A detailed ^{1}H and ^{13}C NMR study of a repeating disaccharide of hyaluronan: the effects of temperature and counterion type

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ABSTRACT

For the first time, a detailed NMR study of the conformation of methyl 2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-β-D-glucopyranoside (disaccharide 1) in aqueous solution is reported. This disaccharide is a repeating unit of hyaluronan, a polysaccharide with widespread biological and pharmaceutical applications. Relatively small changes in temperature, over typical experimental conditions (0–37°C), completely change the appearance of its one-dimensional ^{1}H NMR spectrum at 500 MHz. To determine the underlying cause for this temperature sensitivity, we analyzed ^{1}H and ^{13}C chemical shifts, temperature coefficients ($\Delta\delta/\Delta T$), ^{1}H–^{1}H coupling constants, and interglycosidic ^{1}H–^{13}C coupling constants for **1** as a function of temperature. For comparison, we measured the temperature dependence of ^{1}H chemical shifts and coupling constants for related monosaccharides: glucuronate (GlcUA or U) and *N*-acetylglucosamine (GlcNAc or N), and glucose (Glc). The temperature sensitivity of the ^{1}H spectrum of **1** is caused by relatively larger values of $\Delta\delta/\Delta T$ for some ring protons, rather than a conformational change. The effect is mediated by strong coupling. To detect the presence of long-lived intramolecular hydrogen bonds in the disaccharide, we measured chemical shifts, $\Delta\delta/\Delta T$, and coupling constants for hydroxyl protons of **1**, GlcUA, and GlcNAc in 1:1 H$_2$O–acetone-d_6 at low temperature. We compared ^{1}H NMR parameters for **1**, GlcUA, and GlcNAc in water with published values measured in Me$_2$SO-d_6 and concluded that interactions with water predominated. We found no evidence for long-lived intramolecular hydrogen bonds occurring in **1** in aqueous solution.

INTRODUCTION

Hyaluronan (HA) is a negatively charged polysaccharide found in every mammalian tissue. It plays an important role in processes as diverse as joint lubrication and brain development[1,2]. The conformations of polymeric and lower molecular weight units of HA have been investigated most widely in the solid state[3,4] and in Me$_2$SO (refs 5–7). Its conformation in aqueous solution is still an open question, although models based on mesaurements in Me$_2$SO and Me$_2$SO–water mixtures have been suggested[5,6]. HA is composed of a repeating unit of 2-acetamido-2-de-

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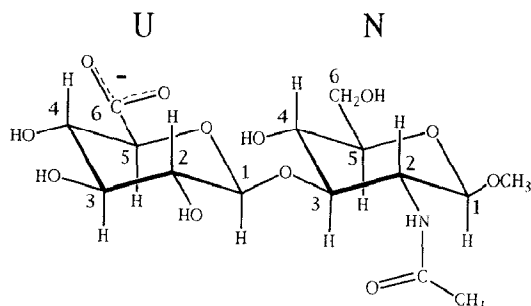
Fig. 1. Methyl 2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-β-D-glucopyranoside (1) with the numbering scheme used.

oxy-3-*O*-(β-D-glucopyranosyluronic acid)-β-D-glucopyranoside (**1**) shown in Fig. 1 *. Fig. 2 shows one proposed secondary structure of sodium HA in Me_2SO. This structure is characterized by four different hydrogen bonds per trisaccharide unit (adapted from ref 5). According to Heatley and Scott[6], if a small amount of water is added to oligomeric HA in Me_2SO, the direct hydrogen bond between the amide proton and the carboxyl group is replaced by a single water molecule bridging both groups. Welti and co-workers[8] investigated low molecular weight sodium HA, chondroitin sulfate, and their constituent monosaccharides in neutral and alkaline (0.4 N NaOD) solutions at 30 and 80°C. They proposed a structure similar to the one shown in Fig. 2 for HA based on a comparison of chemical shifts and vicinal coupling constants of ring protons.

The structure in Fig. 2 was shown by Atkins and co-workers[9] to be compatible with simple molecular model building. However, various interpretations of X-ray data have led authors to propose that two to four of these hydrogen bonds exist in HA in dried films[3,4].

To determine if the amide proton of HA was involved in a hydrogen bond in aqueous solution, Cowman and co-workers[10] measured vicinal coupling constants for the amide protons of low molecular weight (four to twenty disaccharide repeating units) sodium HA in water. The measured $^3J_{HNCH}$ values, all around 9.3 Hz, were independent of chain length, subunit position, or solution pH. This large value is consistent with a *trans* orientation of the amide proton relative to the H-2 proton. This is strong evidence against the existence in water of a stable hydrogen bond between the amide proton and the carboxylate oxygen of the adjacent uronic acid subunit. As previously mentioned, Heatley and Scott[6] modified their original model for HA to take the *trans* orientation into account. They suggested that, as the amount of water increased in a solution of HA, the equilibrium between the

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* Abbreviations used: GlcUA or U = β-D-glucopyranosyluronic acid subunit of **1**, or the α or β anomer of D-glucuronic acid (monosaccharide); GlcNAc or N = methyl 2-acetamido-2-deoxy-β-D-gluco-pyranoside subunit of **1**, or the α or β anomer of *N*-acetylglucosamine (monosaccharide); Glc = α or β anomer of D-glucose.
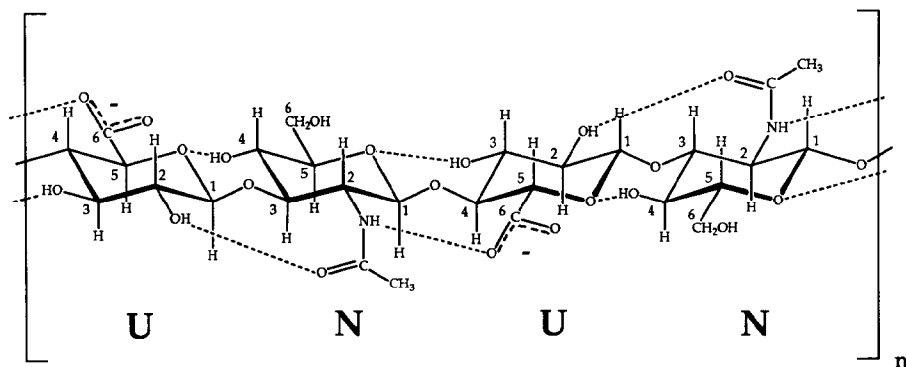
Fig. 2. Secondary structure of HA proposed by several researchers (adapted from ref 5). Proposed intramolecular hydrogen bonds are indicated by dotted lines.

amide proton hydrogen bonding to the carboxyl group and to water molecules would shift to favor solvent–solute hydrogen bonds.

Most recently, Cowman and co-workers[11] compared ^{13}C NMR spectra for sodium HA in the solid state and in aqueous solution (0.15 M NaCl, phosphate buffered to pH 7.1). They attributed the dramatic changes in chemical shift to reorientation of the acetamido group, possibly accompanied by rotation about the glycosidic linkages.

Thus far, then, it appears that the conformation of HA in aqueous solution may differ from the conformation in the solid state or in organic solvents. It is well known[12] that molecules usually crystallize in the most stable conformation, which is often similar to the one found in an organic solvent or the one obtained from energy minimization calculations, which approximate the molecule's conformation in vacuo. However, the conformation in a polar, hydrogen bonding solvent, such as water, may be significantly different. For example, the driving forces for the formation of intramolecular hydrogen bonds will be greatly reduced if water is readily available for hydrogen bonding. The special characteristics of water as a solvent, as well as its physiological relevance, make it important to determine the conformation of biological molecules such as HA in water.

The interactions of carbohydrates and water have been studied extensively because aqueous solutions of small saccharides seldom conform to the behavior predicted by theory[12]. For example, after nearly thirty years of debate, it is still undecided whether the ratio of $\alpha : \beta$ anomers in aqueous solution is determined by an "anomeric" effect or by differential solvation[13]. New NMR techniques[14–17] allow insight into the molecular details of saccharide solvation and greater understanding of the role of hydration in determining saccharide conformation.

Here we present ^{1}H and ^{13}C NMR parameters in water for mono- and di-saccharides related to HA. Our motivation is to provide a firm base for interpretation of spectra of longer HA repeating units in aqueous solution and to emphasize the importance of the solvent in determining conformation.

The specific questions addressed in this report are the effect of temperature and counterion type on the conformation of one of the possible disaccharide repeating units from HA, the methyl β-D-glycoside of β-GlcUA-β-(1 → 3)-GlcNAc. To explore the effect of the β-(U-1, N-3) linkage, analogous measurements were made on the constituent monosaccharides, glucuronate (sodium, potassium, or lithium salts) and N-acetylglucosamine. This study provides, for the first time, complete assignment of ^{1}H signals (including exchangeable protons) for the di- and the mono-saccharides in water, and ^{13}C assignments for the disaccharide.

^{1}H assignments were made from one- and two-dimensional spectra obtained at temperatures from −30 to 37°C. Accurate chemical shifts and coupling constants were verified by simulation of the extremely crowded ^{1}H NMR spectra. Analysis of vicinal coupling constants ($^3J_{HH}$) indicated that both the U and N residues of **1** have the $^4C_1$ conformation. The temperature independence of the $^3J_{H,H}$ values and long range ^{1}H–^{13}C coupling constants strongly suggests that the conformation of **1** is constant over the physiologically relevant temperature range.

While the overall conformation of **1** and its monosaccharides are insensitive to temperature and counterion type, some nonexchangeable proton chemical shifts are more temperature-sensitive than others. The total change in chemical shift for ring protons ranges from 0 to 0.0316 ppm when the temperature rises from 0 to 37°C. The ring protons most sensitive to temperature are at positions N-3, N-2, U-4 and N-1.

Our results suggest that the temperature dependence of the chemical shifts results primarily from solvent–solute interactions rather than from a change in conformation or from interresidue interactions. To explore this possibility, we measured chemical shift and $^3J_{HOCH}$ for hydroxyl protons of these samples in 1:1 mixtures of H_2O–acetone-d_6 at temperatures between −30 and 0°C. The hydroxyl protons at positions U-3 and U-4 show anomalous behavior, which can be explained on the basis of solvent–solute interactions. The lack of concentration dependence of hydroxyl proton NMR parameters supports this explanation.

Glucuronate and N-acetylglucosamine are of interest in their own right because they occur widely in nature. For example, GlcNAc commonly occurs as a constituent of glycopeptides[18], and GlcUA has been implicated in cell–cell adhesion[19]. The ^{1}H NMR spectra for these simple sugars are difficult to assign, not just because many resonances are overlapping, but also because several of the chemical shifts are very sensitive to temperature. Virtual coupling among several protons further complicates analysis[20]. Therefore, the results we present here will be of interest, not only just to those interested in HA, but also to anyone studying glycosaminoglycans and glycopeptides by ^{1}H NMR spectroscopy.

^{1}H chemical shifts at 300 MHz for the methyl α-D-glycoside analogue of **1** in Me$_2$SO-d_6 at 22°C have been published by Heatley and co-workers[21]. The order of assigned shifts for the ring protons differs from that listed in our Table I. The chemical shifts reported for hydroxyl protons for the methyl α-D-glycoside in Me$_2$SO-d_6 are strikingly different from those we have found in H_2O–acetone-d_6

mixtures. These differences are discussed in the Discussion Section under "Hydroxyl protons".

EXPERIMENTAL

Reagents. — Hyaluronan (human umbilical cord) was purchased as the K^+ salt from ICN Biochemicals Co. (Cleveland, OH). *N*-Acetyl-α-D-glucosamine (99%) and D-glucuronic acid (98%) were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). D-Glucose (mixture of anomers) was obtained from Sigma Chemical Co. (St. Louis, MO). The water used in all procedures was distilled and then passed through a Milli-Q apparatus (Waters Associates, Milford, MA).

Column chromatography. — Analytical grade anion-exchange resin AG1-X4 (200–400 mesh, Cl^- form) and Bio-Gel P-2 gel (fine, 65 ± 20 μM wet) were purchased from Bio-Rad Laboratories (Richmond, CA). Dowex-50X8-100 ion-exchange resin was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). AG1-X4 resin was used without any further purification. Dowex-50X8-100, purchased in the H^+ form, was cleaned by conversion to the Na^+ form and then converted back to the H^+ form by sequential application of 1 N NaCl, 1 N NaOH, and 2 N HCl solutions. In a typical procedure, 10 g of resin, swollen after 4 h in water, was shaken for 1 h in 75 mL of the appropriate reagent solution and then rinsed repeatedly with water (about 200 mL) until the pH was neutral (for the H^+ form) or until a test for Cl^- (5% solution of $AgNO_3$) was negative (for the Na^+ form).

Analytical methods. — The carbazole reaction[22] was used to quantify uronic acid. The colorimetric method using 3,6-dintrophthalic acid[23] was used to verify that 1 had virtually no reducing power and was therefore pure methyl β-D-glucopyranoside. Depolymerization of HA was monitored by thin-layer chromatography (TLC) performed on cellulose plates developed with a 2:1:1 1-butanol–acetic acid–water mixture or on silicic acid plates developed with a 2:1 acetone–MeOH mixture. Cellulose plates were stained with 0.3% *p*-aminohippuric acid and 3% phthalic acid in EtOH[24]. Ninhydrin spray reagent (0.2% ninhydrin in EtOH) was applied to cellulose plates to monitor stability during solvolysis of the acetamido group. Silicic acid plates were stained with iodine vapor.

Preparation of 1. — The K^+ salt of HA was converted to the free acid form by passing a solution of the salt (250 mg in 400 mL water) over a 1×12-cm column of Dowex-50X8-100 (H^+) cation-exchange resin (10 g) at a flow rate of 0.6 mL/min/cm^2. The acidic effluent was lyophilized and then dried under vacuum over P_2O_5. This preparation was used as the starting material for preparation of 1, by a modified version of the method described by Inoue and Nagasawa[25]. The free acid form of HA (193 mg) was dissolved in dry Me_2SO (87.5 mL), and then a solution of pyridine–sulfur trioxide (80 mg) in dry MeOH (9 mL) was added. After this mixture had been kept under dry N_2 for 18 h at 95 °C, it was cooled, diluted with an equal volume of water, and its pH adjusted from 3.2 to 6.8 by the addition

of 0.5 N NaOH. The solution was carefully concentrated under reduced pressure at less than 40°C to a volume of ∼ 3 mL, and then diluted with an equal volume of water. The concentrated solution was then applied to a 1 × 47-cm column of AG1-X4 (Cl⁻, 200–400 mesh) anion-exchange resin (50 g) and eluted with a linear gradient of 0 → 0.2 M LiCl (900 mL) at a flow rate of 0.9 mL/min. Fractions (6.3 mL each) were collected and each analyzed for uronic acid content. Those corresponding to the disaccharide were pooled, lyophilized, and desalted by passage over a 1.5 × 45-cm column of Bio-Gel P-2 (200–400 mesh), operating at a flow rate of 0.38 mL/min. The salt-free fractions were collected, pooled, lyophilized, and rechromatographed on an anion-exchange column with a linear gradient of 0 → 0.14 M LiCl (1600 mL) applied at a flow rate of 0.74 mL/min. Each fraction was checked for uronic acid content by the carbazole assay. None of the peaks corresponding to **1** gave a positive ninhydrin reaction, nor did any show detectable reducing power. A ¹H NMR spectrum revealed that the pooled fractions contained > 98% pure β anomer. The pooled fractions were desalted a second time by the method described above. The total yield was 12 mg (7%) of **1** isolated as the Li⁺ salt.

The Na⁺ salt of **1** was obtained from the Li⁺ salt in two steps. First, the Li⁺ salt was converted to the free acid by passage over a Dowex-50X8-100 (H⁺) column. Pooled fractions were lyophilized and then converted to the Na⁺ salt by titration to pH 5.46 with a 3 mM solution of NaOH. This end point was chosen because it is the pH of the same concentration of the K⁺ salt of polymeric HA at the same uronic acid concentration as the solution of **1**.

Preparation of salts of D-glucuronic acid. — D-Glucuronic acid was converted to its Li⁺ and K⁺ salts by titration with a solution of the respective hydroxide. For KOH, a standard solution (0.105 N) was diluted to 5 mM and used as the titrant. For LiOH, a 5 mM solution was prepared from the solid salt under N_2 and degassed with N_2. Titrations took ∼ 1 h at room temperature. Under these conditions, D-glucuronic acid is stable and virtually free from the lactone. Imai and Hirasaka[26] reported that only 1% of D-glucuronic acid is transformed into lactone after 800 min at 25°C.

NMR spectra. — The Li⁺ and Na⁺ salts of **1** were measured in either D_2O or 9:1 H_2O–D_2O mixtures as 18 mM solutions, and in 1:1 H_2O–acetone-d_6 mixtures as 25 mM solutions (except where otherwise noted). Concentrations of all monosaccharides measured were 20 mM in D_2O, and 25 mM in 1:1 H_2O–acetone-d_6 mixtures. The solutions were prepared under N_2. For the purpose of observing hydroxyl protons, samples in H_2O–acetone-d_6 mixtures were prepared according to the protocol described in ref. 27, to minimize catalysis of hydroxyl proton exchange by contaminants on glass. The pH values of samples used were pH 6.5–7.0 (measured by a glass electrode, uncorrected for deuterium).

Acetone (1.5 mM) was used as an internal chemical shift standard for the monosaccharides dissolved in D_2O. In a separate experiment, the chemical shift of acetone was found to be 2.217 ppm relative to DSS [sodium 3-(trimethylsilyl)-1-

propane sulfonate]. This value was constant over the range of temperatures used in this study. For 1 in H_2O or D_2O solutions, ^1H chemical shifts were referenced to the methyl protons of the acetamido group at 2.011 ppm. Over the range of temperatures used in this study, the chemical shift of the methyl group is consistently 0.206 ppm upfield of the acetone signal. All ^1H chemical shifts reported in Table I and Table IV were recalculated relative to DSS. For 1 dissolved in mixtures of H_2O–acetone-d_6, ^1H chemical shifts were referenced to residual acetone or to the methyl protons of the acetamido group. In a separate experiment, the chemical shifts of residual acetone protons and methyl protons of the acetamido group were found to be 2.19 and 2.04 ppm, respectively, relative to DSS. Over the range of temperatures used in measurements in H_2O–acetone-d_6 mixtures, these chemical shifts were constant.

All NMR spectra, except one-dimensional ^{13}C spectra, were obtained at 499.843 MHz on a Varian UNITY spectrometer. ^{13}C spectra of 1 were obtained on a Bruker 500 MHz (^{13}C 125.38 MHz) spectrometer at 4 and 37°C. The ^{13}C chemical shifts reported in Table VI are referenced to the methyl carbon of the acetamido group set to 23.2 ppm[28]. ^{13}C assignments for 1 were made by comparison with chemical shifts reported for N-acetylhyalobiuronic acid and the K^+ salt of polymeric HA[25,29]. Our assignments were verified with ^1H–^{13}C HMQC spectroscopy[30].

Nonexchangeable proton assignments were made based on two-dimensional DQF-COSY[31] and NOESY[32] spectra, using the conventional pulse sequences supplied by Varian Associates (Palo Alto, CA). Coupling constants and exact chemical shifts for ^1H were verified by simulation of the one-dimensional spectra, using the simulation package supplied with the Varian VNMR software. The stereospecific assignment of H-6 and H-6' for the GlcNAc subunit or monosaccharides was made by analogy with the assignments published by Nishida and co-workers[33], which were accomplished by selective deuteration. Hydroxyl proton assignments were made based on two-dimensional HOHAHA (TOCSY) spectroscopy[34,35] with solvent suppression[36], which revealed crosspeaks with their nonexchangeable coupling partners. Integlycosidic $^3J_{HCOC}$ values were measured two ways. Firstly, $^3J_{HCOC}$ values were measured by integrating crosspeaks in HMBC spectra[37] obtained at 4 and 37°C (data not shown). The crosspeak volumes were normalized by dividing by the volume of an equal area of noise in the spectrum. Secondly, we used a new one-dimensional method based on polarization transfer from carbon to proton, with proton detection[38]. It was necessary to use a sequence based on selective excitation of carbon-13 nuclei, because the ^1H spectrum is too crowded to allow selective excitation of the protons involved. Further details are given in the figure captions.

Temperature studies. — One-dimensional ^1H NMR spectra of 1 in D_2O were obtained in one loop for the Li^+ salt (at 21, 15, 14, 0, 37, 29, 27, and 21°C) and in two loops for the Na^+ salt (at 27, 15, 40, 37, and 27°C; then at 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 37, and 15°C). One-dimensional ^1H NMR spectra of 1 in 1:1 H_2O–acetone-d_6 were obtained in one loop for the Na^+ salt (at 0, −12, −25, and 0°C). For the monosaccharide GlcNAc in 2:1 H_2O–acetone-d_5, one-dimensional

^{1}H NMR spectra were obtained in one loop (at -16, -20, and $-16°C$). Typically, experiments were spaced at least 30 min apart to allow equilibration at the new temperature.

Concentration studies. — Measurements of the concentration dependence of chemical shifts of **1** were obtained in two series of experiments. In D_2O, ^{1}H NMR spectra of 20 and 1 mM samples were obtained at 0, 6, and $37°C$. In 1:1 H_2O–acetone-d_6, ^{1}H NMR spectra of 25 and 2 mM samples were obtained at $-30°C$.

RESULTS

The chemical shifts at 0 and 37°C for **1** and the monosaccharides GlcUA, GlcNAc, and Glc are listed in Table I. Scalar [1]H–[1]H coupling constants for these compounds are listed in Table II. Chemical shifts at other temperatures are available upon request. Table III lists changes of ring ^{1}H chemical shifts $(\Delta\delta)$ with temperature for **1** and the monosaccharides. Data for Glc are included in Tables I, II, and III to allow assessment of the effect of acetamido and carboxyl substituents. Tables IV and V list the chemical shifts and vicinal coupling constants, respectively, for hydroxyl protons in the disaccharide and monosaccharides at -30 or $-24°C$ in 1:1 H_2O–acetone-d_6. The temperature coefficients of the chemical shifts $(\Delta\delta/\Delta T)$ are added in parentheses. Table VI lists the ^{13}C chemical shifts for **1** at 4 and 37°C. Figs. 3 and 4 show two regions of the ^{1}H spectrum of **1** as a function of temperature, comparing experimental with simulated spectra. Fig. 5 shows interglycosidic coupling constants mesured by selective excitation of [13]C. Fig. 6 shows the ^{1}H NMR spectrum of hydroxyl protons of **1**, obtained at $-25°C$ on a 2 mM sample dissolved in 1:1 H_2O–acetone-d_6.

DISCUSSION

The spectral regions shown in Figs. 3 and 4 demonstrate how dramatically the one-dimensional ^{1}H spectrum of **1** changes with temperature, especially in the typical experimental range of 20 to 37°C. For example, over the relatively narrow temperature range of 27 to 37 °C, the anomeric protons U H-1 and N H-1 overlap and then separate. Inspection of Table III shows that the greatest changes are observed for protons at positions 3, 2, and 1 on the GlcNAc subunit (N H-3, N H-2, N H-1) and at positions 4 and 3 on the GlcUA subunit (U H-4, U H-3). Fig. 4 illustrates the influence of virtual coupling on the shape of the multiplet signal from the proton at position U-2. Previously, Brisson and Carver[20] suggested that the ^{1}H spectrum of a chitobioside derivative was extremely sensitive to temperature because of virtual coupling, rather than from large conformational changes. Likewise, the example presented in Fig. 4 emphasizes the need for spectral simulation to disentangle virtual coupling from other effects.

TABLE I

¹H chemical shifts (ppm from DSS) ᵃ

Compound	Subunit or anomer	Temperature (°C)	H-1	H-2	H-3	H-4	H-5	H-6	H-6'	CH₃C(O)NH
1, Li⁺ or Na⁺ salt ᵇ	β-GlcUA	0	4.4690	3.3264	3.4777	3.4867	3.7728			
		37	4.4610	3.3320	3.4866	3.5108	3.7150			
	β-GlcNAc	0	4.4473	3.8463	3.7032	3.5275	3.4680	3.9285	3.7722	2.011
		37	4.4702	3.8188	3.7348	3.5152	3.4697	3.9260	3.7604	2.011
GlcUA, Li⁺ or Na⁺ salt ᵇ	α	0	5.2230	3.5588	3.7080	3.4688	4.0768			
		37	5.2255	3.5589	3.7138	3.4890	4.0660			
	β	0	4.627	3.2677	3.4930	3.4830	3.7190			
		37	4.6246	3.2690	3.4890	3.5090	3.7080			
GlcNAc	α	0	5.185	3.861	3.756	3.475	3.849	3.832	3.784	2.037
		37	5.192	3.8645	3.750	3.477	3.8465	3.836	3.773	2.039
	β	0	4.6943	3.6797	3.507	3.442	3.454	3.8992	3.734	2.035
		37	4.702	3.6567	3.5275	3.445	3.453	3.894	3.734	2.037
Glc ᶜ	α	0	5.215	3.515	3.695	3.391	overlap	overlap	3.754	
		37	5.216	3.519	~3.702	3.3964	~3.82	~3.83	3.744	
	β	0	4.6327	3.225	~3.468	3.378	~3.454	3.887	3.706	
		37	4.6285	3.230	~3.475	3.388	~3.452	3.881	3.706	

ᵃ All chemical shifts were measured either by setting the methyl protons of the acetamido group to 2.011 ppm relative to DSS (for **1**) or by setting added acetone (1.5 mM) to 2.217 ppm relative to DSS (monosaccharides). Chemical shifts (except for D-glucose) were verified by simulation of spectra with an average precision of ± 0.0004 ppm. ᵇ Spectra of lithium and sodium salts were virtually identical at the same temperature. ᶜ For D-glucose, chemical shifts were assigned by comparison with published values[39] and also taking into account the ratio of α : β anomers. Resonances from H-5α and H-6α were too overlapped to resolve.

TABLE II

$J_{H,H}$ values (Hz) [a]

Compound	Subunit or anomer	Temperature (°C)	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$	$J_{5,6'}$	$J_{6,6'}$	$J_{NH,H2}$
1, Li⁺ or Na⁺ salt [b]	β-GlcUA	0	7.9	9.5	9.0	9.8				
		37	7.7	9.2	9.0	9.5				
	β-GlcNAc	0	8.7	10.2	8.8	9.9	2.2	5.4	−12.5	9.6 [d]
		37	8.4	10.2	8.4	9.8	2.2	5.4	−12.5	9.7 [c]
GlcUA Na⁺, Li⁺, or K⁺ salt [c]	α	0	3.8	9.7	9.5	10.2				
		37	3.8	9.7	8.8	10.0				
	β	0	8.0	9.7	9.5	9.0				
		37	8.0	9.0	9.1	9.8				
GlcNAc	α	0	3.5	10.8	9.0	9.5	2.0	5.6	−12.0	
		37	3.6	11.0	9.0	9.6	2.5	5.0	−11.5	
	β	0	8.5	10.5	9.6	9.5	1.6	5.5	−12.0	
		37	8.4	10.2	9.0	9.5	1.3	5.0	−11.5	
Glc	α	0	3.7	9.8	9.4	~9.4	~1.7	5.7	−12.6	
		37	3.8	9.8	~9.4	~10.0	1.7	5.5	−12.2	
	β	0	8.0	9.0	9.4	~9.2	~2 Hz	5.5	−12.5	
		37	8.0	9.0	9.4	9.6	2.1	5.7	−12.4	

[a] Values of J were verified by simulation of spectra (except those for D-glucose). Simulation provided a precision of ±0.5 Hz. [b] Spectra of the sodium and lithium salts of 1 had the same values of J. [c] Spectra of the sodium, lithium, and potassium salts of D-glucuronate had the same values of J. [d] Measured at 4°C in 9:1 H₂O–D₂O. [c] Measured at 27°C in 9:1 H₂O–D₂O.

TABLE III

Change in ^{1}H chemical shift $(\Delta\delta)$ [a] at 500 MHz for the nonexchangeable protons of 1 and related monomers when the temperature increases from 0 to 37°C

Compound	Subunit or anomer	H-1	H-2	H-3	H-4	H-5	H-6	H-6'
1, Na$^+$ salt	β-GlcUA	− 0.0080	+ 0.0056	+ 0.0089	+ 0.0241	− 0.0078		
	β-GlcNAc	+ 0.0229	− 0.0275	+ 0.0316	− 0.0123	+ 0.0017	− 0.0025	− 0.0118
GlcUA, K$^+$ salt	α	+ 0.025	0.0001	+ 0.0058	+ 0.0202	− 0.0108		
	β	− 0.0024	+ 0.0013	− 0.0040	+ 0.0260	− 0.011		
GlcNAc	α	+ 0.007	+ 0.0035	− 0.006	+ 0.002	− 0.0025	+ 0.004	− 0.011
	β	+ 0.0077	− 0.023	+ 0.0205	+ 0.003	− 0.001	− 0.0052	0.0
Glc	α	+ 0.001	+ 0.004	+ 0.007	+ 0.0054	n.m. [b]	n.m. [b]	− 0.010
	β	− 0.0042	+ 0.005	+ 0.007	+ 0.0010	− 0.002	− 0.006	0.0

[a] Chemical shifts were verified by simulation of 1D spectra (except for D-glucose) and have a precision of ±0.0004 ppm. Signs indicate direction of $\Delta\delta$: upfield (−) and downfield (+). [b] n.m. = not measurable. For D-glucose, the H-5α and H-6α resonances were too overlapped to allow assignment at 0°C.

Our assignments and J values for α- and β-D-glucose and α- and β-D-GlcNAc are in excellent agreement with those published by Perkins and co-workers[39], with a single exception. We report $J_{H4,H5}$ for β-GlcNAc to be 9.5 Hz, compared to 8.1 Hz reported by Perkins and co-workers.

TABLE IV

Chemical shifts and $\Delta\delta/\Delta T$ (in parentheses) of exchangeable protons of 1 and related monomers in 1:1 H$_2$O–acetone-d_6.

Compound [a]	Subunit or anomer	OH-1	OH-2 or NH	OH-3	OH-4	OH-6
1, Na$^+$ salt	β-GlcUA		6.22 (− 0.010)	6.41 (− 0.009)	6.34 (− 0.005)	
	β-GlcNAc		8.74 (− 0.007)		5.96 (− 0.004)	6.06 (− 0.010)
GlcUA, Na$^+$ salt	α	7.21	6.02	6.21	~ 6.30 [b]	
	β	7.92	6.42	6.32	~ 6.30 [b]	
GlcNAc [c]	α	7.20 (− 0.010)	8.29 (− 0.007)	6.14 (− 0.008)	6.34 (− 0.010)	5.82 (− 0.010)
	β	7.68 (− 0.009)	8.44 (− 0.007)	6.24 (− 0.008)	6.40 (− 0.010)	5.94 (− 0.010)

[a] Samples (25 mM, except where noted below) were measured at −30°C (1) and −24°C (monomers). Chemical shifts were recalculated relative to DSS, either by assigning the residual acetone peak to 2.19 ppm or the methyl protons in the acetamido group to 2.04 ppm. The precision of the reported chemical shifts is ±0.01 ppm. Values for $\Delta\delta/\Delta T$ were measured on sample that was 2 mM in 1 changing the temperature from −25 to 0°C. All resonances shifted upfield (towards the H$_2$O resonance) with increasing temperature. [b] These values were measured for 1:1 (25 mM each) α,β-GlcNAc–α,β-GlcUA (sodium salt) at −24°C, in 1:1 H$_2$O–acetone-d_6. The resonances corresponding to GlcUA OH-4 for both α and β anomers were overlapping. [c] These values were measured for a 25 mM solution of GlcNAc in 1:1 H$_2$O–acetone-d_6, as described in footnote a. Values for $\Delta\delta/\Delta T$ were calculated from spectra obtained at −20 and −16°C.

TABLE V

Vicinal coupling constants (Hz) of exchangeable protons [a]

Compound	Subunit or anomer	$J_{H1,OH1}$	$J_{H2,OH2}$ or $J_{H2,NH2}$	$J_{H3,OH3}$	$J_{H4,OH4}$	$J_{H6,OH6}$
1, Na⁺ salt	β-GlcUA		4.8	3.0	$\Delta\nu_{1/2} = 9$ Hz [b]	
	β-GlcNAc		9.6		$\Delta\nu_{1/2} = 5$ Hz [b]	5
GlcUA, Na⁺ salt	α	4	6	5	overlapping	
	β	6	5	4	broad singlet	
GlcNAc	α	4.5	9.3	5.8	6.0	5.5
	β	6.0	9.3	5.8	5.0	5.5

[a] Samples (25 mM) were as described in footnote a to Table IV. [b] The signals for these hydroxyl protons were broadened and showed no splitting. The linewidths at half-height are indicated.

Overall, replacement of hydroxyl groups by carboxyl or *N*-acetamido groups increased the temperature sensitivity of chemical shifts (Glc vs. **1**, GlcNAc, and GlcUA, Table III). Substitution of a methyl group for a proton also increased the temperature sensitivity of the anomeric proton of β-GlcNAc subunit in **1** relative to the monomer.

Conformation of the rings. — Despite the changes in chemical shifts for many of the ring protons, the ${}^1H–{}^1H$ coupling constants do not change much with temperature ($\Delta J < 0.6$ Hz), indicating that the conformations of the sugar rings are virtually unchanged over the temperature range 0 to 37°C.

Values of ${}^3J_{HH}$ listed in Table II are close to those found by Cano and Foces-Foces[40] for model carbohydrates with fixed ${}^4C_1$ conformation. The two-dimensional NOESY spectrum of **1** at 37°C had the crosspeak intensity pattern characteristic of ${}^4C_1$ conformations for both rings (data not shown: intense crosspeaks between protons at the 1, 3, and 5 positions, and between the 2 and 4

TABLE VI

${}^{13}C$ chemical shifts for **1**, sodium salt [a]

GlcUA	Temperature		GlcNAc	Temperature	
	4°C	37°C		4°C	37°C
C-1	103.8	103.8	C-1	102.6	102.6
C-2	73.4	73.7	C-2	55.1	55.3
C-3	76.2	76.4	C-3	83.7	83.9
C-4	72.5	72.6	C-4	69.5	69.8
C-5	76.8	76.6	C-5	76.1	76.3
COO⁻	176.6	176.3	C-6	61.4	61.8
			$\underline{C}H_3C(O)NH$	23.2	23.2
			$CH_3\underline{C}(O)NH$	175.8	175.6
			$OC\underline{H}_3$	57.9	58.0

[a] Measurements were taken on a 20 mM sample dissolved in D_2O. The chemical shift of $\underline{C}H_3C(O)NH$ was set to 23.2 ppm (28). Assignments of the acetamido carbonyl carbon and carboxyl carbon are based on the assignments in ref. 29.
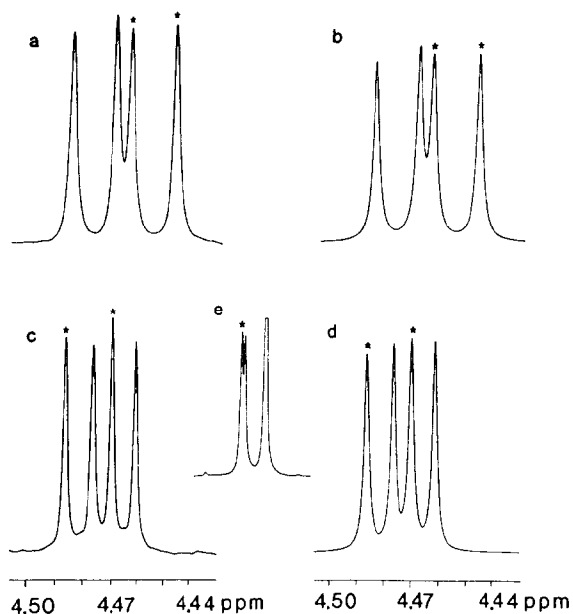
Fig. 3. Region of the 1D ^{1}H NMR spectra at 500 MHz of 1 (sodium salt) showing the anomeric protons at (a) 0°C and (c) 37°C. Simulated spectra corresponding to this region are shown in (b) and (d). The inset spectrum (e) shows the almost complete overlap of the anomeric protons at 27°C. An asterisk marks the downfield resonance of N H-1.
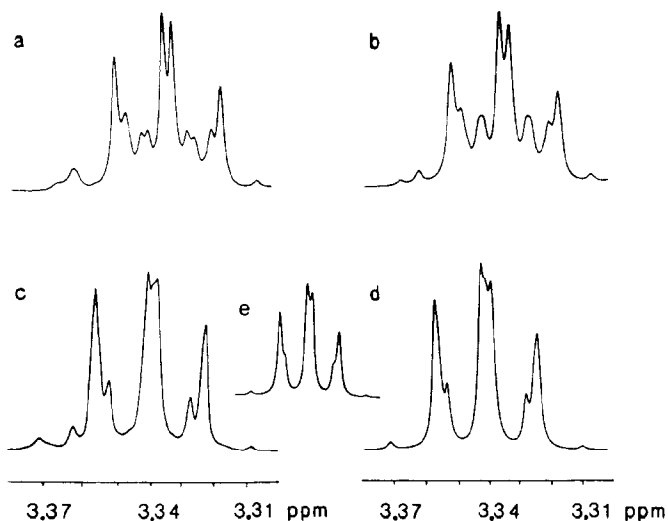

Fig. 4. Region of the one-dimensional ^{1}H NMR spectra at 500 MHz of 1 (sodium salt), showing U H-2, at (a) 0°C and (c) 37°C. Simulated spectra corresponding to this region are shown in (b) and (d). The simulated spectrum in (e) was created using the same parameters as in (d), but the chemical shifts of U H-3 and U H-4 were changed by 0.0006 ppm (upfield) and 0.0012 ppm (downfield), respectively, and $J_{H3,H4}$ was reduced by 0.5 Hz. This demonstrates how precisely simulation can determine these parameters.
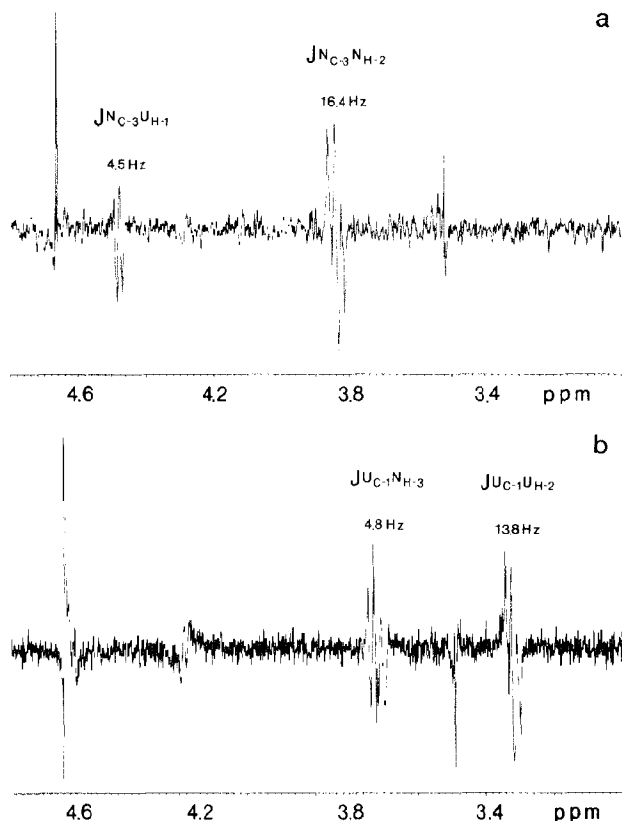
Fig. 5. One-dimensional ^{1}H-detected specta of 1 at 37°C, displaying splitting due to $^3J_{COCH}$ across the glycosidic linkage. These spectra were obtained using the pulse sequence described in ref 38. The sample was 20 mM dissolved in D_2O. The spectral window was 2000 Hz. A selective 90° pulse of 6.4 ms duration was applied to carbons at 83.9 ppm (N-3) or 103.8 ppm (U-1). The spikes at ~ 3.5 ppm and ~ 4.7 ppm arise from protons of the methoxy group and residual H_2O, respectively. (a) Coupling between the carbon at position N-3 and the proton at position U-1 observed after 4,198 transients. (b) Coupling between the carbon at position U-1 and the proton at position N-3 observed after 5,120 transients.

positions). Therefore we conclude that the rings of 1 exist in the $^4C_1$ conformation with no detectable minor conformers. This conclusion is supported by the absence of any unassigned or minor peaks in the ^{13}C spectrum of 1 (discussed below). In constrast, Lamba and co-workers[41] reported that the D-glucuronic acid moiety of chondrosine (2-amino-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-D-galactose) exists in solution as a mixture of $^4C_1$ and boat forms.

The interglycosidic linkage. — As measured with an HMBC experiment, the crosspeak volumes for (N H-3, U C-1) and (U H-1,N C-3) did not change when the temperature went from 0 to 37°C, indicating that there are no major changes in the interglycosidic torsion angles over this temperature range. This indication was confirmed by measuring $^3J_{HCOC}$ at 2°C (4.0 Hz from U C-1 to N H-3; 4.5 Hz from
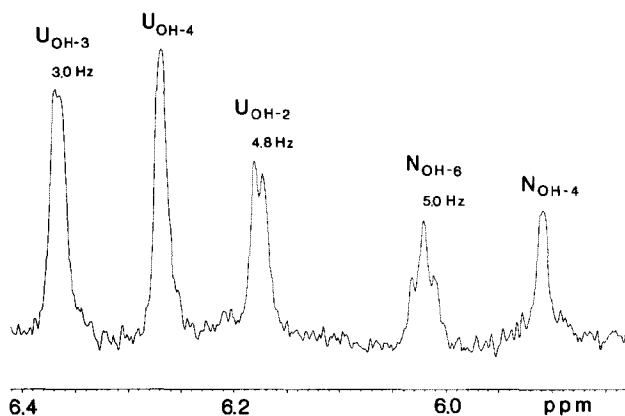
Fig. 6. Region of the one-dimensional ^{1}H NMR spectra at 500 MHz of **1**, showing hydroxyl protons between 6.4 and 5.8 ppm, at $-25°C$. The sample was 2 mM in 1:1 water–acetone-d_6. The 11-echo pulse sequence of Sklenář and Bax[36] was used to suppress the signal from H_2O. The number of transients was 512. The peaks are labelled with their assignments and peak splittings ($J_{H,OH}$).

N C-3 to U H-1) and 37°C (4.8 Hz from U C-1 to N H-3 and 4.5 Hz from N C-3 to U H-1). To the best of our knowledge, there is no complete Karplus equation available that has been customized for C–O–C–H fragments. Tvaroska and co-workers[42] suggested a relationship of the form

$$^3J_{CH} = 5.7 \cos 2\Phi - 0.6 \cos \Phi + 0.5$$

based on 17 points. Based on this equation, J values of ~4.0 to ~4.9 Hz would correspond to dihedral angles of around 30, 138, or 220°. Unfortunately, there are not enough data on model compounds for this particular glycosidic linkage to allow a more definite determination of dihedral angles. It is important to remember that the measured coupling constant may reflect averaging over several possible conformers[42]. At this point, all that we can say is that the distribution of conformers for **1** does not change significantly over the temperature range 0 to 37 ° C. Because the chemical shifts of N H-3 and U H-5 are overlapping, it is not possible to measure accurately an NOE between U H-1 and N H-3 to define the dihedral angles across the glycosidic linkage, without selective deuteration.

Orientation of the hydroxymethyl group. — The problem of relating coupling constants to rotation around the C-5–C-6 bond of D-hexopyranoses has been widely discussed but not solved conclusively. Two physical measurements, optical rotation[44] and $^3J_{HH}^{20,32,39,44-48}$ have been used to estimate the populations of conformers in model compounds. In this manuscript we will use the nomenclature of Sundaralingam[49] for the three predicted conformers: *gg* (*gauche–gauche*), *gt* (*gauche–trans*) and *tg* (*trans–gauche*). These correspond to values of -60, $+60$, and 180°, respectively, for the torsion angle defined by O-5–C-5–C-6–O-6. In this nomenclature, the torsion angle C-4–C-5–C-6–O-6 is stated second. The value of $^3J_{H6',H5}$, where H-6 refers to the upfield hydrogen of the hydroxymethyl group,

reflects the relative populations of *gg*, *gt*, and *tg*. Values reported for $^3J_{HH}$ for the hydroxymethyl group in D-glucose derivatives fall within relatively narrow ranges: 5.0–6.5 Hz for $^3J_{H6',H5}$; 1.5–2.5 Hz for $^3J_{H6,H5}$. Marchessault and Pérez[45] applied statistical analysis to crystal structures of aldohexopyranoses and concluded that for glucopyranoses only two rotamers are significantly populated, *gg* and *gt*, in a ratio of 3:2. The remarkable constancy of this ratio for over 100 different glucopyranoses is believed to result from the 1,3 diaxial (Hassel–Ottar) effect between the hydroxyl group at position 4 and the exocyclic hydroxymethyl group at position 6 (ref 50). Assuming that the same two rotamers are favored in solution, Brisson and Carver[46] predicted that $J_{H5,H6}$ would always have a small value (1–2 Hz), while $J_{H5,H6'}$ would vary from 1–2 up to 8 Hz, depending on the relative populations of the two rotamers.

For both **1** and the monosaccharide β-GlcNAc, measured $J_{H5,H6}$ and $J_{H5,H6'}$ values were 1.8 ± 0.5 Hz and 5.3 ± 0.5 Hz, respectively, consistent with *gg* and *gt* conformers being present in roughly equal proportion[46,47]. The relative proportions are independent of temperature over the range 0 to 37 °C, and unaffected by the presence of the uronic acid moiety. Welti and co-workers[8] reported values for $J_{H5,H6}$ and $J_{H5,H6'}$ of 2.0 and 5.0 Hz, respectively, for high molecular weight HA at 30 and 80 °C, so extension of the disaccharide does not appear to affect the rotamer population distribution of the hydroxymethyl group. This suggests that in polymeric HA, there are no additional restrictions, such as involvement in a hydrogen bond with an adjacent residue, placed on rotation of the hydroxymethyl group.

Orientation of the acetamido group. — The coupling constant between the amide proton and H-2 of the N subunit of **1** was independent of solvent composition (H_2O vs. H_2O–acetone-d_6), counterion type (lithium or sodium), the addition of 0.15 M NaCl, and temperature over the range -30 to 27 °C. For example, J_{NHCH} was 9.6 Hz at -30 and 4 °C, and 9.7 Hz at 27 °C. The orientation of the acetamido group appears to be insensitive to temperature, as it was for the hydroxymethyl group. The values measured are consistent with a *trans* conformation of the acetamido group relative to H-2 of the N subunit, as reported by Cowman and co-workers[10] for many different lengths of HA dissolved in water. The temperature coefficient of the amide proton was 0.007 ppm/ °C, somewhat smaller than for water protons (0.01 ppm/ °C). As the NMR parameters for the amide proton are virtually the same for the monomer β-GlcNAc as for the disaccharide, there is no evidence that, in water or water-containing solutions, its orientation or solvent–solute interactions change. Therefore, we conclude that there is no hydrogen bond between the carbonyl oxygen of the acetamido group and OH-2 of the U subunit, as proposed for longer HA fragments in Fig. 2. This conclusion is supported by measurements of OH-2 (see below).

^{13}C *NMR spectra.* — The observation that ^{13}C chemical shifts are virtually constant over the same temperature range (Table VI) confirms that there is no detectable conformational change for **1** and, furthermore, excludes the possibility

of a slow conformational equilibrium at any temperature in this range. Similarly, for high molecular weight sodium HA in D_2O, Bociek and co-workers[28] reported that ^{13}C chemical shifts were independent of temperature over the range 30 to 80 °C.

Comparison of mono- and di-saccharides. — The data presented in Tables I–III allow comparison of ring proton NMR parameters for 1 and its constituent monomers, β-GlcUA (lithium and sodium salts) and β-GlcNAc. This comparison is very important because it allows us to differentiate between those characteristics that are intrinsic to the monosaccharides and those that appear when the monosaccharides are linked to form one HA repeating unit. Parameters for α anomers are also listed. It is important to emphasize that assignment of the mono- and di-saccharides was done independently, and in all cases (except for Glc) confirmed and refined by simulation to an average accuracy of ± 0.0004 ppm.

In general, the ring protons of the monomers are less temperature sensitive than the corresponding protons of the disaccharide, although the protons most sensitive to temperature are the same: N H-2, N H-3, and U H-4. This may reflect the effect of a polar substituent (carboxyl relative to hydroxymethyl; acetamido relative to hydroxyl). Values of $\Delta\delta/\Delta T$ for ring protons of the N subunit show greater changes upon β (U-1, N-3) linkage into the disaccharide than do ring protons of the U subunit. The direction (sign) of $\Delta\delta/\Delta T$ is the same for the corresponding protons of 1 and its constituent monomers, except for protons U H-3, N H-4, and N H-5. However, for N H-5, the difference in $\Delta\delta$ between monomer and disaccharide is negligibly small, 0.0018 ppm. The similarity in temperature sensitivity indicates that there is no dramatic change in conformation or solute–solvent interactions when the monomers are covalently joined to form the disaccharide. Further support for this conclusion comes from proton–proton scalar coupling constant values (Table II) that are virtually the same for monomers and 1.

The similarity in chemical shifts and coupling constants for monomers and this disaccharide suggests that any secondary structure possible for the disaccharide, such as a hydrogen bond between U OH-2 and the carbonyl oxygen of the acetamido group or between N OH-4 and the pyranosyl oxygen of GlcUA, does not exist. Of course, it is possible that such intramolecular hydrogen bonds require a longer stretch of HA to be favorable. Extensive networks of hydrogen bonds have been observed in crystals of carbohydrates[51].

Effect of counterion type. — We observed that the type of monovalent counterion present had no detectable effect on the conformations of 1 (lithium and sodium compared) or the monosaccharide GlcUA (lithium, sodium, and potassium compared). Nor did the type of counterion present affect the equilibrium ratio of $\alpha : \beta$ anomers (43:57) for the lithium, sodium, and potassium salts of GlcUA at 3°C (measured 2 days and then again 7 days after solutions were prepared).

Hydroxyl protons. — Our measurements of vicinal ^1H–^1H and interglycosidic ^1H–^{13}C coupling constants and ^{13}C chemical shifts establish that the temperature

sensitivity of ring proton chemical shifts is not the result of major conformational changes in **1**.

Then what is responsible for the enhanced temperature sensitivity of specific positions of the sugar rings? Three possibilities are that this behavior might arise from the disruption of intramolecular hydrogen bonds in the disaccharide, from solute–solute interactions, or from solute–solvent interactions. The first and third possibilities, which are complementary, are supported by the report that the chemical shifts of ring protons of compounds similar to **1** are independent of temperature in Me_2SO-d_6 (ref 21).

To determine if intramolecular hydrogen bonds are responsible, we examined the behavior of the hydroxyl protons. It is assumed that involvement of a hydroxyl proton in a hydrogen bond will cause dramatic changes in its ^1H NMR parameters: a downfield chemical shift, a smaller value of $\Delta\delta/\Delta T$, a slower exchange rate, and a coupling constant indicating restricted rotation[15,16,27]. It was not possible to observe hydroxyl protons under the same experimental conditions as the nonexchangeable protons were observed because of their rapid exchange rates. To slow down the exchange of hydroxyl protons, it was necessary to lower the temperature to ∼ −27°C. To prevent the solution from freezing, it was necessary to add an organic cosolvent. Acetone-d_6 was chosen because it is a relatively weak hydrogen bond donor and acceptor[52] and because mixtures of H_2O and Me_2SO freeze at too high a temperature to permit observation of these hydroxyl protons. To take into account the possibility of interresidue interactions in the disaccharide that do not arise directly from the covalent linkage, a solution containing equimolar amounts of both monosaccharides was also examined. NMR parameters for this mixture were the same as those measured for solutions of the individual monomers.

A detailed discussion of the behavior of the hydroxyl protons follows. The results can be summarized thus: although some of the hydroxyl protons of **1** show anomalous behavior (especially U OH-3,4 and N OH-4), other lines of evidence for long-lived intramolecular hydrogen bonds are absent.

Chemical shifts and coupling constants for the hydroxyl protons of **1** (sodium salt) and its constituent monosaccharides are presented in Tables IV and V. Changes in chemical shifts upon creation of **1**, relative to its monomers, were relatively small: ranging from 0.06 ppm to, at most, ∼ 0.4 ppm upfield (for N OH-4). These small changes in chemical shifts suggest that no new hydrogen bonds arise in the disaccharide relative to the individual monosaccharides. The upfield shift for N OH-4, while significant, is in the direction opposite that expected for involvement in a hydrogen bond. For the methyl and allyl α-D-glycoside analogues of **1** in Me_2SO-d_6 at 22°C, Heatley and co-workers[21] reported that U OH-4 appeared more than 2 ppm downfield relative to the other hydroxyl protons. This was interpreted as resulting from a hydrogen bond between U OH-4 and the carboxylate group. They also reported that N OH-4 was shifted downfield by ∼ 1 ppm relative to N OH-6, supporting the existence of a hydrogen bond between N OH-4 and O-5 of the trailing U subunit. In sharp contrast, as can be seen from the

results in Table IV, most of the hydroxyl protons of **1** and the monosaccharides (α and β) cluster around 6.2 ppm in H_2O–acetone-d_6.

Changes in chemical shifts by themselves are difficult to interpret, because so many factors contribute. Evidence for participation in hydrogen bonds may also be obtained from the temperature coefficient ($\Delta\delta/\Delta T$) of the hydroxyl proton chemical shift and from ring–hydroxyl proton coupling constants. Protons involved in hydrogen bonds are expected to have smaller temperature coefficients than those that are freely exchanging with water (~ 0.01 ppm/°C). The only two hydroxyl protons for which $\Delta\delta/\Delta T$ differs significantly from 0.01 are U OH-4 and N OH-4. The former could be involved in a hydrogen bond with its neighboring carboxyl group, and the latter in a hydrogen bond either with O-5 of the trailing U subunit or its own hydroxymethyl group. However, the change in $\Delta\delta/\Delta T$ is rather modest compared to published examples[15,21] where $\Delta\delta/\Delta T$ is 10- or 20-fold times smaller for hydrogen-bonded OH protons than for uninvolved protons.

While the temperature coefficient of hydroxyl protons would reflect both intra- and inter-molecular interactions, concentration-dependent effects would reflect only solute–solute interactions. Upon dilution from 25 to 2 mM, hydroxyl protons of **1** in 1:1 H_2O–acetone-d_6 solution retained the same chemical shifts (± 0.01 ppm), with the exception of U OH-4, which shifted upfield slightly by 0.05 ppm.

As mentioned previously, ring–hydroxyl proton coupling constants could be sensitive to hydrogen bond formation. If there is free rotation about the C–O bond connecting the hydroxyl group to the ring, the coupling constant should be an average value of ~ 5–7 Hz (an average between 0 Hz and 12 Hz). Therefore, most of the hydroxyl protons observed for **1** are undergoing free rotation except for OH-3 of the uronic acid moiety ($J_{H3,OH3}$ 3.0 Hz). (Neither $J_{H4,OH4}$ value in the disaccharide could be determined because of exchange broadening of these signals. Only an upper limit based on the linewidth at half-height can be established.) Based on the conformation obtained from energy minimization and molecular dynamics[53], the hydroxyl group at position U-3 is totally exposed to solvent and not subject to any steric constraints on its rotation. As seen in Fig. 2, one model for secondary structure in HA includes a hydrogen bond between U OH-3 and O-5 for the trailing β-GlcNAc subunit linked $1 \rightarrow 4$. Since formation of such a bond is not possible in the β-(U-1, N-3) linked disaccharide under study here, that cannot be the explanation for the restricted rotation around the C–O bond at position U-3. The rotation is somewhat less restricted in the monomer, sodium D-glucuronate, but not completely isotropic (J 4 Hz). Scott and co-workers[5] report $J_{H3,OH3}$ 3.5 Hz for an HA tetrasaccharide and 3 Hz for sodium D-glucuronate[54] in Me_2SO, so this restricted rotation appears to be intrinsic to the molecule and not determined by interactions with solvent. Furthermore, the temperature coefficient for U OH-3 is close to that for water itself. All this implies that the restricted rotation is not related to hydrogen bonding, either with solvent or with another substituent on the glucuronate.

The coupling constant of 5.0–5.5 Hz for N OH-6 in the disaccharide and the

monomer, and the fact that N OH-6 in both compounds is a triplet, indicate that the hydroxyl group at position 6 is freely rotating, so it is unlikely to be involved as a donor in a hydrogen bond. Although there appears to be free rotation about the C–O bond, there is restricted rotation about the C–C bond of the hydroxymethyl group, as indicated by the unequal values of $J_{5,6}$ and $J_{5,6'}$. However, as neither these coupling constants nor those of the OH-6 methylene protons differ between monomeric β-GlcNAc and **1**, we can find no evidence for a change in hydrogen bonding when β-GlcUA is linked (U-1, N-3) to β-GlcNAc.

For **1**, two of the intramolecular hydrogen bonds indicated in Fig. 2 would be possible: between U OH-2 and the carboxyl oxygen of the acetamido group; and between N OH-4 and O-5 of GlcUA. The near-identity of $^3J_{H2,OH-2}$ in the disaccharide (4.8 Hz) and in sodium D-glucuronate (5.0 Hz) and the similar temperature coefficients of U OH-2 in both compounds indicate that U OH-2 is not involved in a hydrogen bond in the disaccharide. This conclusion is supported by the similarity in ring proton NMR parameters for the mono- and di-saccharides, discussed above.

Hydroxyl protons at position 4 on both residues of the disaccharide exhibit exchange rates enhanced relative to other hydroxyl protons. U OH-4 exhibits the broadest linewidth, indicating the fastest exchange rate. The U OH-4 and N OH-4 signals are broadened by exchange, so their coupling constants with ring protons cannot be determined accurately. The linewidths at half-height place upper limits on $^3J_{H4,OH-4}$ of 5 Hz and 9 Hz for N OH-4 and U OH-4, respectively. The difference in temperature coefficient for N OH-4 relative to that for water (-0.004 ppm/°C vs. -0.01 ppm/°C) is rather modest.

Therefore, for N OH-4, none of the usual lines of evidence presented for intramolecular hydrogen bonds (large downfield shift, reduced exchange rate, greatly reduced $\Delta\delta/\Delta T$) is observed.

In the analogous disaccharide from chondroitin, which is GlcUA linked β-(1 → 3) to methyl-β-D-galactosamine, Heatley and coworkers[21] observed that $\Delta\delta/\Delta T$ for OH-4 of the β-GalNAc subunit was similar to all the other hydroxyls. This suggests that the equatorial orientation of OH-4 in GlcNAc is responsible for its anomalous behaviour. Earlier studies by Lemieux and Brewer[43] on model cyclohexane and pyranose compounds suggested that the orientation of a hydroxyl group at position 4 affects the probability of its involvement in an intramolecular hydrogen bond to the adjacent hydroxymethyl group. Our results suggest that neither U OH-4 nor N OH-4 are involved in intramolecular hydrogen bonds. It is more accurate to say that if they are involved in intramolecular hydrogen bonds, the bonds do not affect their ^{1}H NMR parameters in the expected way. We are currently using *ab initio* calculations to try to determine the underlying mechanism for the exchange rate at these positions.

We have to address the affect of adding acetone to an aqueous solution of the disaccharide. Witanowski and co-workers[52] characterized the ability of a solvent to act as a hydrogen-bond donor or acceptor in terms of solvatochromic parameters.

According to this scheme, water is much more likely to interact with the hydroxyl protons of a saccharide than is Me_2SO or acetone. A direct comparison between the NMR parameters for **1** in D_2O at ambient temperatures and in 1:1 H_2O–acetone-d_6 at ~ –27°C is not possible because of the effect of temperature on the spectra. We were able to determine that the order of chemical shifts of **1** in 1:1 H_2O–acetone-d_6 at –28°C was correctly predicted by applying temperature coefficients to the chemical shifts in D_2O (measured between 0 and 37°C). The one exception was the signal from N H-4, which was predicted to lie downfield of U H-4 and U H-3 in the H_2O–acetone solvent, but actually occurred upfield. In the H_2O–acetone solvent, ring protons U H4, U H-3, and N H-4 occurred as three overlapping peaks between 3.55 and 3.50 ppm and could not be unambiguously assigned. Direct comparison of one-dimensional spectra of **1** at 0°C revealed that the order of chemical shifts and the values of scalar coupling constants were the same in both solvents. Further evidence that acetone did not perturb the structure of **1** was obtained from the amide proton, which exhibited the same coupling constant and temperature coefficient, independent of solvent.

CONCLUSIONS

Although small changes in temperature cause dramatic changes in the appearance of the ^1H NMR spectrum, temperature and counterion type do not affect the dihedral bond angles, including the glycosidic linkage, in the disaccharide repeating unit of hyaluronan. Nor do temperature and counterion type affect the dihedral bond angles for the constituent monosaccharides, β-GlcUA and β-GlcNAc. However, we have observed that the chemical shifts of some ring protons are more sensitive to temperature. In the absence of evidence that **1** forms dimers, we conclude that its interactions with water, whether called hydration, hydrogen bonds, or solute–solvent interactions, are responsible for this temperature sensitivity of ring protons.

Based on the similarity of NMR parameters for hydroxyl protons for **1** compared to the monosaccharides, our conclusion is that this temperature sensitivity arises from the nature of the aglycon substituents rather than formation of long-lived intramolecular hydrogen bonds in the disaccharide. The orientations of the hydroxymethyl group and acetamido group of the GlcNAc subunit are unchanged between monosaccharides and the disaccharide. The chemical shift of the carboxyl carbon of glucuronate is also unaffected by the addition of the β-(U-1, N-3) linked GlcNAc subunit. If intramolecular hydrogen bonds do exist in hyaluronan, they require longer chain lengths. Heatley and coworkers[21] reported ^1H chemical shifts for the methyl α-D-glycoside analogue of **1** in Me_2SO-d_6 at 22°C. Based on more extensive characterization of related compounds, they concluded that N OH-4 was hydrogen-bonded to O-5 of the U subunit and that U OH-4 behaved as though it were hydrogen-bonded to the carboxylate group. In a subsequent study of HA oligomers in Me_2SO-d_6 with small amounts of water

added, Heatley and Scott[6] suggested that intramolecular hydrogen bonds would be in equilibrium with solute–solvent hydrogen bonds, and that the equilibrium would be pushed towards the latter as the amount of water increased. The evidence presented here extends their measurements to physiological amounts of water and verifies that solute–solvent interactions predominate over intramolecular hydrogen bonds.

The driving forces that favor formation of intramolecular hydrogen bonds in organic solvents will be counteracted in water. In water, the enthalpy of forming an intramolecular hydrogen bond should be equivalent to forming a solute–solvent hydrogen bond, while the entropy change for forming an intramolecular hydrogen bond should be unfavorable relative to solute–solvent interactions. Our results highlight the difference between saccharide structures in organic solvents, such as Me_2SO, and in water.

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