^{13}C-NMR Studies of Hyaluronan: Conformational Sensitivity to Varied Environments†

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ABSTRACT: Hyaluronan (HA) samples ranging in size from small oligosaccharides to high molecular weight polymer have been studied by ^{13}C-NMR spectroscopy. In neutral aqueous solutions, the chemical shifts of carbons directly involved in the β-1,3 glucuronidic linkage are found to be sensitive to (1) residue linkage position in short chains, (2) oligomer degree of polymerization, (3) solvent ionic strength, and (4) monovalent *vs* divalent counterions. The carbons of the β-1,4-glucosaminidic linkage show less sensitivity to the above conditions. Thus conformational versatility for HA in aqueous solution is correlated with a chemical shift change primarily in carbons of the β-1,3 linkage. We have also compared the ^{13}C spectrum of HA in neutral aqueous salt solutions to spectra observed in dimethyl sulfoxide (DMSO) solution (ordered 2- or 4-fold HA form) or the solid state (Na^+ counterion, tetragonal 4-fold helical HA form). The solid state spectrum is similar to that found in DMSO but differs substantially from the aqueous solution spectrum. The differences are attributed to (1) rotation of the acetamido group, with concomitant change in hydrogen bonding and average conformation at the β-1,4 linkage, and (2) loss of hydrogen bonds in aqueous solution and consequent change in average conformation at the β-1,3 linkage.

Hyaluronan (HA) is a high molecular weight linear polysaccharide with the repeating disaccharide structure poly[(1→3)-β-D-GlcNAc-(1→4)-β-D-GlcA−] (Figure 1). It occurs in the extracellular matrix and serves such diverse biological functions as organization of cartilage proteoglycans into aggregates, providing a viscoelastic medium in the eye vitreous or joint synovial fluid, maintenance of tissue hydration, and participation in mechanisms controlling cellular activities including adhesion, differentiation, migration, and tissue morphogenesis via specific cell surface receptor interactions.[1−12]

The conformation of HA has been thoroughly analyzed in the solid state by X-ray diffraction study of oriented films.[13−18] Left-handed single helices with 2-, 3-, and 4-fold symmetries, as well as a double helical form, have been identified. The helical structures are stabilized by intrachain hydrogen bonds linking adjacent sugar residues and interchain hydrogen bonds and cation/H_2O bridges. In previous ^{13}C-NMR studies of HA in the solid state, we found evidence for significant conformational differences relative to HA in aqueous solution.[19] Additional evidence for a change in HA conformation upon hydration has been obtained by Brillouin and X-ray scattering, as well as Raman and infrared spectroscopy.[20,21]

The conformation adopted by HA in aqueous solution is the subject of continuing study. HA is a polyanion and exhibits typical polyelectrolyte properties in response to changes in its ionic environment. In dilute aqueous salt solutions, the solvated chain occupies a large, approximately spherical, domain. Its hydrodynamic properties have been explained on the basis of the worm-like chain model, with chains that are highly extended at low molecular weights, with more evident coiling at high molecular weights.[22−25] The relationship between intrinsic viscosity and molecular weight for HA supports the model of a relatively stiff chain.

HA chain stiffness in solution has been attributed to one or more of the following factors: (1) the intrinsically small available conformational space at each of the two glycosidic linkages,[26,27] (2) the tendency of HA chains to self-associate both intramolecularly (chain folding) and intermolecularly in neutral aqueous NaCl solutions,[23,28−31] and (3) the existence of interresidue hydrogen bonds stiffening each glycosidic linkage.[29,32−34]

The main experimental support for the proposal of interresidue hydrogen bonds in HA comes from NMR studies. HA or fragments derived therefrom were analyzed by ^{1}H-NMR in dimethyl sulfoxide (DMSO) solution, so that exchangeable protons could be observed. Interresidue hydrogen bonds were thus shown to exist for HA in that solvent.[35−39] Recent comparative studies of HA alkylammonium salts in DMSO and D_2O show substantial differences. Do the hydrogen bonds persist in aqueous solution? Consideration of periodate oxidation kinetics and amide proton exchange rates in aqueous solution, in conjunction with model building and molecular dynamics simulation, led Scott and coworkers[33,34] to propose the persistence of several hydrogen bonds in aqueous solution. Specifically, one hydrogen bond has been proposed to exist across the β-1,3 linkage, linking the GlcNAc C4OH and GlcA O5. Two hydrogen bonds were proposed to exist across the β-1,4 linkage. The first links GlcA C3OH and GlcNAc O5, and the second is a water-mediated hydrogen bond between the GlcNAc amide NH and GlcA carboxyl oxygen. The proposed hydrogen bonds provide a rigid secondary structure for HA, which has not been reconciled with the hydrodynamic data for HA in aqueous solution.

In the present study, we have sought to provide information about the aqueous solution conformation of HA by directly comparing the ^{13}C-NMR spectroscopic properties in the solid state, DMSO solution, and a series of aqueous solvent conditions differing in ionic composition. We observe evidence for conformational differences among the various conditions, consistent with at least partial loss of the interresidue hydrogen

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† Abbreviations used: HA, hyaluronan; GlcNAc, 2-acetamido-2-deoxy-D-glucose; GlcA, D-glucuronic acid; NMR, nuclear magnetic resonance; DMSO, dimethyl sulfoxide; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; TMS, tetramethylsilane; MD, molecular dynamics; NOE, nuclear Overhauser enhancement.

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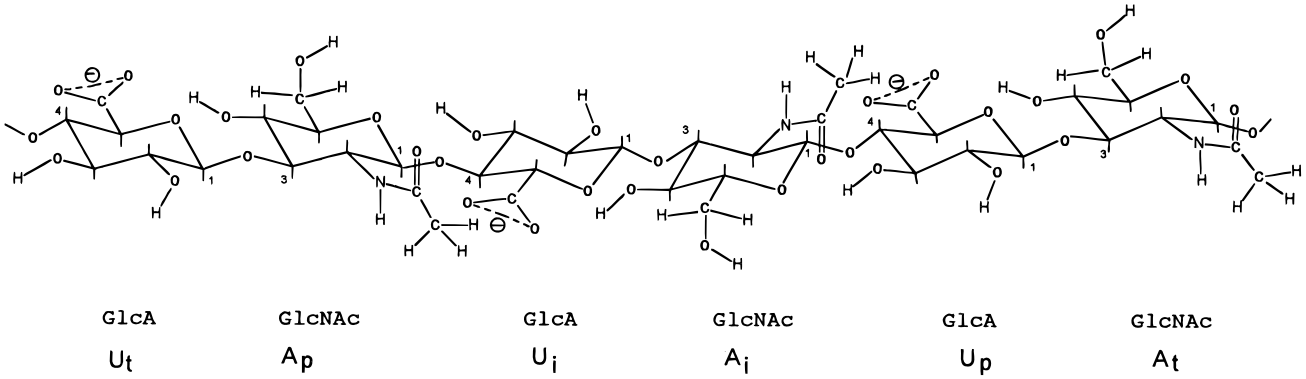


Figure 1. Structure of hyaluronan. The repeat unit is a disaccharide. A hexasaccharide fragment is shown and labeled with shorthand designations, as follows: A, GlcNAc; U, GlcA; X_t, a terminal residue of an oligosaccharide; X_p, a penultimate residue; X_i, an internal residue.

bonds in aqueous solution. The spectra also indicate that the conformational versatility of HA in aqueous solution is associated with changes in the β-1,3 linkage.

Materials and Methods

Sources of polymeric HA were as follows: *Streptococcus zooepidemicus* HA, Sigma Chemical Co.; rooster comb HA, Pharmacia AB; human umbilical cord HA, Sigma Chemical Co.; bovine vitreous HA, Sigma Chemical Co. Only the umbilical cord HA contained chondroitin sulfate as a contaminant, and it was removed by ion exchange chromatography on DEAE-Sephacel.[40] Bovine testicular hyaluronidase (hyaluronate 4-glycanohydrolase, E.C. 3.2.1.35, specific activity 23 613 units/mg) was purchased from Cooper Biomedical. Leech hyaluronidase (hyaluronate 3-glycanohydrolase, E.C. 3.2.1.36) was obtained from Biomatrix, Inc.

For the preparation of low molecular weight HA segments, human umbilical cord HA was digested with bovine testicular hyaluronidase as described elsewhere.[40] Two fractions of HA, differing in molecular weight, were obtained. These were analyzed by polyacrylamide gel electrophoresis[41] to determine the average number of repeating disaccharides in each segment fraction. The sample designated HA_{27} had a weight-average degree of polymerization (disaccharides) of 27; the sample HA_{40} had a weight-average degree of polymerization of 40.

HA oligosaccharides were prepared by digestion of polymeric HA by either testicular hyaluronidase (yielding oligosaccharides of the sequence GlcA−(GlcNAc−GlcA)$_{n-1}$−GlcNAc) or by leech hyaluronidase (yielding oligosaccharides of the sequence GlcNAc−(GlcA−GlcNAc)$_{n-1}$−GlcA). Methods of preparation and analysis have been presented.[42]

For NMR studies, HA species were dissolved in either DMSO-d_6 or 4/1 H_2O/D_2O buffered with 20 mM Na- or K-phosphate, pH 7, and containing added NaCl, KCl, $CaCl_2$, or HCl as described in the text. The HA weight concentration varied in the range 6−15 mg/mL.

NMR spectra were collected at approximately 22 °C on a General Electric GN-300 wide bore spectrometer operating at 300 MHz for ^{1}H and 75.57 MHz for ^{13}C. This instrument was configured with a Nicolet 1280 computer utilizing 20 bits per word. All experiments were done in the Fourier transform mode using quadrature detection. Proton-decoupled ^{13}C spectra were obtained for solution samples using pulsed broadband proton decoupling, with a decoupling power of 1.7 W. A spectral width of 16.7 kHz was observed using a 16 K data block, giving a digital resolution of 2.03 Hz/point. Carbon pulse angles of 27−35° were used. The acquisition time was 0.49 s, with a 500 μs delay between acquisition and the next pulse. Approximately 120 000−300 000 transients were accumulated for each sample. A line broadening function of 2 Hz was applied in all spectra of HA fragments and oligosaccharides, and 4 Hz for HA polymer samples.

In order to observe the exchangeable amide proton of the HA acetamido group with H_2O present in the solution, the 1331 solvent suppression sequence was utilized. Irradiation power was adjusted so that a 90° pulse was 22 μs. Irradiation pulses were 2.5, 7.5, 7.5, and 2.5 μs, with the delay between pulses at 333 μs. The spectral width was ±3012.04 Hz (centered at the irradiation frequency of water) and the acquisition time was 1.36 s. The delay between pulse sequences was 4.0 s. The digitizer was set to 12−16 bits. Usually, 64 accumulations were sufficient.

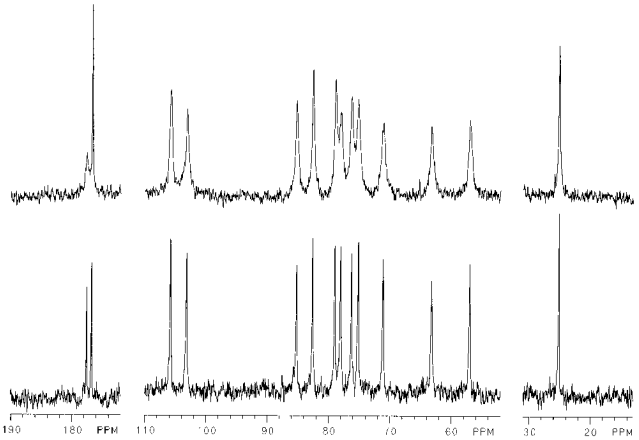


Figure 2. 75 MHz ^{13}C-NMR spectra of HA samples differing in molecular weight. Solvent: 0.15 M NaCl, phosphate buffered to pH 7, H_2O/D_2O = 4/1. Top: streptococcal HA, M_w = 2 × 10^6. Bottom: HA fragment, M_w = 1 × 10^4.

Chemical shifts are reported relative to internal DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate). For aqueous samples, internal acetone (ca. 1 μL in a 500 μL sample) set to 32.907 ppm (^{13}C) or 2.218 ppm (^{1}H) was used as a secondary reference. For samples in DMSO-d_6 or DMSO-d_6/H_2O mixtures, a capillary containing neat TMS (tetramethylsilane), set to 2.635 ppm downfield of aqueous DSS, provided the secondary reference. This value was observed for neat TMS in a capillary in D_2O containing DSS.

Results

Dependence of ^{13}C Chemical Shifts on the Ionic Environment. The 75.57 MHz ^{13}C-NMR spectra of HA have been analyzed as a function of the aqueous solvent composition. As a "standard" condition, HA was studied in neutral aqueous salt solution (0.15 M NaCl, 0.02 M sodium phosphate buffer, pH 7, H_2O/D_2O = 4/1). Figure 2 shows representative spectra for high molecular weight HA (M_w = 2.1 × 10^6) and a lower molecular weight (M_w = 1.6 × 10^4) HA segment preparation derived from the polymer by enzymatic digestion. The polymeric HA spectrum had been earlier reported.[19]

HA polymer or segment samples showed one resonance per carbon atom of the repeating disaccharide structure. (In H_2O/D_2O = 1/1, splitting of the acetamido

Table 1. ^{13}C Chemical Shift Data for Aqueous Solutions of HA Polymer and (GlcA−GlcNAc)$_n$ Fragments[a]

	degree of polymerization (n)						
carbon[b]	2	3	4	7	27	40	5250
$A1_{i,p}$	103.12	103.11	103.12	103.10	103.12	103.09	103.11
$A1_{t\beta}$	97.41	97.40	97.42				
$A1_{t\alpha}$	93.72	93.72	93.73				
$A2_i$		56.97	56.98	56.97	56.97	56.96	56.98
$A2_p$	56.91	56.91	56.93				
$A_{2t\beta}$	58.33	58.29	58.32				
$A2_{t\alpha}$	55.65	55.65	55.66				
$A3_i$		85.13	85.17	85.20	85.18	85.24	85.20
$A3_p$	85.67	85.67	85.68	85.70			
$A3_{t\beta}$	85.05	85.02	85.04				
$A3_{t\alpha}$	82.60	82.59	82.54				
$A4_i$		71.11	71.12	71.15	71.09	71.13	71.06
$A4_{p,t\beta,t\alpha}$	71.25	71.22	71.23				
$A5_{i,p,t\beta}$	78.07	78.05	78.06	78.03	78.00	78.03	78.07
$A5_{t\alpha}$	73.95	73.93	73.95	73.96			
$A6_{i,p,t\alpha}$	63.28	63.24	63.26	63.26	63.23	63.26	63.27
$A6_{t\beta}$	63.44	63.40	63.43				
$A7_{i,p,t\beta}$	177.57	177.56	177.59	177.57	177.59	177.55	177.56
$A7_{t\alpha}$	177.19	177.19	177.20				
$A8_{i,p}$	25.20	25.19	25.21	25.21	25.19	25.19	25.18
$A8_{t\beta}$	24.96	24.94	24.96				
$A8_{t\alpha}$	24.71	24.69	24.71	24.72			
$U1_i$		105.71	105.74	105.73	105.75	105.71	105.78
$U1_{p\beta,t}$	105.54	105.53	105.55	105.55			
$U1_{p\alpha}$	105.63	105.64	105.6				
$U2_{i,p}$	75.19	75.17	75.20	75.20	75.20	75.21	75.20
$U2_t$	75.43	75.42	75.43	75.46			
$U3_i$		76.29	76.29	76.29	76.27	76.30	76.27
$U3_p$	76.35	76.33	76.29				
$U3_t$	78.07	78.05	78.06	78.03			
$U4_i$		82.53	82.54	82.56	82.54	82.55	82.56
$U4_p$	82.60	82.59	82.54				
$U4_t$	74.38	74.38	74.40	74.40			
$U5_i$		78.90	78.91	78.92	78.90	78.91	78.90
$U5_p$	78.97	78.94	78.91				
$U5_t$	78.27	78.28	78.30	78.28			
$U6_{i,p}$	176.70	176.72	176.73	176.68	176.72	176.65	176.69
$U6_t$	178.00	178.03	178.04				

[a] Solvent: 0.15 M NaCl, 0.02 M sodium phosphate, pH 7, $H_2O/D_2O = 4/1$. [b] Carbon designations are as follows: A or U signifies GlcNAc or GlcA, respectively; the number indicates the carbon number in each residue; the subscript indicates the residue position in a short chain (i, p, t, for internal, penultimate, terminal); the α/β subscript refers to the anomeric form of the reducing end residue. For example, $A3_{t\beta}$ means carbon 3 of the β anomeric form of the terminal GlcNAc in a short chain.

carbonyl (GlcNAc C7) resonance can be observed, due to slow exchange of the acetamido NH). Similar ^{13}C spectra, obtained at lower field strengths, were previously reported by Bociek *et al.*,[32] Napier and Hadler,[43] and Toffanin *et al.*[44] The resonances in our spectra were assigned according to the scheme of Bociek *et al.*,[32] as confirmed by the heteronuclear shift correlation data of Hofmann *et al.*,[45] and measured chemical shifts are given in Table 1. Our chemical shift values are in excellent agreement, after consideration of the change to an internal DSS reference in the present study. No significant dependence of chemical shift on the degree of polymerization was noted for chains containing greater than 27 disaccharide units. In the analysis of high molecular weight HA samples from different tissue sources (bacterial, bovine vitreous, human umbilical cord, or rooster comb) in the same aqueous salt solution, no variation in chemical shifts was observed.

The solution ionic strength is known to affect chain rigidity (expressed as persistence length) and coil

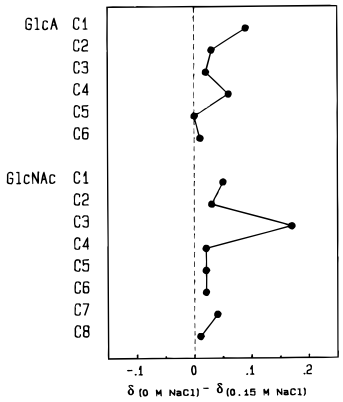


Figure 3. Effect of NaCl on ^{13}C chemical shifts observed for HA segments ($M_w = (1-2) \times 10^4$) in aqueous solution.

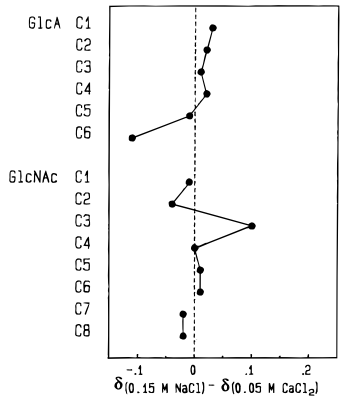


Figure 4. Effect of $CaCl_2$ *vs* NaCl on the ^{13}C chemical shift observed for HA segments ($M_w = (1-2) \times 10^4$) in aqueous solution, at constant ionic strength.

dimensions (radius of gyration) of HA in aqueous solutions, such that chains become more rigid with expanded solvated volume at lower ionic strengths.[22,24,25] In the present work, changing the solvent from 0.15 M NaCl to salt-free aqueous solution was found to have a small effect on the chemical shifts of selected carbons in HA segments. Figure 3 graphically presents the observed change in resonance position for each carbon, as the difference in chemical shift for HA in salt-free solution versus 0.15 M NaCl solution. The greatest changes occurred for GlcA C1 and GlcNAc C3, both of which were shifted downfield in the salt-free solution. Smaller changes were observed for GlcNAc C1 and GlcA C4. Similar results were obtained using an HA hexasaccharide. The same effect was also observed in a comparison of HA in 0.15 M KCl *vs* salt-free solution. There was no significant difference between HA segments or polymer in NaCl or KCl at the same ionic strength.

Calcium ions cause a more pronounced effect on the ^{13}C chemical shifts than sodium or potassium ions. HA segments or polymer in 0.05 M $CaCl_2$ solution showed small changes in the resonance positions of GlcNAc C3 and GlcA C6, relative to NaCl or KCl solution at the same ionic strength. Figure 4 shows the magnitude of these effects. A similarly greater effect of calcium than sodium counterions was found for heparin by Dais *et al.*[46] The change in the carboxylate carbon resonance reflects binding of calcium ion to HA, whereas the linear charge density of single-stranded HA is too low to induce condensation of monovalent counterions. The change in the GlcNAc C3 carbon associated with the β-1,3 linkage suggests a modest conformational change re-

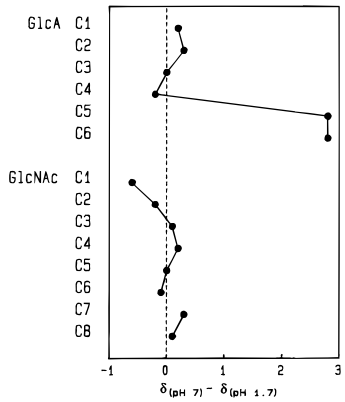


Figure 5. Effect of pH on the ^{13}C chemical shifts observed for HA segments (M_w (1−2) × 10^4) in aqueous solution.

sulting from the more effective screening of charges along the chain in $CaCl_2$ solution.

Acidification of HA solutions affects the chemical shifts of a greater number of carbons. Protonation of the carboxylate group results in large magnitude (ca. 2.8 ppm) upfield shifts of both C5 and C6 of GlcA. This observation also follows from a comparison of the neutral pH data of Bociek *et al.*[32] with the pH 2.6 data of Hofmann *et al.*[45] We have re-analyzed the pH dependence to look for smaller magnitude shifts in other carbons. As seen in Figure 5, several carbons exist in altered environments at low pH, with the greatest change (other than for the GlcA C5 or C6) observed for GlcNAc C1. It is not clear whether this change reflects conformational change or simple proximity to the carboxyl group. Similar effects of acidification were found for HA in either salt-free or salt-containing solution. In the presence of added salt, HA forms a "viscoelastic putty"[47] near pH 2.5. This physical change of the solution was not accompanied by changes in the ^{13}C chemical shifts, other than those expected on the basis of pH change alone.

In the counterion and ionic strength dependence studies described above, no correlation between chemical shift and degree of HA self-association was observed. Self-association of HA chains has been demonstrated in NaCl and possibly $CaCl_2$, but not KCl solution.[23,30,31] The pH 2.5 "putty" state also results from the formation of strong intermolecular interactions.[28] It is possible that rigid associated chains give excessively broad resonances and are therefore unobserved.[48−51] In a quantitative comparison of peak areas, we studied the ring carbon resonances of HA segments averaging 40 disaccharides in length. At constant ionic strength and as a function of the counterion present, the average peak areas showed the following relationship: NaCl < $CaCl_2$ < KCl ≈ H_2O (0.88:0.91:0.98:1.00). Thus it is possible that aggregated chain segments do not contribute to the observed peaks.

The changes in ^{13}C chemical shifts which occur for HA as a function of the solvent ionic composition are not accompanied by a change in the orientation of the substituent amide group. In an earlier study[42] of HA oligosaccharides and segments in salt-free aqueous solution, we showed that the amide proton has a coupling constant $^3J_{HNCH}$ of 9−10 Hz, indicating an approximately *trans* arrangement with respect to the GlcNAc ring proton H2. For the present study, we examined the amide proton region of ^{1}H-NMR spectra for HA segments (averaging 27 and 40 disaccharides in length) in salt-free, NaCl, and KCl solutions. In all cases, the value of $^3J_{HNCH}$ was approximately 9 Hz.

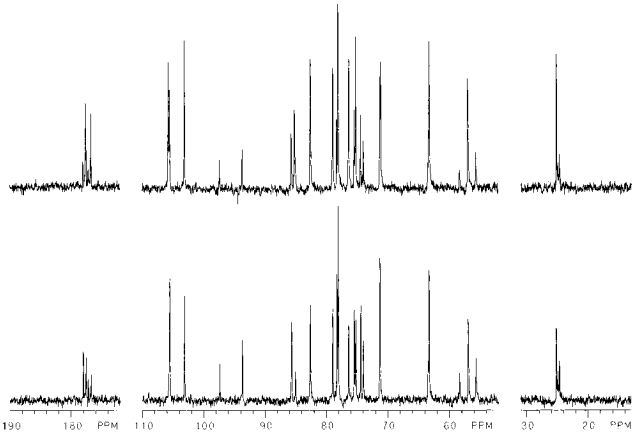


Figure 6. 75 MHz ^{13}C-NMR spectra of HA octasaccharide (GlcA−GlcNAc)$_4$ (top) and HA tetrasaccharide (GlcA−GlcNAc)$_2$ (bottom). Solvent: 0.15 M NaCl, phosphate buffered to pH 7, H_2O/D_2O = 4/1.

Analysis of HA Oligosaccharides: Dependence of Chemical Shift on Residue Position and Degree of Polymerization. Examination of HA oligosaccharides provides additional insight into the conformational sensitivity of the ^{13}C chemical shifts. In the present work, we have most extensively studied oligosaccharides having the structure GlcA−(GlcNAc−GlcA)$_{n-1}$−GlcNAc, where *n* is the number of repeating disaccharide units. Spectra of the tetrasaccharide and octasaccharide are presented in Figure 6, and chemical shift data obtained for all oligosaccharides of this sequence are provided in Table 1.

The oligosaccharide spectra are complicated by the existence of distinct environments for sugar residues in various positions along the chain. Terminal sugar residues participate in only one glycosidic linkage. Penultimate residues participate in two glycosidic linkages but retain unique environments. Interior residues constitute the last structural group. Assignment of the observed resonances to carbon atoms in each type of sugar was a stepwise process.

The carbon resonances attributed to terminal sugar residues in small oligosaccharides were assigned by consideration of the GlcA−GlcNAc disaccharide data of Inoue and Nagasawa[52] and Toffanin *et al.*[44] and the GlcA−(β)GlcNAc−O−Me disaccharide data of Sicinska *et al.*,[53] by the expected glycosidation shifts relative to monosaccharides,[54] and by analysis of relative peak areas for the series of oligomers containing 2−4 disaccharides. The measured chemical shifts for carbons in terminal residues of the oligosaccharides are given in Table 1. These are in very good agreement with the resonance positions published for HA disaccharide and with the terminal residue assignments of Toffanin *et al.*[44] for HA tetrasaccharide. They are generally in good agreement with the data of Livant *et al.*[55] for HA tetrasaccharide.

The penultimate residues of small oligosaccharides differ from terminal residues, mainly as a result of glycosidation shifts associated with the new β-1,4 linkages present. By definition, the tetrasaccharide contains only terminal and penultimate residues and serves best for assignment of resonances due to penultimate sugars. Figure 7 illustrates the chemical shift differences between penultimate and terminal residue carbons in the tetrasaccharide GlcA−GlcNAc−GlcA−GlcNAc(β). The changes are as expected for formation of a single β-1,4 linkage from GlcNAc to GlcA.

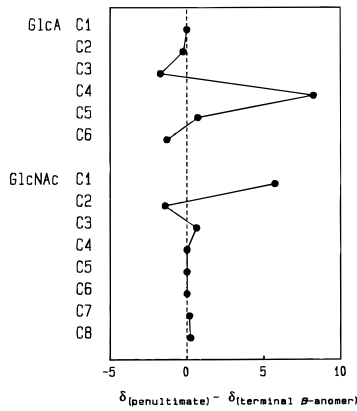


Figure 7. Effect of penultimate *vs* terminal residue position on the ^{13}C chemical shifts for HA oligosaccharides of the sequence $(GlcA-GlcNAc)_n$. A generalized sequence for this structure may also be designated $U_t-A_p-(U_i-A_i)_{n-2}-U_p-A_t$.

Further analysis of the oligosaccharide spectra revealed a difference between penultimate and more interior sugar residue environments for some carbons. Similar phenomena had been observed by Michon *et al.*[56] for α-2,8 linked oligomers of sialic acid and by Benesi and Brant[57] and Heyraud *et al.*[58] for glucose oligomers but was not seen in oligomers of *κ*-carrageenan (random coil form) by Rochas *et al.*[59] Scott and co-workers[38] found similar heterogeneity in ^1H-NMR studies of HA oligosaccharides in dimethyl sulfoxide solution. This was not anticipated in aqueous solution, since under aqueous conditions the circular dichroism properties and amide proton environments (as analyzed by ^1H-NMR) of interior and penultimate disaccharide residues in HA oligosaccharides had been found to be indistinguishable.[42,60,61] Analysis of the chain length dependence of the two environments seen by ^{13}C-NMR shows that certain carbons of penultimate sugar residues exist in environments distinct from those in more interior residues. Thus the tetrasaccharide spectrum contains resonances for only terminal and penultimate residue types, whereas the hexasaccharide and larger oligomers additionally contain resonances typical of more interior sugars (Figure 8). The assignments for all resonances observed are given in Table 1.

Figure 9 graphically displays the differences in chemical shift between penultimate and interior residues in HA hexa- and octasaccharides. Although most carbon resonances show little change, marked differences exist in GlcA C1, GlcNAc C3, and GlcNAc C4. A structural basis for this observation can be suggested by directly comparing the oligosaccharide structural sequences with the chemical shifts of the linkage carbons. The tetrasaccharide has two *β*-1,3 linkages and one *β*-1,4 linkage, whereas the hexasaccharide has three *β*-1,3 linkages and two *β*-1,4 linkages. We label each linkage with the corresponding C1 and C3 or C4 carbon chemical shifts, as follows:

Tetrasaccharide:

105.54 → 85.67 105.54 → 85.05
GlcA → GlcNAc → GlcA → GlcNAc(β)
103.12 → 82.60

Hexasaccaride:

105.53 → 85.67 105.71 → 85.13 105.53 → 85.02
GlcA → GlcNAc → GlcA → GlcNAc → GlcA → GlcNAc(β)
103.11 → 82.53 103.11 → 82.59

The *β*-1,3 linkages at both chain ends are similar,

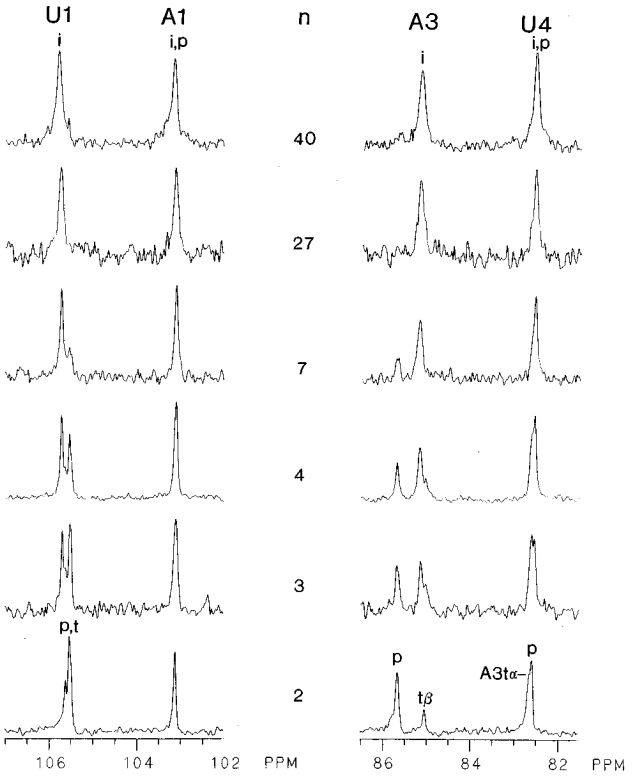


Figure 8. Partial 75 MHz ^{13}C spectra for HA oligosaccharides and fragments of the sequence $(GlcA-GlcNAc)_n$, showing heterogeneity in linkage carbon resonances as a function of residue proximity to chain termini.

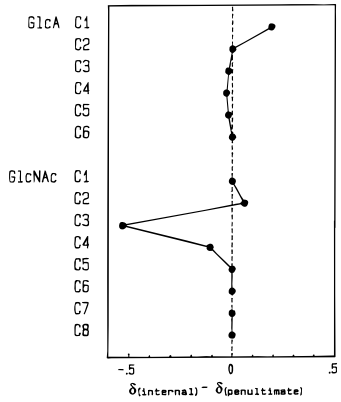


Figure 9. Effect of internal *vs* penultimate residue position on the ^{13}C chemical shifts for HA oligosaccharides of the sequence $(GlcA-GlcNAc)_n$. A generalized sequence for this structure may also be designated $U_t-A_p-(U_i-A_i)_{n-2}-U_p-A_t$.

allowing for the lack of a C1 linkage for the terminal GlcNAc and its effect on C3. These end linkages differ from the more interior 1,3 link of the hexasaccharide. The *β*-1,4 linkages are more nearly invariant along the chains. A similar pattern of chemical shift variability was seen in *ι*-carrageenan oligomers by Greer *et al.*[62]

The chemical shifts of some interior residue carbons in oligosaccharides also show slight further changes with increasing degree of polymerization (Table 1). Only two carbons from interior sugars show a significant correlation between chain length and chemical shift: GlcA C1 and GlcNAc C3. Thus a further modest change in the conformation at the *β*-1,3 linkage may occur with increasing chain length from $n = 3$ to $n > 27$.

We have also examined a set of HA oligosaccharides having the structure $GlcNAc-(GlcA-GlcNAc)_{n-1}-GlcA$. Resonance chemical shifts and assignments are given

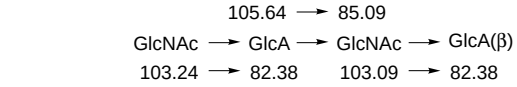
Table 2. ^{13}C Chemical Shift Data for Aqueous Solutions of HA Polymer and (GlcNAc−GlcA)$_n$ Fragments[a]

	degree of polymerization (n)			
carbon[b]	2	3	4	5250
$A1_{i,p}$	103.09	103.11	103.12	103.11
$A1_t$	103.24	103.26	103.28	
$A2_{i,p}$	56.95	56.97	56.95	56.98
$A2_t$	58.04	58.10	58.04	
$A3_{i,p}$	85.09	85.17	85.14	85.20
$A3_t$	76.54	76.57	76.55	
$A4_{i,p}$	71.13	71.13	71.08	71.06
$A4_t$	72.41	72.40	72.37	
$A5_{i,p}$	78.03	78.03	78.00	78.07
$A5_t$	78.53	78.53	78.51	
$A6_{i,p,t}$	63.30	63.26	63.19	63.27
$A7_{i,p,t}$	177.47	177.53	177.55	177.56
$A8_{i,p,t}$	25.12	25.15	25.16	25.18
$U1_{i,p}$	105.64	105.70	105.73	105.78
$U1_{t\beta}$	98.75			
$U1_{t\alpha}$	94.68			
$U2_{i,p}$	75.19	75.21	75.15	75.20
$U2_{t\beta}$	74.57[c]			
$U2_{t\alpha}$	73.85[c]			
$U3_{i,p}$	76.3	76.29	76.23	76.27
$U3_{t\beta,t\alpha}$	76.21[c]			
$U4_i$		82.56	82.54	82.56
$U4_{p,t\beta,t\alpha}$	82.38	82.45	82.42	
$U5_{i,p}$	78.91	78.90	78.91	78.90
$U5_{t\beta,t\alpha}$	79.28[c]	79.36[c]		
$U6_i$		176.69	176.74	176.69
$U6_{p,t\beta,t\alpha}$	176.7	176.77	176.83	

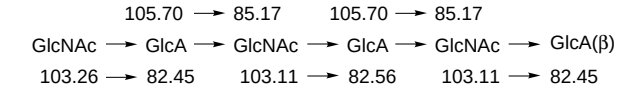
[a] Solvent: 0.15 M NaCl, 0.02 M sodium phosphate, pH 7, $H_2O/D_2O = 4/1$. [b] Carbon designations are explained in Table 1 footnote *b*. [c] Tentative assignments.

in Table 2. The spectra were more simple than those of the previous oligosaccharide set, since in this frame-shifted set the penultimate and interior residues were identical except for small changes at GlcA C4 and C6. In these oligosaccharides, the outermost linkages are the β-1,4 linkages. We found only slight differences between the outer linkages and more interior links, as follows:

Tetrasaccharide:



Hexasaccaride:

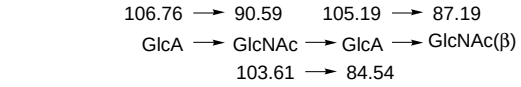


The chemical shift difference observed between the C1 carbons of the nonreducing terminal GlcNAc and internal GlcNAc is directly attributable to a glycosidation shift. Thus no evidence for a significant conformational variation in either the β-1,3 or β-1,4 linkages across the chain was found.

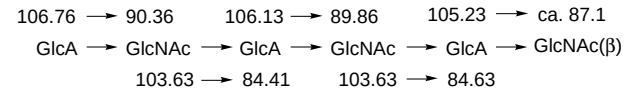
Analysis of HA Oligosaccharides in Dimethyl Sulfoxide-Containing Solutions. Scott[35−38] has provided strong evidence for the existence of an ordered HA conformation in DMSO solution, which is stabilized by interresidue hydrogen bonds. It was therefore of interest to examine the ^{13}C spectrum of HA in that solvent, for comparison with the aqueous solution data. Kvam *et al*[39] have reported the ^{13}C spectra of the tetraethylammonium and tetrabutylammonium salt of HA in DMSO at 60 °C. We were unable to obtain homogeneous solutions of the sodium salt of HA segments or polymer in DMSO at room temperature but successfully studied low molecular weight tetrasaccharide, hexasaccharide, and dodecasaccharide under those conditions. The oligosaccharides gave complicated spectra, with distinct resonances for terminal, penultimate, and interior residue carbons in most cases. By comparing the two smallest oligosaccharide spectra, the resonances which were solely due to interior residue carbons (and therefore present only in the hexasaccharide) were located. These interior resonances agree well with the (noisier) data obtained for the weakly soluble dodecasaccharide. They also agree with the data of Kvam *et al*., except for temperature-dependent shifts in GlcA C4 and GlcNAc C3, and a counterion-dependent change in GlcA C6. Most of the terminal residue carbons were assigned after consideration of linkage differences of the terminal residues and the anomeric equilibrium, which strongly favors the α anomer of GlcNAc at the reducing end. Further assignments were made by studying the chemical shift dependences on solvent composition in DMSO/H_2O mixtures. For this purpose, the hexasaccharide was examined in a mixed solvent containing 3, 7, 14, 24, 45, and 60% H_2O. On the basis of the assignments previously made for the hexasaccharide in purely aqueous solution, and the pattern of changes as a function of increasing DMSO content, the resonances observed in pure DMSO were assigned. The chemical shifts and resonance assignments for oligosaccharides in DMSO are listed in Table 3.

The linkage carbons of the tetrasaccharide and hexasaccharide show greater variability in DMSO than in H_2O:

Tetrasaccharide:



Hexasaccaride:



Although the β-1,4 linkages are all reasonably equivalent, the β-1,3 linkages show a wide range of chemical shifts for the constituent carbons. Examination of Table 3 also reveals multiple environments for carbons of the substituent carboxyl and acetamido groups.

A direct comparison of the ^{13}C-NMR data for HA hexasaccharide in DMSO *versus* H_2O can be used as an aid to interpretation. Figure 10 shows the difference in chemical shift for each *interior* residue carbon of the hexasaccharide, found by comparing DMSO and H_2O solution data. All but one of the resonances move downfield in DMSO, with the most common shift being approximately 1−2 ppm. This movement may represent a general solvent effect, or a problem in setting the proper reference position in the DMSO solution. (The DMSO carbon resonance also appears approximately 2.0 ppm downfield in the pure solvent in comparison with a 40% DMSO, 60% H_2O mixture. The referencing procedure is explained in the experimental section.) From Figure 10 it is clear that several carbons exhibit large magnitude changes relative to the general trend. Such changes are found for GlcNAc C3 and C7 and GlcA

Table 3. ^{13}C Chemical Shift Data for DMSO Solutions of (GlcA−GlcNAc)$_n$ Fragments of HA

	degree of polymerization (n)		
carbon[a]	2	3	6
$A1_{i,p}$	103.61	103.63	103.63
$A1_{t\beta}$	98.70	98.64	
$A1_{t\alpha}$	94.16	94.22	
$A2_i$		58.01	58.1
$A2_p$	58.07	58.09	
$A2_{t\beta}$	59.60	59.57	
$A2_{t\alpha}$	56.79	56.79	
$A3_i$		89.86	89.9
$A3_p$	90.59	90.36	
$A3_{t\beta}$	87.19		
$A3_{t\alpha}$	81.92	81.89	
$A4_{i,p}$	72.55	72.53	72.59
$A4_{t\beta,t\alpha}$	72.90	72.88	
$A5_i$		80.36	80.21
$A5_p$	80.46	80.43	
$A5_{t\beta}$	79.94	79.93	
$A5_{t\alpha}$	75.42	75.43	
$A6_{i,p,t}$	64.66	64.61	64.62
$A7_{i,p}$	175.57	175.65	175.56
$A7_{t\beta}$	174.00	174.0	
$A7_{t\alpha}$	173.41	173.40	
$A8_{i,p,t\beta}$	26.69	26.72	26.69
$A8_{i,\alpha,t}$	26.28	26.30	
$U1_i$		106.13	106.1
$U1_p$	105.19	105.23	
$U1_t$	106.76	106.76	
$U2_i$		76.45	76.36
$U2_p$	76.01	76.03	
$U2_t$	76.96	76.95	
$U3_i$		77.50	78.0
$U3_p$	78.09	78.06	
$U3_t$	76.57	76.58	
$U4_i$		84.41	84.4
$U4_p$	84.54	84.63	
$U4_t$	75.59	75.57	
$U5_i$		79.10	79.2
$U5_p$	79.27	79.29	
$U5_t$	79.27	79.35	
$U6_i$		175.04	174.98
$U6_p$	174.84	174.74	
$U6_t$	175.67	175.75	

[a] Carbon desginations are explained in Table 1.

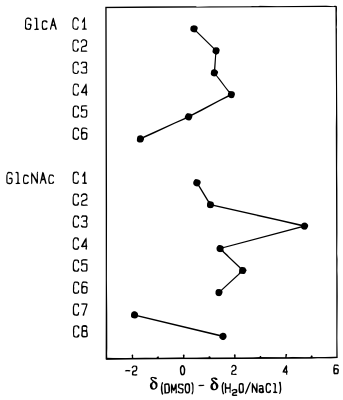


Figure 10. Effect of solvent—DMSO *vs* buffered aqueous salt solution—on the ^{13}C chemical shifts for internal residues of HA hexasaccharide, (GlcA−GlcNAc)$_3$.

C6, with probable significant changes in GlcNAc C1 and GlcA C1 and C5. The same pattern of chemical shift changes is observed in the data of Kvam *et al.*[39] for HA salts at 60 °C. The solvent-sensitive carbons fall into the following groups: linkage carbons (especially those of the 1−3 linkage), acetamido carbonyl carbons, and carboxyl group-associated carbons. The substantial changes in their electrostatic environments in DMSO relative to H_2O presumably reflect solvational, conformational, and hydrogen bonding differences.

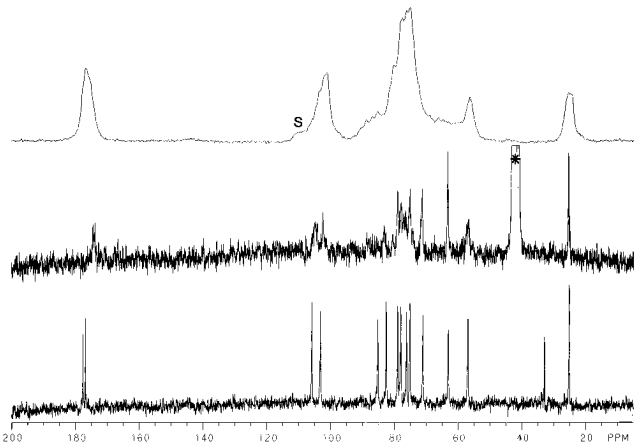


Figure 11. 75 MHz ^{13}C spectra of HA, comparing solid state, DMSO solution, and aqueous solution. Top: solid state CPMAS spectrum of streptococcal NaHA. The peak labeled (s) is a spinning sideband. Middle: solution spectrum of HA dodecasaccharide (partially aggregated) in DMSO. The peak labeled with an asterisk is due to the solvent. Bottom: solution spectrum of HA segment ($M_w = 1 \times 10^4$) in 0.15 M NaCl, 0.02 M phosphate buffer, pH 7.

Comparison of HA Conformation in Solution and the Solid State. In view of the substantial ^{13}C-NMR chemical shift differences observed between HA oligosaccharides in aqueous and DMSO solvent systems, we have re-examined our previously published ^{13}C-CPMAS spectrum obtained for dry NaHA in a 4-fold helical form.[19] Figure 11 compares spectra obtained for the HA segment in aqueous solution, HA dodecasaccharide in DMSO, and the HA polymer in the solid state. The DMSO and solid state spectra are remarkably similar to one another but differ from the aqueous solution spectrum. The main differences occur in the carboxyl and carbonyl carbons, and in the linkage carbons. The C1 carbons of GlcA and GlcNAc are shifted slightly upfield in DMSO and the solid, while the aglycone carbons are shifted substantially downfield.

Discussion

This study is part of an effort to determine the conformation of HA in physiological solution, and to investigate its flexibility, dynamics, and sensitivity to environmental perturbations. The present work reports the ^{13}C chemical shifts for HA samples in both aqueous and organic solvent systems and compares these data with previous work on the solid state ^{13}C properties of HA.

The conformation of HA is reasonably well established for the solid state and for solution in DMSO. In the solid state at low relative humidity, NaHA adopts a condensed 4-fold left-handed helical form. Each disaccharide repeat has two intramolecular hydrogen bonds. The first exists between the amide NH of GlcNAc and a carboxylate oxygen of GlcA across the β-1,4 glycosidic linkage. The second stabilizes the β-1,3 linkage, being from GlcNAc C4OH to the GlcA O5. In DMSO, there exist 2−4 hydrogen bonds per disaccharide. The two well-estabilished[39] hydrogen bonds are the same as

those observed for NaHA in the solid state. Weaker experimental evidence coupled with model building has been applied to suggest adoption of a 2-fold helix in DMSO, with two additional hydrogen bonds per disaccharide. One joins GlcA C3OH to GlcNAc O5 across the β-1,4 linkage, and the second joins GlcA C2OH to the GlcNAc amide CO across the β-1,3 linkage. Using a more conservative analysis of the DMSO data, the condensed 4-fold helix seen in the solid state would also be compatible with the experimental evidence in DMSO solution. Direct comparison between the ^{13}C spectra of NaHA in the solid state and in DMSO solution shows marked similarities (Figure 11).

NaHA in aqueous solution shows a different characteristic ^{13}C spectrum. Relative to DMSO solution or dry NaHA, the largest differences in chemical shift exist for carbons of the β-1,3 glycosidic linkage, and the substituent amide and carboxylate groups (Figure 11). Smaller changes occur for carbons of the β-1,4 linkage. These data suggest altered average conformations about the two linkages, and changes in the environments of the bulky substituent groups in aqueous solution.

The solvent change from DMSO to H_2O is correlated with an increase in HA chain mobility. We have observed that the spin−lattice relaxation times at 75 MHz for linkage carbons of an HA tetrasaccharide change from approximately 150−170 ms in DMSO to approximately 270−320 ms in H_2O (Cowman, Feder-Davis, and Hittner, unpublished observations).

The environment of the amide substituent is already known to differ for HA in aqueous solution *versus* DMSO solution. Thus the coupling constant $^3J_{HNCH}$ measured by ^{1}H-NMR suggests a nearly *trans* arrangement of the amide proton with the ring proton H2 in aqueous solution (recently confirmed by proton−proton NOE measurements[63]), but a substantially rotated orientation in DMSO. In the *trans* orientation, a direct hydrogen bond from the amide proton to the carboxylate oxygen of the adjacent sugar residue cannot form across the β-1,4 linkage. This same amide orientation also precludes a hydrogen bond linking the amide CO with a GlcA C2OH across the β-1,3 linkage. (As discussed above, this hydrogen bond may also be absent in DMSO solution). Scott and co-workers[33,34] have proposed a water-mediated hydrogen bond from the amide NH to the carboxylate, but absence of the hydrogen bond from the C2OH to amide CO, in aqueous solvent. The slow exchange rate of the amide proton is in accord with this proposal. Our ^{13}C data are also compatible with this proposal in that we see little variation in the chemical shifts of carbons of the 1,4 linkage in aqueous solution but provide no direct support for a stable water-mediated hydrogen bond across the β-1,4 linkage.

If the conformation at the β-1,3 linkage differs between HA in aqueous solution and in DMSO or the solid state, it is plausible that all hydrogen bonds crossing that linkage are disrupted by solvent competition in H_2O. Such a model is supported by our studies on the sensitivity of the ^{13}C chemical shifts to variations in aqueous solvent ionic composition, to HA fragment chain length, and to residue position in short chains. Thus the greatest variability in chemical shifts is observed for GlcA C1 and GlcNAc C3. In this respect, our data are in accord with the results of Bociek *et al.*,[32] who found the carbons of the 1,3 linkage most sensitive to alkali. These changes occur despite the constancy of the amide proton coupling constant and thus the substituent group orientation. Chain expansion is most strongly correlated with a downfield shift of both carbons of the β-1,3 linkage, rather than the β-1,4 linkage. We further observe the existence of a distinct average conformation for linkages which occur at the ends of oligomeric chains, only if the linkages are β-1,3 and not β-1,4.

Our interpretation of the ^{13}C chemical shift data as signifying greater conformational changes in the β-1,3 linkage of HA than in the β-1,4 linkage is in direct conflict with the recent molecular dynamics (MD) simulations of Holmbeck *et al.*[63] In their unrestrained MD simulations, the 1,4 linkage showed greater flexibility than the 1,3 linkage. However, the structures generated were not compatible with interproton distances calculated from their NOE data. MD simulations restrained by the NOE data also indicated two conformations for the 1,4 linkage, but only one for the 1,3 linkage. Conformational energy minimization calculations have provided a slightly different view. In the recent work of Holmbeck *et al.*,[63] as well as earlier work by Potenzone and Hopfinger,[26,27] the β-1,4 linkage exhibits an inherently smaller available conformational space than the β-1,3 linkage. Our ^{13}C data are in better accord with these calculations, in that chemical shift variability resides predominantly with the β-1,3 linkage.

The magnitude of conformational changes occurring at the β-1,3 linkage in response to changes in solvent ionic composition or chain molecular weight may be small. Bock *et al.*[64] have correlated the distance of separation of the glycosidic and aglycone protons with linkage carbon chemical shift variations. A variation of approximately 0.5 ppm in carbon chemical shift may correspond to a change of approximately 0.05 Å in the interproton distance. A similar analysis of the magnitude of conformational difference existing between HA in aqueous solution and the solid state or DMSO solution is complicated by the rotation of the amide group, which may also affect the linkage carbon electrostatic environments.

In summary, the ^{13}C spectrum of HA has been shown to be sensitive to changes in HA conformation. The conformations adopted in DMSO solution and for NaHA in the solid state appear to be similar by this criterion. The aqueous solution conformation differs, having an altered amide orientation, a probable alteration in the β-1,3 glycosidic linkage conformation, and a possible alteration in the β-1,4 linkage conformation. On the basis of the chemical shift variability, the β-1,3 linkage appears to be the major source of conformational alteration for HA in aqueous solution. We also observe evidence for a difference in average conformation for β-1,3 linkages occurring at the ends of oligomeric chains, relative to more interior linkages. These results suggest the absence of any stabilizing hydrogen bond across the 1,3 linkage of HA in aqueous solution. Nevertheless, the rigidity of isolated single-stranded HA chains is best attributed to limited conformational freedom at both glycosidic linkages. The overall hydrodynamic properties of HA depend on both the isolated chain rigidity and the well-documented tendency of HA chains to self-associate in physiological solutions.

Acknowledgment. Portions of this data were described in the Ph.D. dissertation of Hittner,[65] and were presented at the 4th Chemical Congress of North America and 202nd National Meeting of the American Chemical Society, New York, 1991.

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