Table I, are in close agreement with those published. The 360-MHz spectrum of the δ 3.53–4.14 region is shown in Fig. 1. The assignments are as previously given[18] and were confirmed by double resonance and two-dimensional chemical shift cor-

TABLE I

^{1}H-N.M.R. DATA FOR β-*N*-ACETYLNEURAMINIC ACID, B POLYSACCHARIDE, AND (*O*-Ac$^-$)-C POLYSACCHARIDE

Hydrogen	*NeuNAc*	*B*	*(O-Ac$^-$)-C*
	Chemical shifts	*(δ)*[a]	
3*a*	1.89	1.72	1.72
3*e*	2.32	2.62	2.74
4	4.08	[b]	[b]
5	3.95	[b]	[b]
6	4.07	[b]	3.82
7	3.57	3.87	3.62
8	3.77	4.11	3.98
9	3.63	4.05	3.87
9′	3.86	[b]	3.75
Coupling constants (Hz)			
$J_{3a,3e}$	13.06	12.1	12.3
$J_{3a,4}$	11.62	11.8	11.5
$J_{3e,4}$	4.97	4.5	4.5
$J_{4,5}$	10.21	[b]	[b]
$J_{5,6}$	10.37	[b]	10.1
$J_{6,7}$	1.31	<1	1.2
$J_{7,8}$	9.12	3.5	9.0
$J_{8,9}$	6.30	4.0	6.2
$J_{8,9'}$	2.63	4.0	2.3
$J_{9,9'}$	11.76	12.2	10.3

[a]From Me_4Si, taking the *N*-acetyl resonance as δ 2.07. [b]Resolution insufficient to allow determination of the n.m.r. parameters.

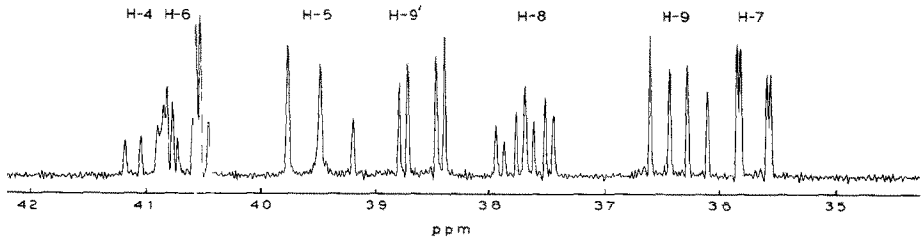
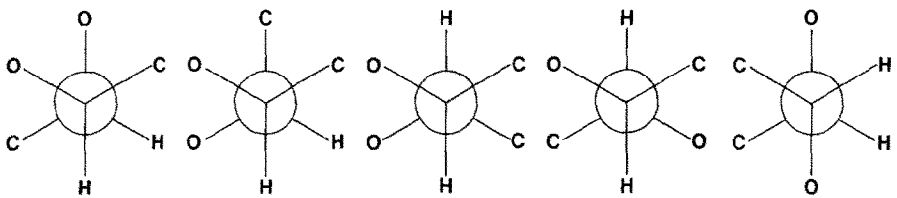
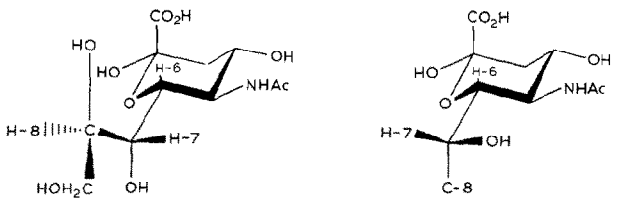


Fig. 1. 360-MHz ^{1}H-n.m.r. spectrum of a solution of NeuNAc in D_2O; only the region δ 3.53–4.14 is shown.

relation experiments. The ring coupling constants, $J_{3a,4}$, $J_{4,5}$, and $J_{5,6}$ are typical of axial–axial hydrogen interactions and confirm the assignment of the ring conformation as $^2C_5$. The coupling constants observed for the C-7–C-9 side-chain are averages over internal rotation that is fast on the n.m.r. timescale, although the barriers to rotation are such that the side-chain exists in staggered conformations. Haasnoot *et al.*[19] have formulated predictive rules for vicinal H–H coupling constants in carbohydrate systems based on the *gauche* or antiperiplanar nature of the coupled hydrogens and the relative disposition of the oxygen substituents. These predictions explain the magnitudes of the observed coupling constants given in Table I. Thus, $J_{6,7}$ is measured as 1.3 Hz and fits structure **1**, and $J_{7,8}$ is 9.1 Hz and is in agreement with structure **3**, with structure **2** being excluded on the basis of the J value; structure **4** is excluded because it corresponds to the unnatural optical isomer. The important deduction is that H-7 and H-8 are antiperiplanar. The total conformation, by adding structures **1** and **3**, is therefore structure **6**. There exists a second *gauche* rotamer obtained by a rotation of structure **1** through 120°, thus giving structure **5**. This arrangement, however, no longer has the two hydrogens anti-





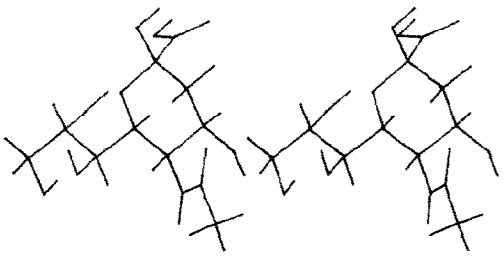


Fig. 2. Relaxed stereo-images of the energy-minimised NeuNAc structure, as calculated using the molecular mechanics approach.

periplanar to two oxygens as in **1**, and the predicted[20] $J_{6,7}$ value would be 5.5 Hz. This rules out a *gauche* conformation, as illustrated in structure **7**.

As a test of the molecular mechanics procedures, NeuNAc was built and minimised over its torsional space. Charges were computed by using CNDO-MO followed by a full torsional minimisation by linear search of 5 bonds over 1-degree increments (5400 conformations), and the results are summarised in Fig. 2. The final minimised structure and the degree of flexibility were in good agreement with the literature conclusions from the n.m.r. and molecular orbital calculations[21].

The conformation deduced in the present study differs in one minor respect from the crystal structure[22] and from the theoretical molecular orbital study[21]. Both crystal and molecular orbital structures show the N-H bond of the acetamido group to have a torsional angle with respect to the C-5–H-5 bond of ~180°, whereas the present molecular mechanics study yields a value near 0°. This apparent anomaly has been investigated and, using the molecular mechanics force-field, we find a difference of only 1.25 kcal.mol^{-1} between the two forms (with that shown in Fig. 2 being of lower energy), indicating that, at ambient temperatures, both conformations will be substantially populated. The discrepancy from the crystal structure is explained on the basis of the known large magnitude of crystal packing forces, and the deviation from the previous molecular orbital calculations is due to the well documented inadequacy of these calculations for predicting conformational detail.

Conformation of (O-*Ac*$^-$)-*C polysaccharide.* — The ^{1}H-n.m.r. spectrum of this material is shown in Fig. 3, and the n.m.r. parameters, where they can be resolved, are also given in Table I. Because of the viscous nature of the solution, the relaxation times (T_2) of the nuclei are shorter and this results in broader lines. This effect has been lessened by measuring the spectrum at 70°. Again, computer resolution enhancement was necessary; under these conditions, by analysis of the coupling patterns and through the use of spin decoupling, the bands due to H-7 and H-8 are assigned. The signal for H-8 is easily assigned because, apart from H-4, it is the only nucleus coupled to three other protons. Thus, irradiating the signal at δ 3.98 caused no effect at either of the H-3 resonances but removed the 9.0-Hz coupling on the band at δ 3.62, thereby confirming the assignments of H-7 and H-8.

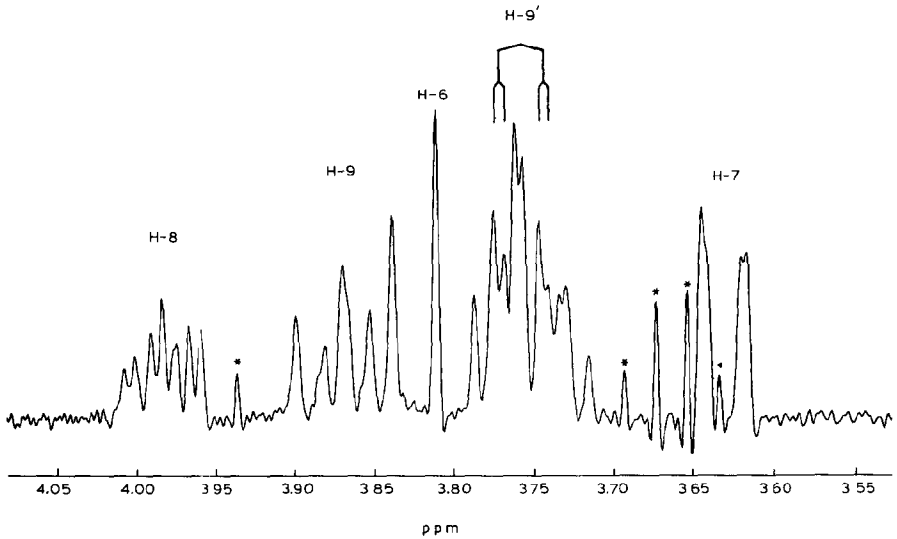


Fig. 3. 360-MHz ^{1}H-n.m.r. spectrum (δ 3.55–4.05) of the (O-Ac$^-$)-C polysaccharide; this region excludes H-3.

The coupling constant $J_{7,8}$ is typical of the antiperiplanar arrangement of H-7 and H-8 as in NeuNAc, and $J_{6,7}$ is similar to the monosaccharide value, indicating the same total conformation.

Conformation of the B polysaccharide. — This material was examined under the same conditions as for the (O-Ac$^-$)-C polysaccharide, and the ^{1}H-n.m.r. spectrum is shown in Fig. 4. The assignments of the resonances again follow from application of the techniques used on the other polysaccharide. The H-3 resonances show coupling constants similar to those of the other substances and confirm that the ring conformation is unchanged. The other protons give rise to three separate bands with considerable overlap. To highest frequency is a two-proton band, followed by a signal from a single hydrogen, and a four-proton complex band at low-

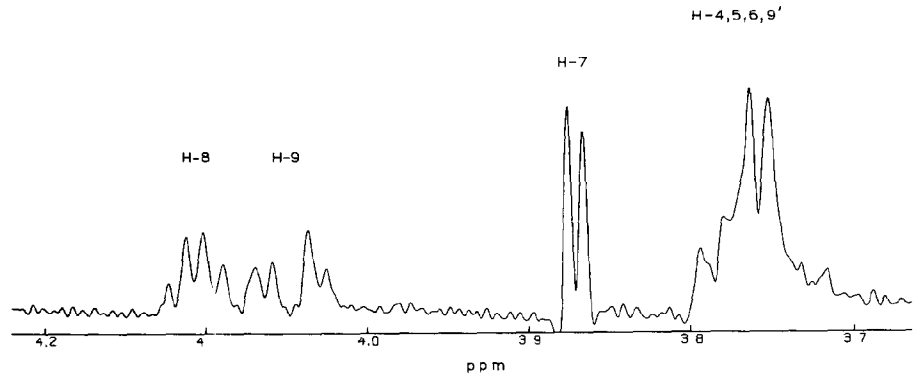
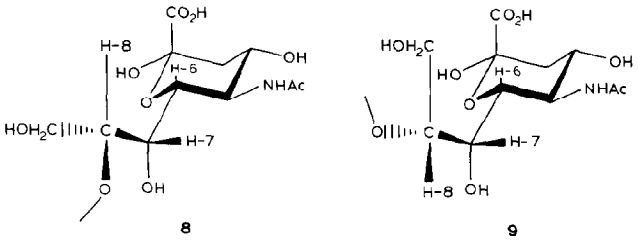


Fig. 4. 360-MHz ^{1}H-n.m.r. spectrum (δ 3.7–4.2) of the B polysaccharide.

est frequency. On resolution enhancement, the signal at δ 4.11 can be seen to possess three couplings, indicating that it must be due to H-4 or H-8. The couplings do not match those for H-3(*a*) and, thus, the band cannot be due to H-4. The band assigned to H-8 shows a quartet structure, indicating three moderate couplings of similar magnitude (3–4 Hz). The next band at δ 4.05 shows two couplings, one of ~12 Hz typical of a geminal interaction, and is therefore assigned to one of the non-equivalent protons in the C-9 methylene group (the two protons are expected to have different chemical shifts because of the chiral nature of the molecule). The single proton resonance at δ 3.87 has only one coupling constant of 3.5 Hz and, as this is repeated in the band due to H-8, it is assigned to H-7, with $J_{7,8}$ 3.5 Hz. The coupling constant $J_{6,7}$ is not resolved and, as in the previous molecules, must be <1 Hz. The complex band centred at δ 3.75 therefore arises from H-4, H-5, H-6, and H-9 (one proton).

The conformational inference from the measured coupling constants is that H-6 and H-7 have approximately the same dihedral angle as in NeuNAc, but that the conformation about the C-7–C-8 bond is different and, as the coupling constant of 3.5 Hz is close to that in **2**, the two hydrogens are now *gauche*. Thus, by adding together rotamers **1** and **2**, the complete conformation is either **8** or **9**.



Attempts have been made to support the n.m.r. conclusions by using molecular mechanics calculations. NeuNAc models were joined together to form disaccharides linked either (2→9)-α or (2→8)-α, using the graphics facilities of the Wellcome Molecular Modelling system. The charges were suitably adjusted to account for the ether linkage, and both disaccharides were subjected to the linear

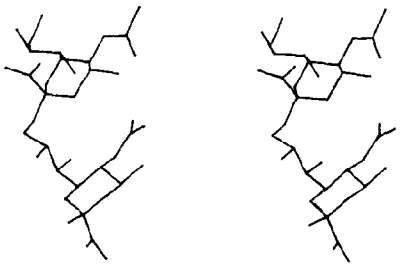


Fig. 5. Relaxed stereo-images of the minimised energy structure of a (2→9)-α-linked disaccharide of NeuNAc, showing the antiperiplanar arrangement of HO-7 and HO-8.

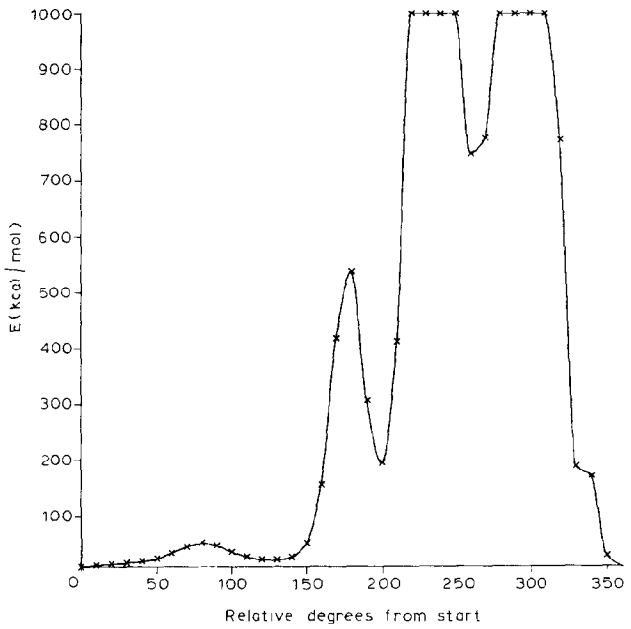


Fig. 6. Plot of energy against the C-7–C-8 torsional angle, showing secondary minima at ~130° and ~200° corresponding to *gauche* conformers. Start torsional angle = −53.4°.

search procedure already mentioned. It was necessary to spin the chosen 12 bonds by a 5-degree increment to reduce the time for the calculation.

The minimum energy for the (2→9)-α-linked C disaccharide occurs at an antiperiplanar conformation (Fig. 5), and the energy values of the rotational barrier around the C-7–C-8 bond after minimisation are plotted in Fig. 6. Although there is a second minimum at the forward *gauche* position, which is 12 kcal/mol above the first, the barrier to interconversion is high (~50 kcal/mol), and it is therefore not likely that the *gauche* forms will be accessible to the conformational space of the (2→9)-α-linked C disaccharide.

The minimum energy position for the (2→8)-α-linked disaccharide is an antiperiplanar conformation (Fig. 7), and Fig. 8 gives the rotational information

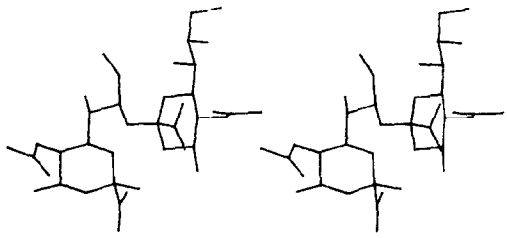


Fig. 7. Relaxed stereo-images of the minimised energy structure of a (2→8)-α-linked disaccharide of NeuNAc, showing the antiperiplanar arrangement of HO-7 and HO-8.

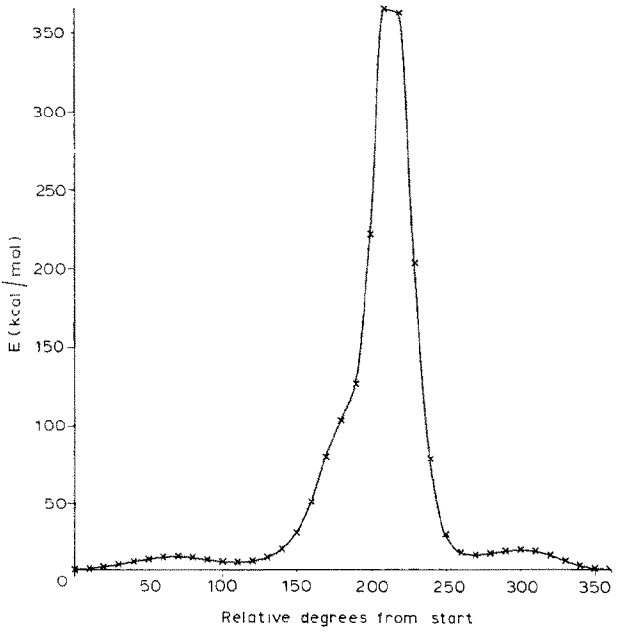


Fig. 8. Plot of energy against the C-7–C-8 torsional angle, showing accessibility of secondary minima at ~120° and ~270° to *gauche* conformers. Start torsional angle = −45°.

around the C-7–C-8 bond. Again, the antiperiplanar position is at a minimum but, in this case, the barrier to interconversion is low (~10 kcal/mol), suggesting that the antiperiplanar and *gauche* conformers are in equilibrium. A third, accessible, local energy minimum, corresponding to approximately the second *gauche* conformation, is also seen at ~270°. N.m.r. studies of the corresponding polymers of each disaccharide have indicated that H-7 and H-8 are antiperiplanar in the (2→9)-α-linked C polysaccharide, and *gauche* in the (2→8)-α-linked B polysaccharide. If it is assumed that the action of polymerisation favours the *gauche* conformation, then the calculated barriers are in accord with the n.m.r. results insofar as the *gauche* (2→8)-α-linked arrangement would be allowed but not the *gauche* (2→9)-α-linked conformation.

Based on the disaccharide structures (Figs. 5 and 7) and on the conformational differences between the B and C polysaccharides, molecular mechanics calculations have been used to support the conclusions of experiments on the differing ability of the B and C polysaccharides to undergo internal esterification[12,13]. Thus, in order to bring one of the carboxyl oxygens to within reacting distance of an adjacent HO-8 group in the (2→9)-α-linked C disaccharide, three bond twists are required and the energetics of this process are unfavourable. Conversely, only one bond needs to be rotated in the (2→8)-α-linked B disaccharide in order that the carboxyl group of one residue and HO-9 of the adjacent residue are brought into proximity, entropically a much more favourable process.

TABLE II

^{13}C-N M R. SPIN–LATTICE RELAXATION-TIMES (NT_1 VALUES, S)

Carbon atom	*B (5.9T)*	*B (9.4T)*	*(O-Ac$^-$)-C (5.9T)*
C-3	0.16	0.56	0.17
C-4	0.16	0.44	0.14[a]
C-5	0.17	0.37	0.16
C-6	0.17	0.43	0.14
C-7	0.15	0.42	0.19[a]
C-8	0.16	0.43	0.17
C-9	0.19	0.35	0.20

[a]Assignments may be reversed, although that shown is preferred.

Polysaccharide flexibility. — Dynamic information is derived from interpretation of ^{13}C spin–lattice relaxation-times (NT_1) which are given in Table II for the various species as 5% solutions in D_2O at 27° and at different field strengths.

Each distinguishable ^{13}C nucleus has an associated spin–lattice relaxation-time, NT_1, made up from various contributions by which the nucleus can lose energy to the lattice[15]. It is known that, for carbons directly bonded to one or more hydrogens, the dipole–dipole mechanism is the predominant cause of relaxation. This has been checked for dextrans, because NT_1^{DD} is also responsible for the nuclear Overhauser effect, which can be obtained in a separate measurement[23].

Measurement of the NT_1 values at two field strengths shows that there is a field dependence, and therefore that the molecule is tumbling slower than the motional narrowing limit[16]. The results for the B and (*O*-Ac$^-$)-C polysaccharides are listed in Table II. The constancy of the NT_1 values for C-4, C-6, C-7, and C-8 of the B polysaccharide repeating-unit indicates that there is no segmental motion for the backbone of this species. This result is similar to that observed[24] for NeuNAc. At 5.9T, C-9 of the B polysaccharide has an NT_1 longer than that of the rigid backbone, but at 9.4T this becomes shorter than those of the carbons in the rigid part. This demonstrates that C-9 has some extra degree of motion over and above the overall molecular tumbling.

For the (*O*-Ac$^-$)-C polysaccharide, increased T_1 values are observed for C-7, C-8, and C-9, something not observable for the (*O*-Ac$^+$)-C polysaccharide, which consists of a mixture of non-*O*-, 7-*O*-, 8-*O*-, and 7,8-di-*O*-acetylated residues. These longer T_1 values demonstrate extra segmental motion for C-7, C-8, and C-9, all involved in the chain backbone.

The NT_1 values for C-3 are generally longer than those for the rigid parts of the molecule and this may be a result of increased molecular flexibility due to ring-puckering motions. If it is assumed that the molecule is tumbling isotropically, and this is likely to be true for a coiled polymer, then there is a single rotational correlation time, τ_R.

Taking r_{CH} = 1.09 Å, ω_H (at 9.4T) = 25.96 × 10^8 rad.s^{-1}, and using the NT_1

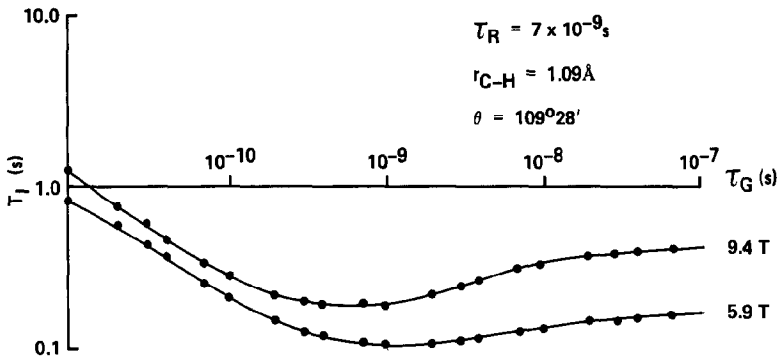


Fig. 9. Variation of the dipolar T_1 for a ^{13}C nucleus in a ^{13}C–^1H fragment with one degree of internal motion characterised by a correlation time τ_G, in an otherwise rigid molecule with a correlation time $\tau_R = 7 \times 10^{-9}$ s, $r_{CH} = 1.09$ Å, and $\theta = 109°28'$.

values for the rigid parts of the polysaccharide (see Experimental section), one obtains: B polysaccharide (5.9 T), $\tau_R = 6.7 \times 10^{-9}$ s; B polysaccharide (9.4 T), $\tau_R = 7.1 \times 10^{-9}$ s; and (*O*-Ac$^-$)-C polysaccharide (5.9 T), $\tau_R = 5.6 \times 10^{-9}$ s. The model developed by Doddrell *et al.*[16] has been applied to the internal rotation of the pendant CH_2OH (C-9) of the serogroup B polysaccharide. NT_1 for C-9 has been calculated as a function of τ_G, given $\tau_R = 7 \times 10^{-9}$ s, and the results are shown in Fig. 9. Applying this specifically to the B polysaccharide gives, at 5.9T, $\tau_R = 6.7 \times 10^{-9}$ s and $\tau_G = 1.3 \times 10^{-10}$ s; and at 9.4T, $\tau_R = 7.1 \times 10^{-9}$ s and $\tau_G = 0.7 \times 10^{-10}$ s; assuming that $\theta = 109°28'$.

The consistency of these correlation times is obvious from the constancy obtained for the B polysaccharide at the two field strengths. The agreement for τ_G is clearly not so good as for τ_R, but the results at both field strengths indicate that the CH_2OH group has an internal rotational correlation time 50–100 times faster than the overall molecular tumbling.

The molecules under consideration are homopolymers of sialic acid and hence the NT_1 values measured represent an average for all the monosaccharide units throughout the chain, including end groups. Consequently, increased flexibility at the ends of the polymer chain could invalidate the motional model used. This situation is unlikely, because of the high molecular weight which effectively gives the end groups little relative weight. Also, if a particular carbon in different sialic acid residues along the chain possessed a variety of T_1 values, the relaxation data for that carbon would not fit the observed, single exponential decay.

DISCUSSION

The findings presented here have immunochemical implications. Internal esterification takes place with ease in the B polysaccharide, with some difficulty in the (*O*-Ac$^-$)-C polysaccharide, and not at all in the (*O*-Ac$^+$)-C polysaccharide[12,13].

This is consistent with the evidence that the formation of a 6-membered ring by esterification between the carboxylic acid and the hydroxylic group of neighbouring sialic acid residues is thermodynamically more favourable for the B polysaccharide than for the (*O*-Ac$^-$)-C polysaccharide. This reaction results in a loss of antigenicity, as indicated by a gradual decrease in the capacity of the polysaccharide to react with corresponding antibodies. However, given the molecular restrictions pertaining to esterification of the C polysaccharide, only the B polysaccharide would be expected to undergo such a change under normal physiological conditions.

The second important conclusion refers to the relative flexibility of the B and C polysaccharides in solution. The lack of segmental motion for the former (except for C-9) is compatible with some form of three-dimensional structure, whereas the C polysaccharide behaves as a random coil in solution. Immunologically, these results suggest that the B polysaccharide bears conformational epitopes and that group C determinants are sequential.

Similar experiments on the molecular dynamics of the B and C polysaccharides have been reported by Egan *et al.*[25,26], and their results for the molecular correlation times, τ_R, are in the same range as those derived here. An exact comparison cannot be drawn because of the different experimental conditions used. For example, their solution concentration was four times higher than in the present study, with a related increase in viscosity. However, by measuring spectra at 37° compared to our 27°, this may have been somewhat counterbalanced. In addition, their experiments were performed at ~2.4T and ~6.4 T (corresponding to 100 and 270 MHz for ^{1}H observation) and are thus probing aslightly different time-scale. The main difference between the two sets of results is that we observe differences in the ^{13}C relaxation times for the side-chain carbons of the (*O*-Ac$^-$)-C polysaccharide. It appears that the previous study used the (*O*-Ac$^+$)-C polysaccharide, which is a mixture of partially acetylated species, giving rise to a loss of peak resolution and chemical shift changes on *O*-acetylation that lead to assignment difficulties. This may account for their inability to differentiate T_1 values for the side-chain and hence derive relative flexibility information on the two polymers.

It should be remembered that n.m.r. NT_1 measurements only probe fluctuations that occur on the time-scale of the Larmor frequency, and that slower segmental motion may not affect T_1. Both experimental NT_1 values, ours and those of Egan *et al.*[25,26], indicate that the B and C polysaccharides possess internal flexibility.

The idea of a conformational determinant for the B polysaccharide has been advanced before, although there is no clear evidence. However, B polysaccharide of low molecular weight or colominic acid (*i.e.* 5–20k molecular weight) shows a remarkably poor capacity to react with antibody[13],a fact that is not compatible with an orthodox, sequential polysaccharide determinant where a penta- or hexa-saccharide is sufficiently large to define an epitope[27]. The data presented here and in a future paper[13] can be taken as experimental evidence, albeit indirect, in support of conformational epitopes. Data available either from n.m.r. studies or molecular

modelling are insufficient to establish the nature of the secondary or tertiary structure adopted by the B polysaccharide. So far, it does not appear to involve hydrogen bonding[28], and probably it is held together by very weak, short-range interactions. In this context, Mandrell and Zollinger[29] have pointed out the considerable decrease in avidity of group B-specific antibodies with increasing temperature, whereas that of anti-C antibodies changed very little. We interpret these results in terms of a loss of the three-dimensional structure and therefore of conformational determinants of the B polysaccharide with the increase in temperature, whereas the sequential determinants of the C polysaccharide remain unaffected. However, it must be stressed that there is no n.m.r. evidence for a temperature-dependent conformational change of the B polysaccharide.

Carbohydrate antigens of bacterial origin often show structural similarities to cell-surface components of the host. In this situation, it would be advantageous if the immune system of the animal were to produce antibodies directed against conformational determinants, thus preventing autoimmune cross-reactivity. This seems to occur for type III group B streptococcal polysaccharide[30], where sialic acid is important for the antigenic conformation. The same situation may apply to the *N. meningitidis* group B polysaccharide, *i.e.*, if the antibodies were to recognise a straightforward (2→8)-α-linked sequential determinant, as seems to be the case with a specific horse anti-B serum[10], they would cross-react with gangliosides[31] and sialopeptides[32] bearing this linkage, whereas antibodies recognising an epitope defined by the secondary or tertiary structure of the polysaccharide would not react with them. Careful immunochemical characterisation of more antisera and monoclonal antibodies, ideally of human and murine origin, may well resolve this point.

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