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^{1}H- and ^{13}C-NMR studies of solutions of hyaluronic acid esters and salts in methyl sulfoxide: comparison of hydrogen-bond patterns and conformational behaviour

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ABSTRACT

The ^{1}H- and ^{13}C-NMR spectra of the ethyl and benzyl esters and the tetrabutylammonium and tetraethylammonium salts of hyaluronic acid {→2)-β-D-Glc*p*A-(1 → 3)-β-D-Glc*p*NAc-(1→}$_n$ in Me_2SO-d_6 have been assigned using 1D and 2D techniques. The chemical shifts of the resonance of GlcNAc C-3 suggest that the relative orientations of the monosaccharides at the (1 → 3) linkage in the esters and salts are different. Small differences in the chemical shifts of the resonance GlcA C-4 suggest only a slight conformational variation around the (1 → 4) linkage. The ^{13}C-NMR data also suggest similarities in conformation between the esters in Me_2SO-d_6 and the salts in water. The chemical shifts of the ^{1}H resonances for NH and OH groups and their temperature dependence for the esters and salts in Me_2SO reveal markedly stronger inter-residue hydrogen bonds between the carboxyl and NH groups and between HO-4 of GlcA and O-5 of GlcNAc for the salts. The $^3J_{2,NH}$ values indicate a slightly different orientation for the acetamido group. For solutions in Me_2SO, the higher segmental flexibility of the esters is supported by the line widths, whereas the reduced viscosity for the tetrabutylammonium salt showed a sigmoidal concentration dependence and suggests association of chains which could contribute to the segmental rigidity. The linear concentration dependence for the benzyl ester suggests a higher overall flexibility without chain association.

INTRODUCTION

Hyaluronate {→4)-β-D-Glc*p*A-(1 → 3)-β-D-Glc*p*NAc-(1→}$_n$ behaves like a fairly stiff coil in aqueous solutions[1–3]. On the basis of rheology[1] and X-ray studies of the acid form[4], it has been suggested that several inter-residue hydrogen bonds

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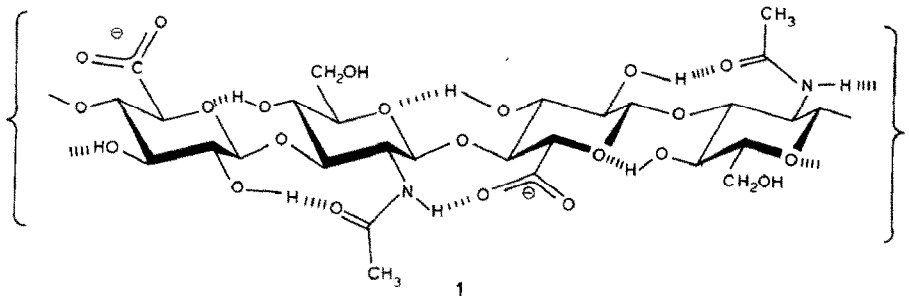

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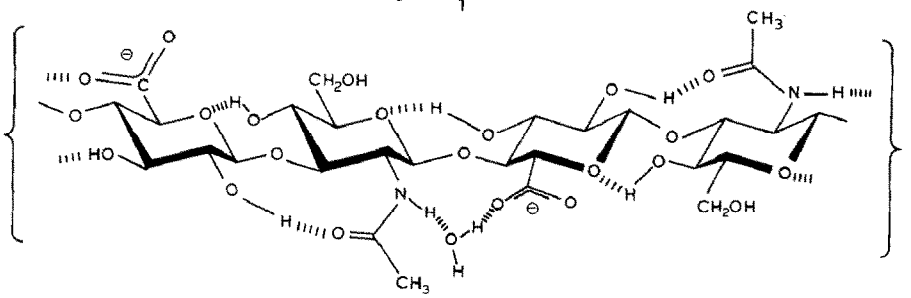

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exist, which may contribute to this stiffness[5]. These bonds can also explain the low reactivity of hyaluronate towards periodate oxidation[6].

Several NMR studies have focussed on the role of the amide group in the secondary structure of oligosaccharides[7–10] derived from hyaluronic acid. On the basis of ^{1}H-NMR studies of a solution of the sodium salt of the hexasaccharide in Me_2SO-d_6, it was concluded[8] that there is an intramolecular hydrogen bond between HO-2 of GlcA and the C=O of GlcNAc, and another between the NH and the carboxyl group (see **1**). However, similar studies[9] of aqueous solutions of hyaluronate and its oligosaccharides revealed no evidence for strong, direct hydrogen bonds between the NH and COO^- groups in water. Subsequently, it was demonstrated[10] that when water was added stepwise to a solution of the hexasaccharide in Me_2SO-d_6, the NH signal gradually shifted towards the value found in an aqueous solution, and the saturation-transfer experiments suggested a slow exchange of the NH protons in the internal residues. Thus, the existence of a water-bridged hydrogen bond between the NH and the COO^- groups in aqueous solutions of hyaluronate was proposed (see **2**), which requires slight modification of the previously suggested[1,7,10] secondary structure **1**.

The ^{1}H- and ^{13}C-NMR spectra of hyaluronate in aqueous solutions have been assigned for different pH values and temperatures[11–13]. However, for solutions in Me_2SO-d_6, the ^{1}H-NMR spectra of the oligosaccharides have been assigned only partially due to the overlap of several signals[8], and the studies of the polymer have dealt solely with the acetamido resonances[7,14]. No NMR data on esters of hyaluronic acid have been published. The present study was aimed at a complete assignment

of the ^{1}H- and ^{13}C-NMR spectra for the benzyl and ethyl esters and the tetraethylammonium and tetrabutylammonium salts dissolved in Me_2SO-d_6.

Association tendencies have been proposed for the chains of hyaluronate in water[1,5,15], although other results[2] suggest that the presence of aggregates may reflect the manner in which the sample was prepared. However, the presence of associated species in solution in Me_2SO-d_6 has not been reported hitherto. Therefore, viscosity measurements and NMR studies have been undertaken in order to compare the conformations of the salts and esters of hyaluronic acid in solution in Me_2SO.

EXPERIMENTAL

Methods.—NMR spectra were recorded at 4.70 T (^{1}H, 200 MHz; ^{13}C, 50.3 MHz) or 7.05 T (^{1}H, 300 MHz; ^{13}C, 75.4 MHz) with a Bruker AC 200 or AM 300 WB instrument, respectively. Unless otherwise noted, 5-mm tubes (o.d.) were used. Chemical shifts for solutions in Me_2SO-d_6 are referenced indirectly to Me_4Si by using the solvent signal, which varied with the temperature. The following relationships were found by linear regression of six values of the chemical shift determined for the resonances of the methyl group of Me_2SO-d_6 with respect to Me_4Si in the range 24–80°: ^{1}H $(2.508 - t3.036E - 4)$ ppm, ^{13}C $(39.692 + t3.417E - 4)$ ppm, where t is the temperature. For solutions in D_2O, ^{13}C shifts at 60° are referred to 1,4-dioxane, for which a shift of 66.44 ppm relative to Me_4Si was determined in Me_2SO-d_6, giving comparable shift scales in water and Me_2SO-d_6. These shifts are systematically ~ 1.1 ppm lower than those reported[11,12], where they were referred via sodium 4,4-dimethyl 4-silapentanesulfonate to Me_4Si in a capillary (see also the note added in proof in ref. 12).

^{1}H–^{1}H correlation spectroscopy (COSY) was performed at 200 MHz with the second pulse being $\pi/4$ (COSY 45). The sample concentration was 32 mg/mL, the spectral width was 1500 Hz (7.5 ppm), and the repetition time was 1.2 s. In the F_2 and F_1 dimensions, 1024 and 512 data points were used, respectively, with zero-filling in F_1. Centered sine-bell window multiplication was used in each dimension. NOESY of the same sample was performed in the phase-sensitive mode with a mixing time of 100 ms, using the same spectral widths as for COSY, but a Lorentzian–Gaussian transformation was applied prior to Fourier transformation with a line broadening (LB) and Gaussian broadening (GB) of -8 and 0.5, respectively (Bruker terminology).

Heteronuclear (^{1}H–^{13}C) 2D chemical shift correlation spectroscopy was performed with ^{13}C detection (75 MHz), typically using 40 mg of sample/mL in 10-mm (o.d.) tubes. The applied pulse sequence gave proton decoupling in each dimension. For the benzyl ester, the repetition time was 2 s. Spectral widths of 8000 and 2400 Hz, and 4096 and 512 data points were used in F_2 and F_1, respectively. Zero-filling was applied in F_1. Lorentizian–Gaussian transformation was applied in each dimension, with LB -5 and GB 0.15 in F_1 and LB -5 and GB

0.05 in F_2. For the other compounds, somewhat smaller spectral widths and shorter repetition times were used. All pulse sequences for the 2D experiments involved standard Bruker software.

Simulation and fitting of the ^{1}H-NMR spectrum of benzyl hyaluronate (carbohydrate part) was performed with a modified version of the LAOCN3 programme[16].

Reduced viscosities (η_{sp}/c, where c is the concentration of the polysaccharide) were determined at 40° in a Schott Geräte AVS 440 viscometer, using an Ubbelohde 0_c capillary.

Materials.—The benzyl and ethyl esters, synthesised under homogeneous conditions[17], and the tetrabutylammonium and sodium hyaluronates were obtained from FIDIA (Abano Terme, Italy). Molecular weights were determined by low-angle laser-light scattering as described[18] and weight-average molecular weights ($\bar{M}_w$) of the samples used for NMR studies were 20500, 47000, and 34100 for the sodium, tetrabutylammonium, and benzyl hyaluronates, respectively. The tetraethylammonium salt was prepared from the sodium salt by conversion into the acid form, autohydrolysis at 90° for 3 h, neutralisation with aq tetraethylammonium hydroxide to pH 6.0, and lyophilisation. Molecular weights were not determined for the tetraethylammonium and ethyl hyaluronates. The samples of benzyl and tetrabutylammonium hyaluronate, used for the viscosity measurements, had molecular weights of 140000 and 211000, respectively. Samples were dissolved in, and diluted with, 0.25 M tetrabutylammonium bromide (Fluka) in dry Me_2SO (Merck) and filtered through a membrane filter (0.5 μm, Millipore) prior to the viscosity measurements, which were performed twice for each dilution.

RESULTS AND DISCUSSION

Assignment of NMR spectra.—The ^{1}H resonances of the esters were assigned on the basis of COSY 45 experiments. Fig. 1 shows a part of the contour plot obtained for the benzyl ester. The NH and OH groups were identified from the COSY cross-peaks with their CH partners. The chemical shifts for the CH resonances were confirmed by 2D ^{1}H–^{13}C chemical shift correlation spectroscopy with ^{1}H decoupling in each dimension, as shown in Fig. 2 for the benzyl ester. The protons of the phenyl ring gave a complex, higher-order pattern centered at ~7.36 ppm for which no detailed assignment was performed. In order to facilitate determination of the coupling constants, a ^{1}H-NMR spectrum of the benzyl ester in Me_2SO-d_6 was obtained after adding D_2O to remove signals from exchangeable protons. The temperature (65°) was chosen so as to shift the water signal to a zone with no other ^{1}H signals. Spectral simulation and fitting of shifts and couplings was then performed, and the best fit values are given in Table I. The chemical shifts deviate slightly from those found for solutions in dry Me_2SO-d_6 (Table I), possibly due to changes in the hydrogen bonds in the presence of added D_2O.

The ^{1}H–^{1}H COSY plots of the tetra-alkylammonium salts were dominated by the signals of tetraethylammonium and tetrabutylammonium ions, which made the

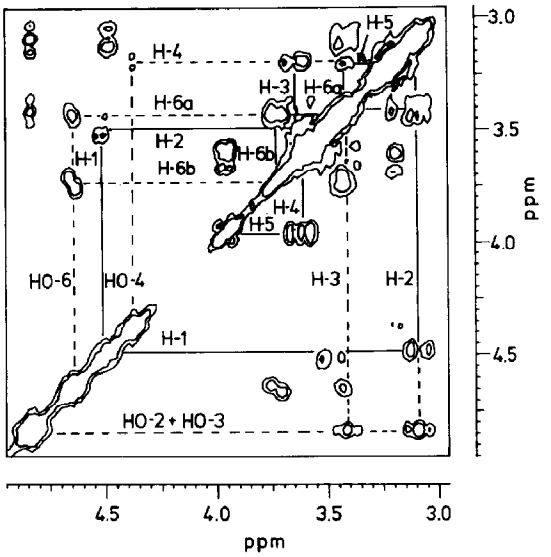


Fig. 1. Expansion of the $^{1}H-^{1}H$ chemical shift correlation spectroscopy (COSY 45) contour plot for a solution of benzyl hyaluronate in Me_2SO-d_6 at 40°. Couplings between C-linked protons are indicated by fully drawn lines; couplings involving NH or OH protons are indicated by dashed lines. GlcNAc and GlcA couplings are indicated above and below the diagonal, respectively.

plots difficult to assign. However, all the ^{13}C signals were well separated and allowed the assignment of the ^{1}H resonances by 2D $^{1}H-^{13}C$ chemical shift correlation spectroscopy on a sample of reduced chain length, as shown in Fig. 2.

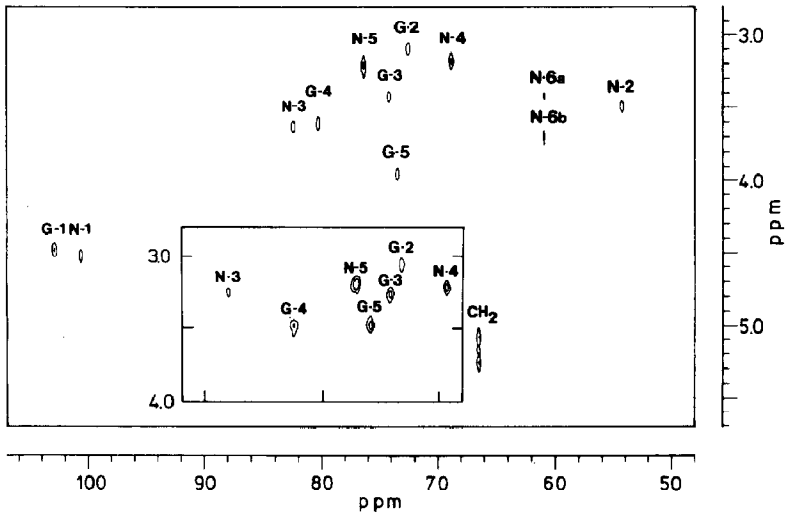


Fig. 2. A $^{1}H-^{13}C$ chemical shift correlation spectroscopy contour plot obtained for a solution of benzyl hyaluronate in Me_2SO-d_6 at 40°. G and N denote GlcA and GlcNAc correlations, respectively. The phenyl and acetyl signals are omitted from the plot. The insert shows part of the plot obtained for a solution of tetraethylammonium hyaluronate in Me_2SO-d_6 at 60°.

TABLE I

^{1}H-NMR data (δ in ppm, J in Hz) for solutions of benzyl, ethyl, and tetraethylammonium hyaluronates in Me_2SO-d_6

Residue	Benzyl ester					Ethyl ester		Tetraethylammonium salt	
		δ [a]	δ [b,c]	J [c]			δ [d]		δ [e]
GlcA	H-1	4.47	4.446	$J_{1,2}$	7.7	H-1	4.46	H-1	4.15
	H-2	3.09	3.103	$J_{2,3}$	8.4	H-2	3.09	H-2	3.08
	H-3	3.41	3.423	$J_{3,4}$	8.6	H-3	3.41	H-3	3.26
	H-4	3.62	3.646	$J_{4,5}$	9.3	H-4	3.57	H-4	3.47
	H-5	3.96	3.958			H-5	3.87	H-5	3.47
	$-CH_2-$	5.16 [f]	5.16 [f]	$J_{A,B}$	−12.7	$-CH_2-$	4.15		
	Ph	7.36	7.36			$-CH_3$	1.24		
GlcNAc	H-1	4.52	4.515	$J_{1,2}$	8.4	H-1	4.51	H-1	4.63
	H-2	3.51	3.485	$J_{2,3}$	8.5	H-2	3.48	H-2	3.47
	H-3	3.65	3.622	$J_{3,4}$	7.7	H-3	3.65	H-3	3.25
	H-4	3.19	3.176	$J_{4,5}$	9.1	H-4	3.20	H-4	3.21
	H-5	3.22	3.207	$J_{5,6a}$	5.3	H-5	3.23 [g]	H-5	3.19
				$J_{5,6b}$	0				
	H-6a	3.43	3.422	$J_{6a,6b}$	−10.9	H-6a	3.43	H-6a	3.50
	H-6b	3.73	3.712			H-6b	3.72	H-6b	3.76
	Ac	1.75	1.75			Ac	1.76	Ac	1.76

[a] Obtained at 40° for a solution at 32 mg/mL. [b] Obtained at 65° for a solution (32 mg/mL) containing D_2 (60 mg/mL). [c] Best-fit values obtained by simulation and iteration with a LAOCN3 programme ($J_{A,B}$ wa measured from the spectrum). [d] Obtained at 40° for a solution at 30 mg/mL. [e] Obtained at 65° for a solutic at 30 mg/mL; signals for Et_4N^+ ions are omitted. [f] Centre of the AB dd. [g] From heterocorrelatic experiment at 75°.

The three peaks for GlcNAc C-5 and GlcA C-2,3 were easily identified in the plots (cf. Fig. 2), which facilitated the assignment of the resonance for GlcA C-5, the chemical shift of which depends on the presence or absence of the neighbouring charged group. The derived ^{1}H chemical shifts are given in Table I and the ^{13}C assignments in Table II. The chemical shift data for the tetrabutylammonium salt were similar to those of the degraded tetraethylammonium salt despite the different molecular weights, suggesting that the ^{1}H and ^{13}C chemical shift data of the salts are truly representative of the polymer.

The ^{13}C-NMR spectra of tetrabutylammonium and tetraethylammonium hyaluronate dissolved in D_2O were assigned in order to allow a comparison with the data for solutions in Me_2SO-d_6 (see Table II). The data for the salts accorded with those reported[12] for aqueous sodium hyaluronate. The assignments were confirmed by 2D heterocorrelation spectroscopy (plot not shown). Apparently, the rather lipophilic tetra-alkylammonium counterions do not alter the conformation of the polysaccharide in water, as observed from the ^{13}C chemical shifts.

The chemical shift of the resonance of GlcA C-6 is sensitive to the solvent and the presence or absence of charge on the carboxyl group. The chemical shift of the C-5 resonance was less sensitive to the neighbouring charge, but its identification

TABLE II

^{13}C-NMR data [a] (δ in ppm) for solutions of hyaluronates at 60°

GlcA residues										
Hyaluronate	C-1	C-2	C-3	C-4	C-5	C-6	CH_2	C-1′	C-2′,6′	C-3′,4′,5′
Benzyl ester	102.9	72.6	74.1	80.2	73.5	167.3	66.4	135.4	128.0	128.3
Ethyl ester	102.9	72.6	74.3	80.5	73.5	167.5	61.1	13.9 (CH_3)		
Bu_4N^+ salt	103.1	73.0	74.0	82.4	75.6	169.8				
Et_4N^+ salt	103.0	73.0	74.0	82.3	75.6	169.8				
Bu_4N^+ salt (D_2O)	103.6	73.1	74.2	80.5	76.9	174.3				
GlcNAc residues										
Hyaluronate	C-1	C-2	C-3	C-4	C-5	C-6	CH_3	C=O		
Benzyl ester	100.5	54.4	82.5	68.9	76.4	61.0	23.0	169.7		
Ethyl ester	100.8	54.4	82.6	68.9	76.4	61.1	23.0	169.6		
Bu_4N^+ salt	100.7	54.5	88.2	69.2	76.9	61.3	22.9	172.0		
Et_4N^+ salt	100.6	54.4	87.8	69.2	76.9	61.2	22.9	171.9		
Bu_4N^+ salt (D_2O)	100.9	54.8	83.5	69.2	76.0	61.2	23.0	175.3		

[a] For solutions in Me_2SO-d_6 unless stated otherwise; signals for Bu_4N^+ and Et_4N^+ omitted.

was crucial for an unambiguous assignment of the remainder of the spectrum (cf. Table II). The chemical shift in the literature for the resonance of GlcA C-5 of the aqueous acid form[13] corresponds to 73.7 ppm (see Experimental for comments on choice of reference) which agrees well with the value (73.5 ppm, cf. Table II) found for the esters. For the salt, the ^{13}C chemical shifts of the resonances of GlcA C-6 and C-5 are lower for solutions in Me_2SO-d_6 than in water, but the values were lowest for the esters. All other chemical shifts were similar (within 0.5 ppm) for solutions of the ester in Me_2SO-d_6 and the acid form (pH 2.6) in water[13].

Conformational behaviour.—Phase-sensitive NOESY experiments with the benzyl ester showed cross-peaks for 1,3-diaxial protons of both rings (Fig. 3), which confirmed the $^4C_1$ conformation for the GlcNAc and GlcA residues. However, because of the partial overlap of the signals for H-1 and also those of GlcNAc H-3 with GlcA H-4, the inter-residue cross-peaks could not be identified unequivocally.

The resonance of GlcNAc C-3 appeared at 88.2 ppm for a solution of the tetrabutylammonium salt in Me_2SO-d_6, whereas it appeared at 83.5 ppm for a solution in D_2O, which is closer to the shifts of the corresponding resonances of the benzyl and ethyl esters (82.5 and 82.6 ppm, respectively) in solution in Me_2SO-d_6. Since the chemical shift of the resonance of GlcNAc C-3 is sensitive to changes in conformation, as shown[12] for the alkali-induced conformational transition of sodium hyaluronate, the differences observed suggest a different orientation of groups around the (1 → 3) linkage. A parallel regarding the orientation at the (1 → 4) linkage is provided by the resonance of GlcA C-4. For a solution of the tetrabutylammonium salt in Me_2SO-d_6, this resonance appeared at 82.4 ppm, whereas for solutions of the benzyl ester in Me_2SO-d_6 and the tetrabutylammo-

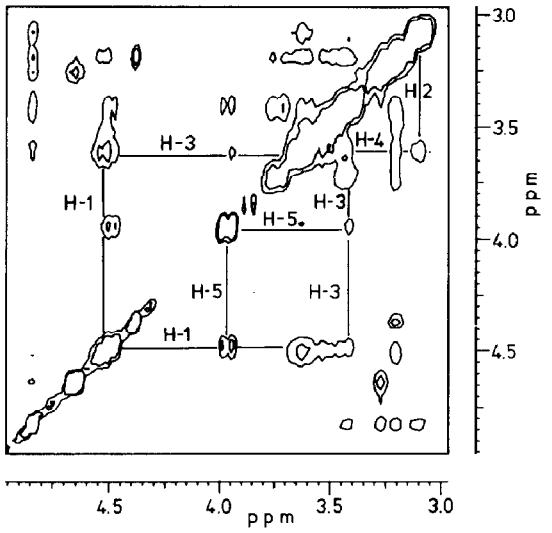


Fig. 3. Contour plot for a 2D $^1H-^1H$ NOE spectroscopy (NOESY) experiment recorded in the phase-sensitive mode for a solution (32 mg/mL) of benzyl hyaluronate in Me_2SO-d_6 at 40°. A mixing time of 100 ms was used. GlcNAc and GlcA NOESY inter-residue cross-peaks are indicated above and below the diagonal, respectively.

nium salt in water, it appeared at 80.2 and 80.5 ppm, respectively (Table II). These values suggest a similar relative orientation of the residues at both glycosidic bonds for solutions of the esters in Me_2SO and the salts in water, but also that the latter behave differently in solution in Me_2SO-d_6. All the ^{13}C chemical shifts, except those of the resonances of GlcA C-5 and C-6, and the acetamido carbonyl group of the ester in Me_2SO-d_6, are similar to those of the salt in water. This finding indicates that the water-bridged hydrogen bond between the NH and the carboxyl group, proposed[10] for the aqueous hyaluronic acid (cf. **2**), has little or no influence on the chemical shifts of the resonances of the surrounding carbon nuclei.

The temperature dependence of the chemical shifts of the OH and NH resonances are given in Fig. 4. The assignment for the tetraethylammonium salt accords with values[8] for the internal residues of solutions of oligosaccharides in Me_2SO. For the NH group, the chemical shift data for solutions of the tetraalkylammonium salts in Me_2SO (8.9–9.4 ppm, Fig. 4) correspond well with the values (9.2 ppm[8] and 9.3 ppm[7]) reported for solutions in Me_2SO-d_6 of the hexasaccharide at 25° and for other polymer salts, respectively. These values have been attributed to a strong $NH \cdots OCO^-$ hydrogen bond of internal residues[5,19]. The chemical shifts for the NH resonances of the esters (7.1–7.5 ppm, cf. Fig. 4) suggest weak hydrogen bonding, since they are comparable to those found for the GlcNAc reducing ends of solutions of hyaluronate oligosaccharides in Me_2SO (7.8 ppm) for which this bond is absent[8]. This assumption is supported further by the observed $^3J_{2,NH}$ values. The value (8.1 Hz) found for the benzyl ester at 80° was similar to that (8.2 Hz, ref. 8) of the β anomer of the GlcNAc reducing end of

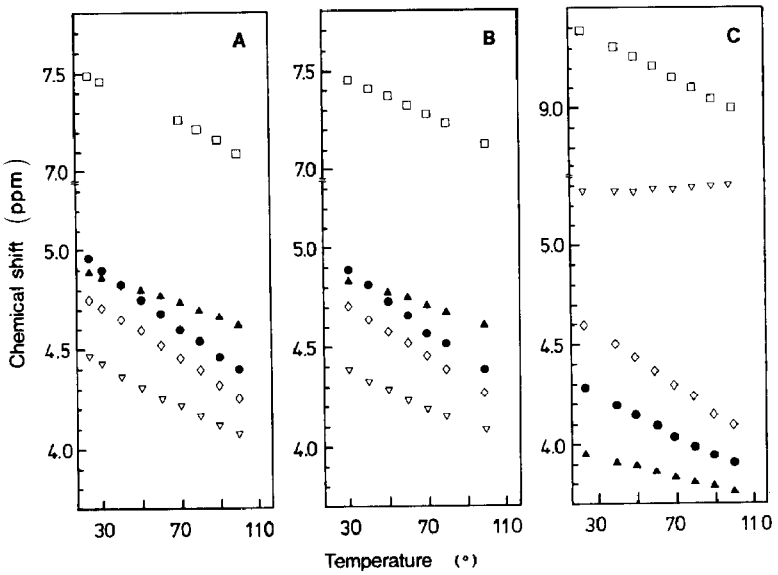
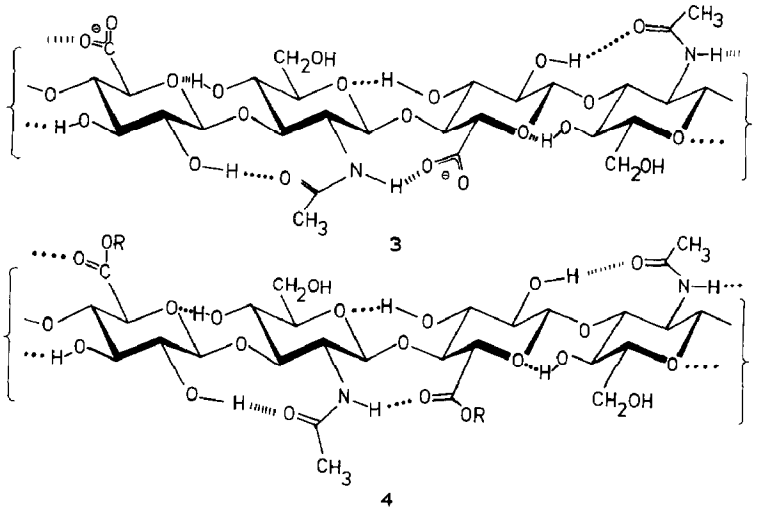


Fig. 4. Chemical shifts of the ^{1}H resonances of the NH and OH groups of hyaluronates in Me_2SO-d_6 *versus* temperature: A, benzyl ester (32 mg/mL); B, ethyl ester (30 mg/mL); C, tetraethylammonium salt (3.2 mg/mL). GlcNAc: □, NH-2; ▽, HO-4; ◇, HO-6; GlcA: ▲, HO-2; ●, HO-3. For the benzyl ester, the NH signal overlapped the phenyl signal for a part of the temperature range studied.

hyaluronate oligosaccharides dissolved in Me_2SO, suggesting a CH–NH dihedral angle of ~ 148° (ref. 20). Whereas solutions of the oligosaccharides in Me_2SO gave a $^3J_{2,NH}$ value of 6.2 Hz for the internal residues (corresponding[8] to a dihedral angle of 137°), the value for a solution of the tetraethylammonium salt in Me_2SO was 5.4 Hz, indicating[20] a dihedral angle value of 132.5°. Hence, both the *J* values and the chemical shifts for the NH resonance reveal that the hydrogen bond between the NH and the COO groups is weak for the esters, but relatively strong for the salt in solutions in Me_2SO.

The differences in the ^{13}C-NMR spectra of solutions of the esters and the salts in Me_2SO (Table II) may imply that hydrogen bonds involving hydroxyl groups are also different. Generally, intramolecular hydrogen bonds give temperature-independent shifts, and the stronger the bond, the higher the chemical shift of a hydroxyl resonance. Although absolute chemical shifts may be difficult to predict, a comparison of relative chemical shifts may provide useful information regarding the relative strengths of the hydrogen bonds for these similar molecules. For a solution of the tetraethylammonium salt in Me_2SO, the resonance of GlcNAc HO-4 had a rather high chemical shift which increased slightly with increasing temperature, indicating a strong, intramolecular hydrogen bond between GlcNAc HO-4 and GlcA O-5. The chemical shifts of the other OH and NH resonances, however, decreased with increasing temperature (Fig. 4). This behaviour suggests an equilibrium between an intramolecular hydrogen bond and a hydrogen bond to the solvent. The GlcA HO-2, also involved in the hydrogen bond pattern at the



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(1 → 3) linkage, has the lowest δ value of all of the hydroxyl groups, even lower than that of GlcNAc HO-6 (Fig. 4C). This finding suggests that the hydrogen bond involved (HNC=O · · · HO-2) is weak for the salt, but this weakness is probably compensated by the strong hydrogen bond between GlcNAc HO-4 and GlcA O-5. In contrast, the chemical shifts of the resonance GlcA HO-2 is the highest and that of GlcNAc HO-4 is the lowest in the esters (Fig. 4), suggesting that the hydroxyl groups form strong (||||) and weak (· · · · ·) hydrogen bonds, respectively, as shown in **3** and **4**.

Polymer-chain association.—Viscosity measurements on solutions of polysaccharides provide information on the macroscopic dimension and relative stiffness. Reduced viscosities were determined for solutions of tetrabutylammonium and benzyl hyaluronate in Me_2SO at 40° in a capillary viscometer, using samples of comparable average chain length. The results (Fig. 5) gave an intrinsic viscosity, $[\eta]$, of 1.29×10^2 (L/kg) for the ester. The polymer salt gave a much larger reduced viscosity in the high-concentration range but, on dilution, the viscosity dropped dramatically. The values of η_{sp}/c and ln η_{rel}/c in the high-concentration range did not coincide when extrapolated to zero concentration. Therefore, an intrinsic viscosity value could not be determined for the tetrabutylammonium salt. However, experiments performed with a different capillary at 30° demonstrated the presence of a limiting slope in the high-concentration range, for which intrinsic viscosities could be obtained[21]. Hence, the sigmoidal shape of the viscosity curve (Fig. 5) suggests a dissociation upon dilution, where the associated state seems to involve ordered species, and not just "random" aggregates. Further support for the hypothesis of association is given by the low-angle laser-light scattering determination of the molecular weight which was performed at low concentrations, i.e., in the range where the viscosity approaches its lower limit, giving $\overline{M}_w$ values close to those obtained for the ester.

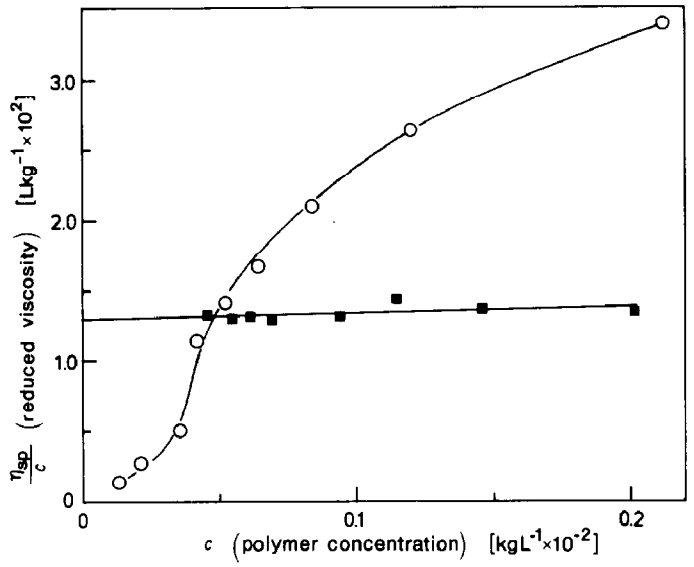

Fig. 5. Reduced viscosity (η_{sp}/c) versus polymer concentration (c) for solutions of tetrabutylammonium hyaluronate ($\bar{M}_w$ 211000; ○) and benzyl ($\bar{M}_w$ 140000; ■) hyaluronate at 40° in Me_2SO containing 0.25 M tetrabutylammonium bromide.

These results also suggest that the association of the polyanionic chains in Me_2SO will be favoured at the high concentrations used for NMR studies. The line widths of the NMR signals reflect the correlation times characteristic of the various motions of the molecule. The line width at half height of the ^1H signal for Ac was measured for solutions of benzyl and tetrabutylammonium hyaluronates in Me_2SO at 75° and gave values of 5.0 and 7.0 Hz, respectively. The greater line width for the salt reflects a reduced segmental mobility, which may well occur for species in an associated state. Furthermore, the ^1H-NMR spectra of solutions of the tetraethylammonium salt in Me_2SO-d_6 at 3.2, 5.2, and 11.2 mg/mL showed slightly lower (0.05 ppm) chemical shifts for the NH group at the highest concentration, whereas those of the OH resonances were the same within experimental error. More striking, however, was the increased line width of the NH and OH signals with increase in concentration of the sample (spectra not shown), indicating that the rate of intermolecular exchange was increased. The fact that the chemical shifts were largely unaffected indicates that the chain conformation for associated species remains unaltered.

From the $^3J_{2,NH}$ value (6.2 Hz) for solutions of the hyaluronate oligosaccharides in Me_2SO-d_6, it has been suggested[8] that the NH · · · OCO hydrogen bond exists in a rapid equilibrium with a solvent-bound state. The value (5.4 Hz) for the tetraethylammonium salt corresponds to a lower dihedral angle[20], and suggests a shift of the equilibrium towards the intramolecular hydrogen bond in the polymer. The presence of cooperative hydrogen bonding in the associated species may also explain why the GlcNAc HO-4 bond has a positive, but small temperature

coefficient for the polymer. The negative temperature coefficients observed for the chemical shifts of the HO-4 resonance in the GlcNAc residues[8] of the oligosaccharide suggest that these molecules are too short to give rise to long stretches of cooperative secondary structure.

The solubility of ionic polysaccharides in different solvents depends on the type of counterion present[22], and is also related to the fraction of counterions "condensed" on the polymer[23]. For solutions in water at 25°, no monovalent counterions will condense on the hyaluronate, leaving only long-range electrostatic interactions of the polyion and the diffuse cloud of counterions. This view accords with the observed invariance of the ^{13}C shifts of aqueous solutions of hyaluronate with different counterions. However, the relative permittivity of Me_2SO is lower than that of water, implying that condensation will occur. The bulky and highly hydrophobic tetrabutylammonium ions will condense onto the polymer, enhance its hydrophobicity[5,10], and increase its solubility. The observed tendency to form associates at higher concentrations of polysaccharide (see Fig. 5) is not inconsistent with the enhanced solubility. The tetrabutylammonium ions might participate in intermolecular ion bridges or may favour intramolecular hydrogen bonding by screening electrostatic repulsions. The viscosity results for the ester (Fig. 5) do not indicate any association. The observed chemical shifts and line widths of the OH and NH ^1H resonances did not vary with the concentration of the sample in the range 10–32 mg/mL (corresponding to 22–70 mM in repeating units), and these results are as expected for non-associating species.

The results presented provide more details to the model previously postulated[5,7] and presented in **1** for the salt dissolved in Me_2SO. The esters appear to have many conformational similarities with the salts in aqueous solution, but it remains to be demonstrated whether the hydrogen bond patterns shown in **4** for the esters are valid also for the aqueous solutions.

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