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Note

A detailed ^{1}H and ^{13}C NMR study of a repeating disaccharide of hyaluronan: the effect of sodium and calcium ions

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Hyaluronan (HA), a polyanionic polysaccharide consisting of repeating glucuronate and *N*-acetylglucosamine residues, exists surrounded by ions in its physiological milieu. For example, the average concentration of sodium is 300 mM in bovine hyaline cartilage, or roughly twice that of typical extracellular fluid [1]. It has been shown that salts can modify strongly the properties of HA [2,3] and its constituent monomers [4,5]. For example, Van Damme et al. [2] reported that binding of HA to lysozyme is most efficient at pH 7.5 and 10–15 mM NaCl. Self-association of hyaluronate segments in aqueous solution requires a sodium concentration of 150 mM [3]. Several physical techniques (NMR [6–13], X-ray [14–17], CD [8,18], and IR [9,14]) have been employed to determine if salts interact with uronate residues non-specifically (via electrostatic interaction) [7] or through chelation by several ligands acting in concert. Most authors have postulated the existence of chelation sites. A review of studies conducted on HA in the solid and liquid states leads to the conclusion that the oxygens of the carboxyl groups on HA and of water molecules always take part in coordination of metal cations, while other oxygens around the sugar ring are sometimes involved as well. The type of

Abbreviations: U, β-D-glucopyranosyluronic acid subunit of **1**; GlcUA, α or β anomer of D-glucopyranosyluronic acid; N, methyl 2-acetamido-2-deoxy-β-D-glucopyranoside subunit of **1**; GlcNAc, α or β anomer of *N*-acetylglucosamine; HA, hyaluronan; Note: all sugars named in this manuscript are in the D-hexopyranose form.

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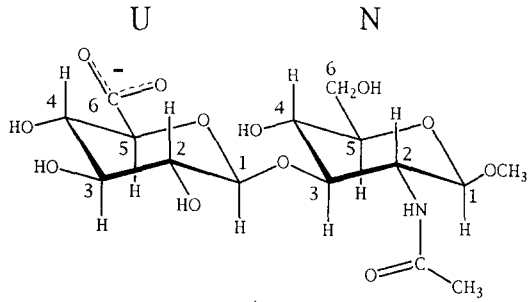
counterion and anomeric configuration at C-1 determines the exact chelation pattern. The configuration of specific OH groups (axial or equatorial) also influences complex formation. For example, Gould and Rankin [17] reported that potassium D-galacturonate formed a more stable complex with calcium ions than did potassium D-glucuronate.

Here we describe how the ^{1}H and ^{13}C NMR spectra of components of HA are affected when NaCl or $CaCl_2$ is added. Careful examination of these changes provides information about the nature of the interactions of cations with HA. The disaccharide studied, methyl 2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-β-D-glucopyranoside (**1**) is one of two possible disaccharide repeating units of HA.



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To avoid the issue of competition between sodium and calcium, we only examined the effect of adding NaCl to the sodium form of **1**, and $CaCl_2$ to its calcium form. In a previous study [19] we concluded that the temperature sensitivity of the ^{1}H spectrum of **1** in water is caused by solute–solvent interactions. The results reported below suggest that sodium and calcium ions have only subtle effects on the environment of **1**, either by chelation or by some interaction with the solvent which affects **1**. These ions do not cause a detectable change in the conformation of **1**.

We found that scalar ^{1}H coupling constants ($^{3}J_{HH}$) of both the sodium and calcium forms of **1** were virtually constant (within an experimental error of 0.5 Hz) with varying temperature (0–37 °C) and varying salt concentration (from no added salt to 0.45 M NaCl or 0.225 M $CaCl_2$). The magnitudes of the coupling constants correspond to a $^{4}C_1$ conformation [19,20] for the hexapyranose rings of the disaccharide, as well as for both GlcNAc anomers. D-Glucuronate has been investigated extensively by ^{1}H and ^{13}C NMR spectroscopy in D_2O solutions containing $Zn(OAc)_2$ [6] and $CaCl_2$ [7]. In both studies, $^{3}J_{HH}$ values were characteristic for unperturbed $^{4}C_1$ ring conformation. It appears that addition of monovalent or bivalent cations to disaccharide **1**, as well as to its constituent monomers, leaves their ring conformations unchanged. Also, increasing the temperature did not perturb the $^{4}C_1$ ring conformation or the orientation of the hydroxymethyl group of **1**.

Although the addition of NaCl or $CaCl_2$ did not alter $^{3}J_{HH}$ values for **1**, proton chemical shifts were affected (Table 1). In general, ring protons were shifted downfield upon addition of salt. $CaCl_2$ produced a greater chemical shift displacement ($\delta\Delta$) than did NaCl. Ring protons of the U subunit were more sensitive to salt than were ring

Table 1
Change in ^{1}H chemical shifts ($\delta\Delta$, ppb from DSS) [a] of **1** and GlcNAc (β or α) upon addition of NaCl or $CaCl_2$ at 37 °C

Solvent [b]	Compound of anomer	U H-1	U H-2	U H-3	U H-4	U H-5	CH_3	N H-1	N H-2	N H-3	N H-4	N H-5	N H-6	N H-6′
0.45 M NaCl	**1**	+23.6	−4.9	+17.4	−5.4	+18.8	+6.6	+17.6	+1.4	+17.6	+2.6	+10.8	−0.8	+3.5
0.225 M $CaCl_2$	**1**	+29.5	−0.9	+24.4	+6.4	+40.0	+8.2	+21.9	+3.0	+20.0	+11.4	+17.0	+2.8	+5.3
0.45 M NaCl	β						+6.2	+17.0	+5.3	+20.5	+1.4	+15.4	+2.4	+4.3
	α						+4.8	+5.5	+0.8	+9.4	+2.5	+9.9	+3.0	+4.4

[a] Chemical shifts were verified by simulation of one-dimensional spectra to a precision of ±0.0004 ppm. Signs indicate the direction of $\Delta\delta$: − for upfield and + for downfield. NaCl and $CaCl_2$ were added to **1** in its sodium and calcium forms, respectively.
[b] All in D_2O.

protons of the N subunit. The magnitude of $\delta\Delta$ was roughly proportional to the amount of salt added. Although these salt-induced chemical shift changes are small ($\delta\Delta \leq 40$ ppb), they are not negligible when interpreting ^{1}H spectra of HA and related compounds. Because the added salts shifted the ring ^{1}H signals downfield by a variable amount, changes in the order of signals occur. Different sensitivities to salt addition are responsible for changes in the order of glucuronate protons at positions-3 and -4, and for the total overlap of the anomeric protons of **1** at 37 °C in a 0.45 M solution of NaCl in D_2O. For both U and N subunits of disaccharide **1**, the protons most sensitive to salt addition were at positions-1, -3, and -5, but the order depended on the counterion. For the sodium form of **1**, the order from most to least sensitive was: U H-1 > U H-5 > N H-3 ≥ N H-1 > U H-3 > N H-5; whereas for the calcium form, the order was: U H-5 > U H-1 > U H-3 > N H-1 > N H-3 > N H-5. The observation that the type of cation affects the salt sensitivity suggests that mono- and bi-valent cations added in excess may form different complexes with **1**. Angyal [21] noticed that complex formation between metal ions and saccharides caused a downfield shift of the proton vicinal to an oxygen atom involved in chelation. The strongest complex was detected between lanthanide ions and *epi*-inositol, which possesses an ax–eq–ax sequence of hydroxyl groups. Angyal suggested that this was the most favorable arrangement for chelating an ion. He also reported that for the same ionic radius, a more highly charged cation caused greater downfield shifts of the ^{1}H signals. The changes in ^{1}H chemical shifts that we observe are consistent with this observation, although the di- and mono-saccharides related to HA do not have this ax–eq–ax sequence of hydroxyl groups.

Previously we observed that ^{1}H spectra of **1** detected in D_2O were virtually the same for its sodium and lithium forms with no added salt [19]. Now we conclude that replacing a monovalent counterion (sodium) with a bivalent one (calcium) also has no effect on the ^{1}H spectrum. The differences in proton chemical shifts between both forms of the compound (detected in D_2O) did not exceed 0.0024 ppm, which is within the experimental variability of 0.0003–0.0024 ppm for spectra recorded on different preparations. In the sodium and calcium forms of **1**, the order of the ring proton chemical shifts was the same. In contrast to our results, Braud et al. [8] found a dramatic difference between the ^{1}H spectra of sodium and calcium forms for some fragments of heparin dissolved in D_2O.

The addition of salts to **1** in D_2O solution caused only modest changes in ^{13}C chemical shifts: about half of the signals stay unchanged, while others move by about ± 0.1 ppm. The greatest variation, shielding by 0.3 ppm, was observed at position-5 in the U subunit in both forms of **1**. It should be noted that NaCl shifted the carboxyl carbon of the U subunit upfield by 0.1 ppm, while $CaCl_2$ left it unchanged. If the cations were complexing directly with the carboxyl group, one would expect a greater change in the $^{13}COO^-$ chemical shift, unless the interaction somehow does not perturb the immediate environment. According to previous studies [7,22] the carboxyl carbon would be deshielded by at least 0.4 ppm if the uronate subunit were involved in a non-specific, electrostatic interaction with a cation.

For comparison with the disaccharide, we also investigated the effect of added NaCl on the monosaccharide GlcNAc (Table 1). The other monomer, GlcUA, was investigated by Whitfield and Sarkar [6], as well as by Jaques et al. [7]. The direction,

magnitude, and order (H-3 > H-1 > H-5) of the shift displacements for β-GlcNAc are identical to those for the N subunit of **1**. This indicates that replacements of hydroxyl groups at positions-1 and -3 on GlcNAc by the methoxyl group and the β-D-glucuronate subunit, respectively, do not have much influence on saccharide–sodium ion interactions. Similarly, Angyal [21] demonstrated that replacement of the hydroxyl proton in position-3 by a methyl group only slightly reduced the ability of *epi*-inositol to complex counterions. In contrast to these observations, Symons et al. [23] reported that replacement of a hydroxyl group at C-1 of glucose, to form the methyl β-glucoside, eliminated calcium coordination.

We found that most of the ring protons that exhibit strong sensitivity to salt show more moderate sensitivity to temperature (Table 2). The largest temperature-induced chemical shift ($\delta\Delta = 32.2$ ppb) was observed for N H-3 in the sodium form, whereas the largest salt-induced chemical shift ($\delta\Delta = 40.0$ ppb) was observed for U H-5 in the calcium form. Analysis of the data listed in Table 2 shows that the order of temperature sensitivity of ring protons (in the range 15 to 32 ppb) is the same for all samples studied: N H-3 > N H-2 > U H-4 > N H-1. This fact suggests that the addition of salt, whether mono- or di-valent, does not change the fundamental mechanism underlying temperature sensitivity.

Water is a unique solvent system characterized by a strong molecular network of hydrogen bonds with tetrahedral coordination and extremely high cohesive energy [24,25]. It is well documented [24,26] that salts modify the structure of water. For example, the addition of salts effects the hydrophobic effect, the rates of some reactions, the solubility of neutral organic compounds, hydrogen-bonding capability [27], and bond-stretching frequencies [28]. We detected that an addition of NaCl (0.45 M) increases the population of α-GlcNAc at 37 °C by about 3%. For the other constituent monomer of HA, GlcUA, Jaques et al. [7] reported that addition of $CaCl_2$ shifted the α:β ratio towards the α form as well. The mechanism of the influence of cations on mutarotation has not yet been established [29]. Inspection of salt-induced chemical shifts for α- and β-GlcNAc reveals that protons at positions-1, -3, and -5 in both anomers are the most deshielded by the addition of NaCl, although the exact order of salt sensitivities is different. For the β anomer, it is N-3 > N-1 > N-5, whereas for the α anomer, it is N-5 > N-3 > N-1. Generally, chemical shift displacement for these protons upon addition of NaCl is 2.5 times smaller for the α anomer relative to β, but the direction of the changes is the same. For the β anomer of GlcNAc, all hydroxyl groups are in the equatorial orientation. This allows for a better fit to a tetrahedral arrangement of water molecules [30] than does the arrangement of hydroxyls on the α anomer. This difference in hydration for β vs. α anomers could explain the difference in chemical shift displacements observed.

1. Summary

We have shown that the addition of salt causes small but measurable changes in the ^{1}H and ^{13}C chemical shifts of a disaccharide **1**. The observed changes are not consistent with a simple electrostatic interaction between the carboxyl group of the uronate residue

Table 2
Change in ^{1}H chemical shifts ($\delta\Delta$, ppb) [a] of **1** upon increase of temperature from 0 to 37 °C

Solvent	Form of **1**	U H-1	U H-2	U H-3	U H-4	U H-5	CH_3	N H-1	N H-2	N H-3	N H-4	N H-5	N H-6	N H-6′
D_2O	sodium	−6.3	+8.1	+9.8	+24.6	−4.6	+1.6	+24.1	−24.8	+32.2	−10.9	+3.4	+0.2	−11.4
0.45 M NaCl [b]	sodium	−11.8	+5.0	+7.6	+22.3	−8.9	+0.6	+19.6	−26.1	+24.6	−11.4	+0.6	−1.2	−12.1
D_2O	calcium	−6.3	+10.1	+9.5	+23.5	−4.4	+1.4	+22.1	−23.7	+29.5	−10.8	+2.8	−0.7	−9.9
0.225 M $CaCl_2$ [b]	calcium	−11.7	+4.1	+6.2	+21.7	−10.5	+0.5	+15.8	−24.1	+22.8	−10.8	−1.7	−2.3	−3.5

[a] Chemical shifts were verified by simulation of one-dimensional spectra to a precision of ±0.0004 ppm. Signs indicate the direction of $\Delta\delta$: − for upfield and + for downfield.
[b] In D_2O.

and the cation. Changes in ionic strength and temperature affect the ^1H spectrum of **1** independently, which should be taken into account for NMR studies of HA. We believe that the results reported here will be of interest not only for those interested in behavior of HA in its physiological milieu, but also to anyone studying glycosaminoglycans by NMR spectroscopy.

2. Experimental

Reagents.—Hyaluronan (human umbilical cord) was purchased from ICN Biochemicals Co. (Cleveland, OH) as the potassium salt. 2-Acetamido-2-deoxy-α-D-glucose (99%) and D-glucuronic acid as the sodium salt monohydrate (99%) were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). NaCl (99.999%), NaOH (volumetric standard, 0.1988 N), and $Ca(OH)_2$ (98% ACS Reagent) were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). NaCl dihydrate (99.5% MicroSelect for molecular biology) was purchased from Fluka Chemical Co. (Ronkonkoma, NY). Water used in all procedures was distilled and then passed through a MilliQ apparatus (Waters Associates, Milford, MA).

Analytical methods.—The carbazole reaction [31] was used to quantify uronic acid. The colorimetric method using 3,6-dinitrophthalic acid [32] was used to verify that disaccharide **1** had virtually no reducing power and was therefore the methyl-β-D-glucopyranoside. Potentiometric titration of the free acid was used to prepare the disaccharide as the sodium or calcium salt, as described below. ICP and neutron activation was used to quantify calcium and sodium concentrations, respectively.

Preparation of compounds.—The sodium salt of disaccharide **1** was prepared from the free acid by titration as described elsewhere [19]. The calcium salt of disaccharide **1** was prepared, under nitrogen, by titration of the free acid with a 10.39 mM solution of $Ca(OH)_2$. Sodium glucuronate was used as a reference solution to estimate the end-point of titration. Water free from CO_2 was used to prepare solutions of the disaccharide acid, the titrant, and D-glucuronic acid. This water was prepared from MilliQ water in four cycles, with the last cycle carried out in a glove bag. During each cycle, the water was degassed for 20 min in an ultrasonic bath under vacuum and then purged with nitrogen for 20 min. Water prepared in this manner had a pH 7.03–7.07, stable in an open beaker in a glove bag for at least 1 h. The disaccharide was converted to the calcium salt by titration to pH 7.11. This end-point was chosen because it is the pH of sodium D-glucuronate at the same concentration as that of the calcium salt of **1**. The $CaCl_2$ solution added to the NMR sample tube was prepared from crystalline $CaCl_2$ dihydrate that had been dissolved in D_2O and then lyophilized.

NMR spectra.—The spectra of the sodium and calcium salts of **1** and spectra of GlcNAc were measured as 20, 8 (i.e., 16 mM per disaccharide unit), and 20 mM, respectively, at 0 and 37 °C in D_2O or in the indicated aqueous solution of NaCl or $CaCl_2$. The solutions handled under nitrogen were always prepared from the same stock solution of saccharide and corresponding salts. The disaccharide **1** in its sodium form was measured in D_2O and in D_2O solutions of NaCl of 0.15, 0.30, and 0.45 M. Disaccharide **1** in its calcium form was measured in D_2O and in D_2O solutions of $CaCl_2$

of 0.075 and 0.225 M. The pH values of the samples clustered around 7.6 ± 0.2 (measured by a Micro Combination pH Electrode, uncorrected for deuterium). Acetone (1–7 mM) was used as an internal chemical shift standard. In a separate experiment, ^{1}H chemical shifts of acetone relatively to DSS [sodium 3-(trimethylsilyl)-1-propane sulfonate] were measured. All ^{1}H NMR spectra were recorded at 499.843 MHz. ^{13}C spectra were measured either at 125.38 or 125.697 MHz (depending on the spectrometer used) at 0 and 37 °C. ^{13}C and ^{1}H assignments for disaccharide **1** and GlcNAc were made by comparison with our previously published assignments [19]. Values for both the coupling constants and chemical shifts for ^{1}H spectra were verified by simulation of the one-dimensional spectra, using the simulation package supplied by Varian Associates (Palo Alto, CA). Chemical shifts and coupling constant values are not shown in order to save space, but are available upon request.

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