Carbohydrate Research, 139 (1985) 35–46
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

^{1}H- AND ^{13}C-N.M.R. ASSIGNMENTS AND CONFORMATIONAL ANALYSIS OF SOME MONOSACCHARIDE AND OLIGOSACCHARIDE SUBSTRATE-ANALOGUES OF LYSOZYME

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(Received June 8th, 1984; accepted for publication, November 21st, 1984)

ABSTRACT

The ^{1}H- and ^{13}C-n.m.r. spectra of solutions of GlcNAc, β-GlcNAc-(1→4)-GlcNAc, and β-GlcNAc-(1→4)-β-GlcNAc-(1→4)-GlcNAc in D_2O at 50° are interpreted in terms of the conformations, using a combination of 1D- and 2D-n.m.r. spectroscopy and spectra simulation techniques. Two preferred orientations of the hydroxymethyl group were found for each of these saccharides. The conformations have been compared with those found from X-ray crystallographic data and conformational energy calculations.

INTRODUCTION

The proposed catalytic mechanism of lysozyme is mainly based upon observations derived from X-ray crystallographic data[1,2], conformational energy calculations[3–6], and physical organic chemistry[7–11]. The conclusions which emerge from these data have been summarised[12–15].

Recently, progress has been made in confirming and making new assignments for the ^{1}H-n.m.r. spectrum of hen egg-white lysozyme[16–19] and assignments now exist for various proton resonances throughout the molecule. More particularly, assignments have been made for some residues in each of the six subsites comprising the active-site region. This, taken in combination with a full assignment for several substrate analogues, would allow some detailed mechanistic studies to be undertaken. We now report an interpretation of the ^{1}H- and ^{13}C-n.m.r. spectra of the substrate analogues, 2-acetamido-2-deoxy-D-glucose (GlcNAc), di-*N*-acetylchitobiose (DiGlcNAc), and tri-*N*-acetylchitotriose (TriGlcNAc), using a combination of 1D- and 2D-n.m.r. spectroscopy and spectra simulation techniques.

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0008-6215/85/$ 03.30

EXPERIMENTAL

Typically, spectra were recorded on ~68mM solutions of the appropriate sugar in D_2O, pH* 7.0 (meter reading uncorrected for deuterium isotope effect) at 50°. The ^{1}H-n.m.r. spectra were recorded at 270 or 469 MHz, using spectrometers with Oxford Instruments superconducting magnets. Each machine was interfaced to a Nicolet 293B pulse controller and 1180 computer. The ^{13}C-n.m.r. experiments were performed at 67.9 MHz. The 1D n.O.e. experiments, used to determine some specific coupling constants (see Results), were carried out at 469 MHz. The coupling constants were measured directly from the spectra with the assumption that the first-order coupling approximation was valid.

2D ^{1}H,^{1}H Homonuclear-correlated spectroscopy was carried out using the Jeener pulse sequence; 512 t_1 increments were collected using identical sweep widths of ±900.9 Hz in both the F_1 and F_2 frequency dimensions. The data matrix was defined by 2048 data points in the F_2 frequency dimension, and the F_1 frequency dimension was zero-filled twice to improve the spectral definition. The ^{13}C,^{1}H heteronuclear-correlated experiments were performed using pulse sequences already described[20,21], the utility of which has been demonstrated[22] for carbohydrates. Spectral simulations were performed using the standard Nicolet 7 spin simulation program NTCSIM. The simulated spectra of each monosaccharide unit were convoluted with a line broadening of 2.5 Hz. The choice of vicinal coupling constants used for the simulations has been described[23,24]. Equation *1* was used to calculate the vicinal coupling constants for gg, gt, and tg rotamers:

$$^3J_{H,H} = 13.22\cos^2\theta - 0.99\cos\theta + \sum\Delta X(0.87 - 2.46\cos^2\{\varepsilon\theta + 19.9\Delta X\}), \quad 1$$

where θ is the dihedral angle, ε takes the values ±1 depending on the orientation of the substituent with respect to its geminal proton substituent, and ΔX is the Huggins electronegativity.

RESULTS

GlcNAc. — Assignment of the ^{1}H- and ^{13}C-n.m.r. spectra of solutions of the α- and β-GlcNAc in D_2O have been presented[25]. Simulated ^{1}H-n.m.r. spectra, based on these chemical shift data, with the vicinal coupling constants for the ring protons derived from ref. 24 and those for $J_{5,6}$ measured directly, show good agreement with experimental spectra both with respect to chemical shifts and intensities. A heteronuclear-correlated experiment confirmed the ^{13}C assignments, with the exception of the resonances for C-3 and C-5 for α-GlcNAc which were the reverse of those reported previously[25].

DiGlcNAc. — The approach used for the specific identification of the ^{13}C resonances proceeded on the basis of established empirical correlations in use for carbohydrates[26] in combination with our n.m.r. experiments. Our conclusions accord with those published[27].

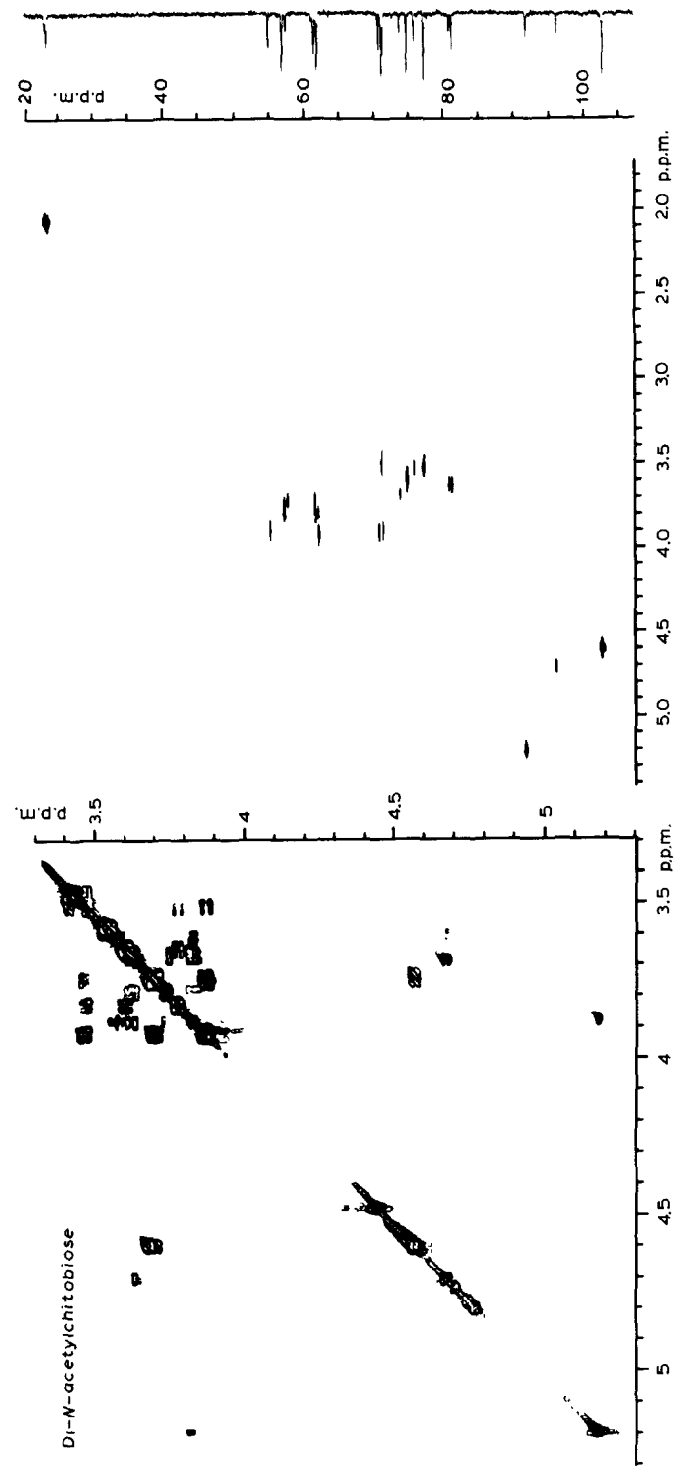

Fig. 1. 2D Homonuclear-correlated spectrum (469 MHz) and 2D heteronuclear spectrum of a solution of DiGlcNAc (270 MHz for ^{1}H and 67.9 MHz for ^{13}C) in D_2O at pH 7 and 50°.

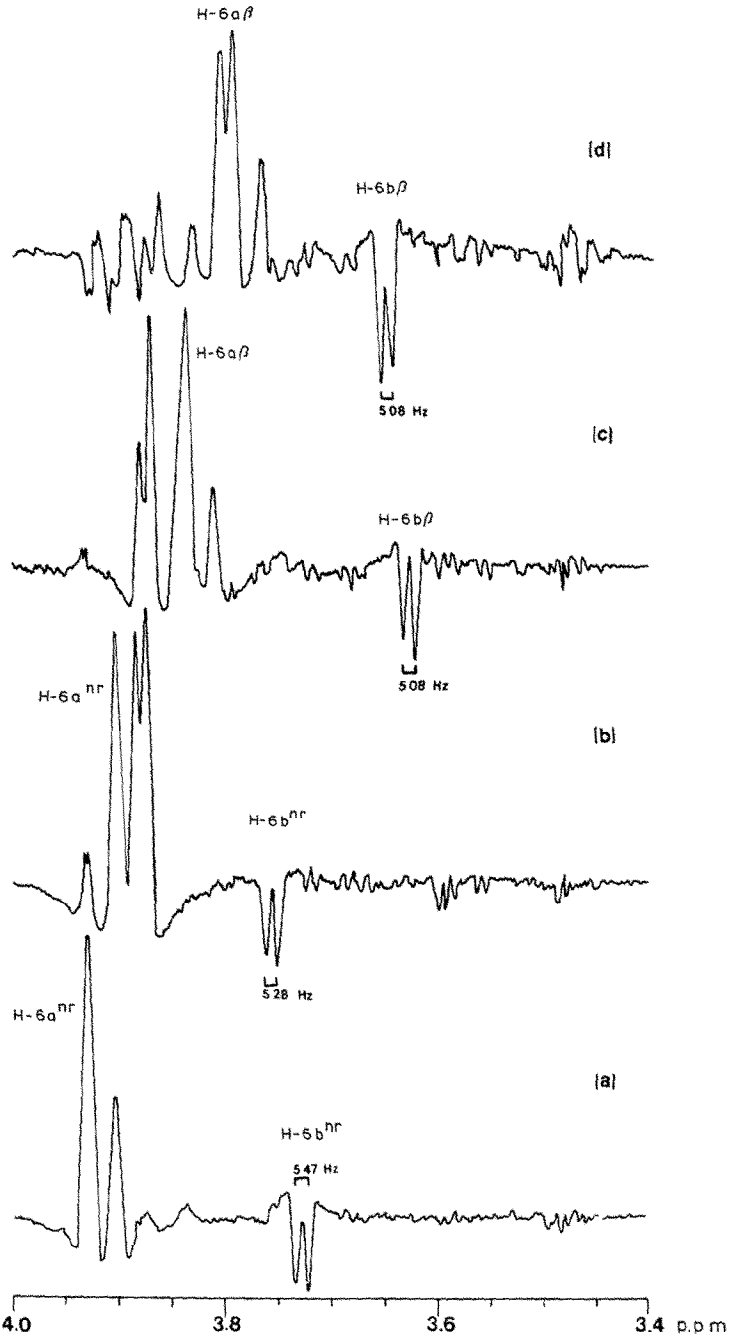

Fig. 2. 1D ^{1}H-N.O.e. difference spectra for DiGlcNAc (solution in D_2O at pH 7, 50°) obtained by selective irradiation of a single transition of one of the hydroxymethyl groups: irradiation of (*a*) the downfield transition of the doublet at 3.917 p.p.m. corresponding to a H-6 of the non-reducing unit, (*b*) the upfield transition of the same proton as in (*a*), (*c*) the downfield transition for the doublet at 3.82 p.p.m. corresponding to a H-6 of the β reducing-moiety, (*d*) the upfield transition of the same proton as in (*c*). Only the corresponding perturbed transition is shown. The experiment allows measurement of both the value and the sign of the coupling constant. The geminal coupling constant is negative. The observed vicinal coupling constants are indicated; for each, the first-order approximation was valid

TABLE I

N.M.R. DATA FOR DiGlcNAc

Unit	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6a*	*H-6b*
1. ^{1}H Chemical shifts (p.p.m.) used for the final simulation[a]							
Reducing α	5.190	3.863[b]	3.870[b]	3.619	3.868	3.778	3.659
	(0.0)	(−0.001)	(+0.119)	(+0.143)	(+0.023)	(−0.057)	(−0.117)
Reducing β	4.700	3.677[b]	3.666[b]	3.606	3.502	3.820	3.636
	(0.0)	(+0.013)	(+0.144)	(+0.164)	(+0.045)	(−0.075)	(0.099)
Non-reducing α[c]	4.594	3.749	3.575	3.466[b]	3.497[b]	3.912	3.734
	(+0.159)	(+0.071)	(+0.054)	(+0.038)	(+0.051)	(−0.014)	(−0.006)
Non-reducing β[c]	4.585	3.736	3.569	3.463[b]	3.485[b]	3.912	3.737
	(+0.150)	(+0.071)	(+0.078)	(+0.035)	(+0.039)	(−0.014)	(−0.003)

2. Coupling constants (Hz) used for the final simulation[d]

Unit	$J_{1,2}$[e]	$J_{2,3}$[e]	$J_{3,4}$[e]	$J_{4,5}$[e]	$J_{5,6a}$[f]	$J_{5,6b}$[f]	$J_{6a,6b}$[f]
Reducing α	3.4	10.6	9.3	9.8	2.1	4.7	−12.2
Reducing β	8.7	10.1	9.3	9.8	2.0	5.3	−12.1
Non-reducing	8.7	10.1	9.3	9.8	2.0	5.5	−12.3

3. ^{13}C Chemical shifts (p.p.m.)

Unit	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
Reducing α	91.68	54.84	70.56	81.19	71.21	61.35
Reducing β	96.03	57.36	73.77	80.75	75.79	61.35
Non-reducing	102.70	56.85	74.73	71.02	77.14	61.84

[a]The shifts from the respective monomers are given in brackets. [b]The intensities are very sensitive to chemical shifts, because of the presence of virtual coupling. [c]The chemical shift of the non-reducing unit is sensitive to the anomeric configuration of the reducing moiety. [d]The following long-range coupling constants[36,37] were introduced into the simulations, because the correct, apparent line-widths for some of the resonances, in particular that for H-1 of the α anomer, could not be reproduced without them: α anomer $J_{2,4} = J_{3,5} = 1$, $J_{1,3}$ 0.5, $J_{2,5}$ 0.9 Hz: β anomer $J_{1,3} = J_{2,4} = J_{3,5} = 1$, $J_{2,5}$ 0.9 Hz. [e]Calculated from additivity relationships. [f]Experimentally determined.

The ^{1}H chemical shift data obtained from both the homonuclear- and heteronuclear-correlated experiments for DiGlcNAc, presented as contour plots, are shown in Fig. 1. The only ambiguity centres around the assignment for the magnetically non-equivalent geminal protons of the hydroxymethyl substituent. The assignment of these two protons is discussed below. The observed vicinal coupling constants for H-6a,6b, using both 1D n.O.e. experiments (Fig. 2) and direct measurements, are recorded in Table I and were used to analyse the fractional rotamer populations.

The calculation was performed first with the assumption that H-6a was associated with the small coupling constant and H-6b with the larger value, and second with these values reversed. The first assumption gives rise to rotamer populations of 70% gg and 30% tg, whereas the latter predicts a negative population for

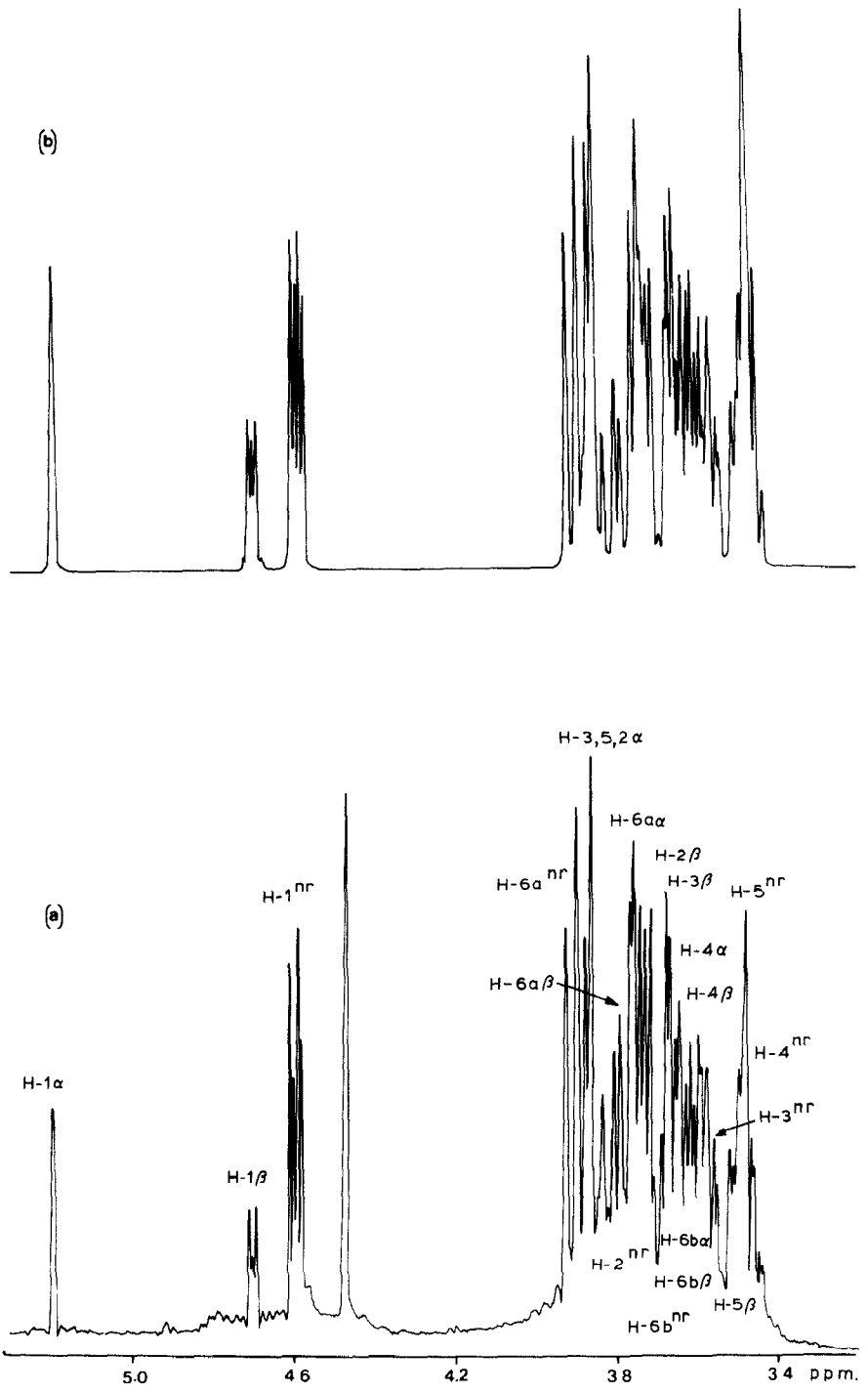

Fig. 3. ^{1}H-N.m.r. spectra of DiGlcNAc: (*a*) experimental spectrum (469 MHz, for a solution in D_2O at pH 7 and 50°), (*b*) simulated spectrum, obtained with the chemical shift values of Table I.

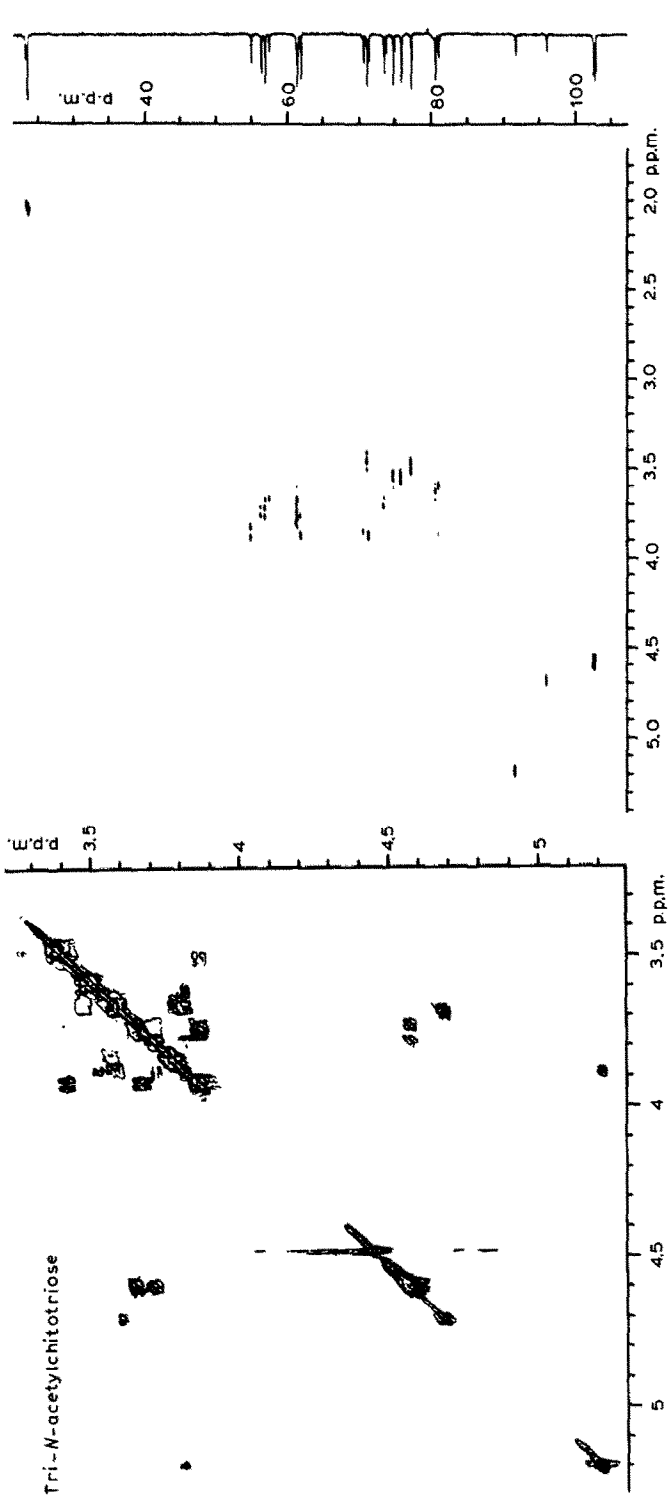

Fig. 4. 2D Homonuclear-correlated spectrum (469 MHz) and 2D heteronuclear-correlated spectrum of TriGlcNAc (270 MHz for ^{1}H and 67.9 MHz for ^{13}C) for a solution in D_2O at pH 7.0 and 50°.

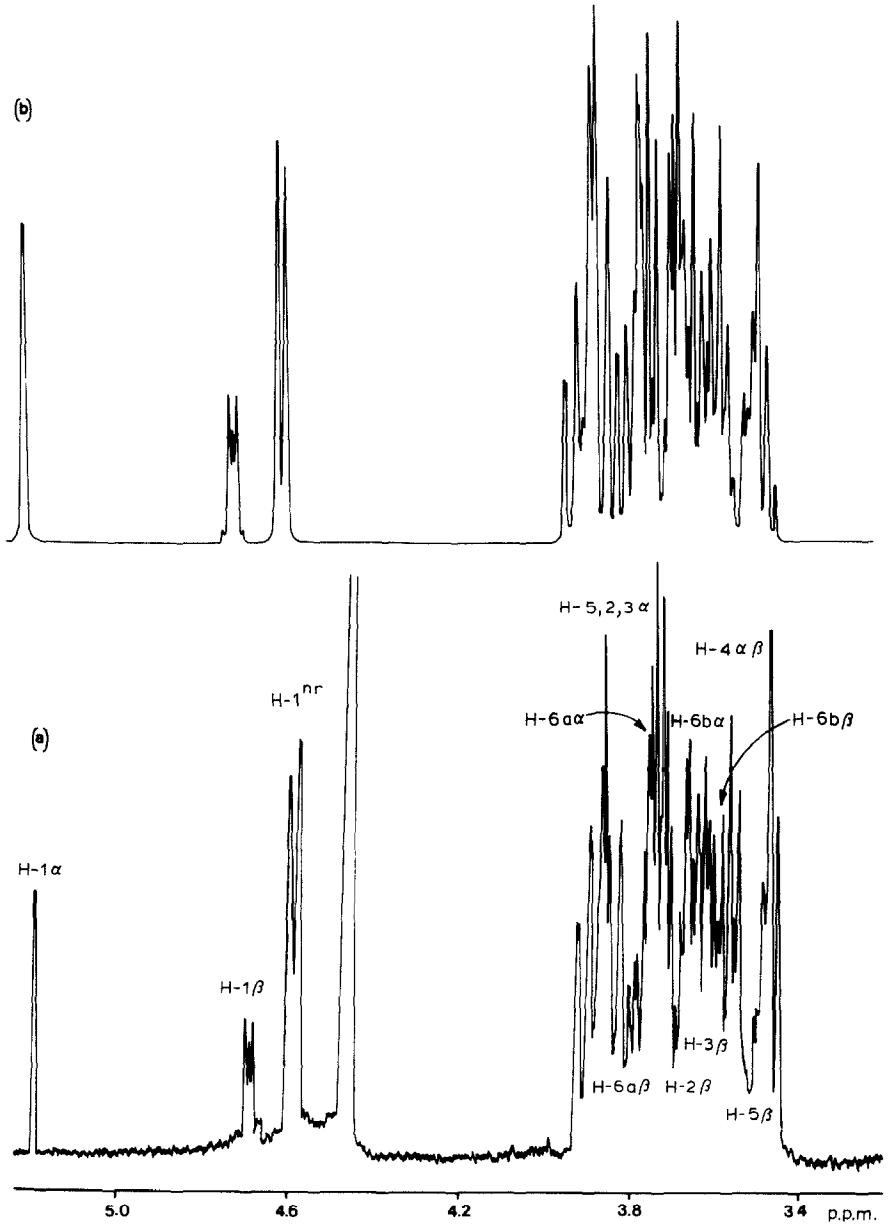

Fig. 5. ^{1}H-N.m.r. spectrum of TriGlcNAc: (*a*) experimental spectrum (469 MHz, solution in D_2O at pH 7 and 50°; only assignments of protons from α and β reducing-units are reported here for the sake of clarity; (*b*) simulated spectrum, obtained with the chemical shift values of Table II.

TABLE II

^{1}H CHEMICAL SHIFTS (p.p.m.) USED FOR THE SIMULATION OF TriGlcNAc[a]

Unit	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6a*	*H-6b*
Reducing α	5.190	3.870	3.863	3.598	3.881	3.771	3.670
Reducing β	4.700	3.680	3.667	3.590	3.498	3.820	3.647
Non-reducing unit (middle)	4.589	3.765	3.716	3.629	3.553	3.843	3.650
Non-reducing unit (terminal)	4.585	3.736	3.568	3.461	3.490	3.916	3.744

[a]The coupling constants were the same as those used for the simulation of DiGlcNAc.

^{13}C CHEMICAL SHIFTS (p.p.m.)

Unit	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
Reducing α	91.71	54.89	70.54	80.99	71.28	61.3
Reducing β	96.08	57.44	73.73	80.55	75.84	61.3
Non-reducing unit (middle)	102.51	56.31	73.44	80.55	75.75	61.43
Non-reducing unit (terminal)	102.70	56.87	74.74	71.04	77.17	61.87

the tg rotamer and hence can be eliminated. Therefore, both H-6a and H-6b can be assigned. The final chemical shifts and coupling constants used for the simulations are recorded in Table I. The simulated and experimental spectra show good agreement both with respect to intensities and chemical shifts (Fig. 3).

TriGlcNAc. — The above approach was used for the assignment of the ^{13}C spectrum of TriGlcNAc. The 2D heteronuclear-correlated experiment (Fig. 4) revealed that the ^{13}C resonance frequency for C-4 of both the β reducing moiety and the middle unit were identical. However, the assignment for the corresponding protons was made by comparing the relative intensities of the peaks in the cross section at the frequency of the C-4 resonance. This was possible because the proportion of the β anomer in the reducing unit is <40% for all the saccharides discussed here[25]. The same procedure was used to distinguish the chemical shifts of the signals for C-5 for the β reducing unit and middle unit. Assignment of C-6 for the terminal non-reducing unit was straightforward, but the resonances of the other three carbon atoms overlapped. Assignment of their corresponding protons H-6a and H-6b was made from the homonuclear-correlated experiment. The proposed assignments for both ^{1}H and ^{13}C are recorded in Table II. The simulated proton spectra based on these assignments show quite good agreement with experiment, both with respect to chemical shifts and intensities (Fig. 5).

DISCUSSION

The solution data reported for GlcNAc are consistent with the $^4C_1$(D) confor-

mation for both anomers as previously[25] found. Two X-ray analyses[28,29] for α-GlcNAc reported similar chair conformations. From the X-ray data, the hydroxymethyl group was found to have the tg[28] or gg[29] conformation. Energy calculations for α-GlcNAc[30] propose the tg conformer as the energetically most stable rotamer because of the possibility for intramolecular hydrogen-bonding between O-4 and O-6. However, it has been concluded from optical rotation studies[31] that the gt rotamer would preponderate in aqueous solutions by analogy with that found for the majority of crystal structures of other saccharides[32,33].

Interpretation of the coupling constant data for the hydroxymethyl group in terms of rotamer populations, using the method described above, shows that the equilibrium in aqueous solution favours the gg conformation (75%) over the tg conformation, with the gt conformation existing to a negligible extent. However, the n.m.r. data cannot distinguish between an equilibrium of rotamers or a single rigid conformation of a definite dihedral angle different from ±60° or 180°; in this analysis, two assumptions are made, namely, that interconversion between these rotamers is fast on the n.m.r. time-scale and that gg, gt, and tg are the only stable rotamer conformations. We have chosen to interpret the data in terms of an equilibrium, because the anticipated barrier height to rotation[30] of ~3 kcal.mol^{-1} is not large in comparison with the thermal energy, kT.

Analysis of coupling constants of the hydroxymethyl group of DiGlcNAc indicates that no significant change is observed on going from the monomer to the dimer. The X-ray data obtained for DiGlcNAc[34] indicated that the gg rotamer was preponderant for both units. Our solution data indicate a ratio of 70% gg and 30% tg in dynamic equilibrium, and suggest that the preponderating rotamer corresponds to that existing in the crystal. The observed coupling constants for the remaining protons are consistent with the $^4C_1$(D) conformation for both the rings, as expected.

The conformational energy calculations for the DiGlcNAc[30] have distinguished two stable conformers (3 and 4 of ref. 30) which have left- and right-handed helical twists, respectively. These conformations, which are close to the energy minimum, derive much of their stability from hydrogen bonds. However, each conformer is stabilised by a different hydrogen-bonding scheme. Both involve hydrogen bonding between the acetamido carbonyl group of the non-reducing unit as acceptor and the hydroxymethyl group as donor. In conformer 3, the hydroxymethyl group of the reducing unit adopts the gg rotamer, whereas in conformer 4 it is the tg rotamer. With our data, we cannot determine whether most of the molecules exist in the energy well that corresponds to conformer 3 in aqueous solution or whether, at any given time, a substantial population exists in another energy well, conformer 4.

A comparison of the proton assignments for DiGlcNAc with the respective monomers (Table I) indicates significant shifts, both upfield and downfield. All of the downfield shifts can be accounted for by the effect of the glycosidic linkage[35]. The hydroxymethyl protons of the non-reducing unit showed very small downfield

shifts. This was not the case for the corresponding protons of the reducing moieties. In this instance, the shifts were upfield, indicating that, on average, these protons are in close proximity to the acetamido side-chain of the non-reducing unit and experience the magnetic anisotropy of the carbonyl group.

The interpretation of the ^{1}H-n.m.r. spectrum of TriGlcNAc is difficult because of severe overlapping of resonances. However, there is reasonably good agreement between the simulated and the experimental spectra (Fig. 5). As the coupling constants used in this simulation were identical with those used for DiGlcNAc, the close agreement between the spectra suggests that the conformations of the residues in TriGlcNAc are similar to those in DiGlcNAc. This is reasonable, as the central residue can assume an orientation similar to that of the non-reducing unit of DiGlcNAc. Similarly, the orientation of the terminal residue can be expected to maximise intra-residue interactions with the middle residue.

ACKNOWLEDGMENTS

We thank Dr. C. M. Dobson for his interest and encouragement, and Dr. F. M. Poulsen (Carlsberg Laboratories, Denmark) for gifts of DiGlcNAc and TriGlcNAc. One of us (M.D.) thanks The Royal Society and the C.N.R.S. for financial support.

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