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^{1}H-N.M.R. SPECTRA OF GLYCOSAMINOGLYCAN MONOMERS AND DIMERS IN SOLUTION IN METHYL SULPHOXIDE AND WATER

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ABSTRACT

^{1}H-N.m.r. spectra of glycosaminoglycuronan monomers and dimers in solution in methyl sulphoxide-d_6 have been investigated; N-H and O-H resonances were observed and partially assigned. Their temperature-dependence suggests hydrogen-bonding to the solvent, with the notable exception of that of HO-4 of sodium D-glucuronate, which was consistently downfield and relatively temperature-insensitive. The concentration-dependence of this signal indicates that the corresponding hydroxyl group is involved in the formation of a dimer. Signals for N-H and O-H were observed for aqueous solutions, especially at subzero temperatures.

INTRODUCTION

The main features of the primary structures of the glycosaminoglycans of connective tissues (chondroitin sulphates, keratan sulphate, heparan sulphates, *etc.*) are well established, but little is known of the secondary and tertiary structures. The main discussion has arisen from X-ray data[1,2] for solid samples. The secondary or tertiary structures in solution may control the physiological functions of glycosaminoglycans. We have applied ^{1}H-n.m.r. spectroscopy to glycosaminoglycans, in seeking to acquire knowledge of possible secondary and tertiary structures *in solution*, as well as of the less well-established details in the primary structure.

^{1}H-N.m.r. spectra have been obtained for three solutions of glycosaminoglycans in D_2O, and ^{13}C-n.m.r. spectra are also available on many compounds[4,5]. However, in order to elucidate secondary and tertiary structures, it is necessary to study the easily exchangeable protons (*i.e.*, O*H* and N*H*) and this can be done by using non-aqueous solvents. The signals for the hydroxyl protons of carbohydrates are characterised by chemical shifts and temperature dependences that indicate their involvement

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in hydrogen bonding, either intramolecular or with the solvent[6,7]. Inter- and intramolecular associations involving OH, NH and other groups should be diagnosable by comparing the spectra of monomers with those of the polymers.

Since the polymers are almost insoluble in methyl sulphoxide and even the solubility of some of the monomers is low, Fourier-transform methods must be used. A high field is essential, in order to resolve the many signals, from OH, NH, and CH groups, which often overlap. We have determined the ^{1}H-n.m.r. spectra of sodium salts of uronic acids and extended the published[10] observations on 2-acetamido-2-deoxyhexoses. The spectra of the disaccharides, especially *N*-acetylchondrosinate, have been studied also in a logical progression to completely attributed spectra of polymers. The spectra of aqueous solutions have been studied because of their physiological relevance.

EXPERIMENTAL

The 2-acetamido-2-deoxy derivatives of D-glucose, D-galactose, and D-mannose and the sodium salts of D-galacturonic and D-glucuronic acids were commercial samples. Methyl (methyl 4-*O*-methyl-α-D-glucopyranosid)uronate, which was a gift from Dr. P. Kovacs (Bratislava), was converted into the sodium salt by hydrolysis in sodium carbonate solution. The product was freeze-dried, and its purity was assessed by the carbazole–sulphuric acid reaction and by electrophoresis[8].

Chondrosine, prepared by acid hydrolysis of chondroitin 4-sulphate, followed by ion-exchange chromatography, was *N*-acetylated[9], to produce *N*-acetylchondrosinate. We thank Dr. A. H. Olavesen for a gift of this material.

Spectra were obtained by using a Varian SC300 (300-MHz) spectrometer with a Fourier-transform facility. Perkin–Elmer R32 (90-MHz) instruments were used to obtain several of the spectra for solutions in H_2O.

RESULTS

Sodium D-*glucuronate and derivatives.* — The spectrum at 20° of a 1% solution of sodium D-glucuronate in Me_2SO-d_6 is shown in Fig. 1(*a*). The number of well-resolved doublets for hydroxyl groups indicates that both α and β anomers are present, together with minor components. The small amount of water is not sufficient to delete the hydroxyl peaks by rapid exchange, although some show exchange-broadening, notably that at δ 6.75. Treatment with D_2O removed the hydroxyl signals, leaving the H-1 doublets downfield of the signals for the other methine protons. The doublet at δ 5.01 ($J_{1,2}$ 3 Hz) was assigned to the α form, and that at 4.31 ($J_{1,2}$ 9 Hz) was assigned to the β form. Using spin-decoupling and these initial identifications, the assignments listed in Table I were established. The signals for H-2α, H-3β, H-4α, and H-4β overlap in the vicinity of δ 3.1; the signal for H-3α is obscured by the H_2O peak in the spectrum shown, but was resolved when the H_2O peak was reduced in intensity by the addition of D_2O. The coupling constants are typical of

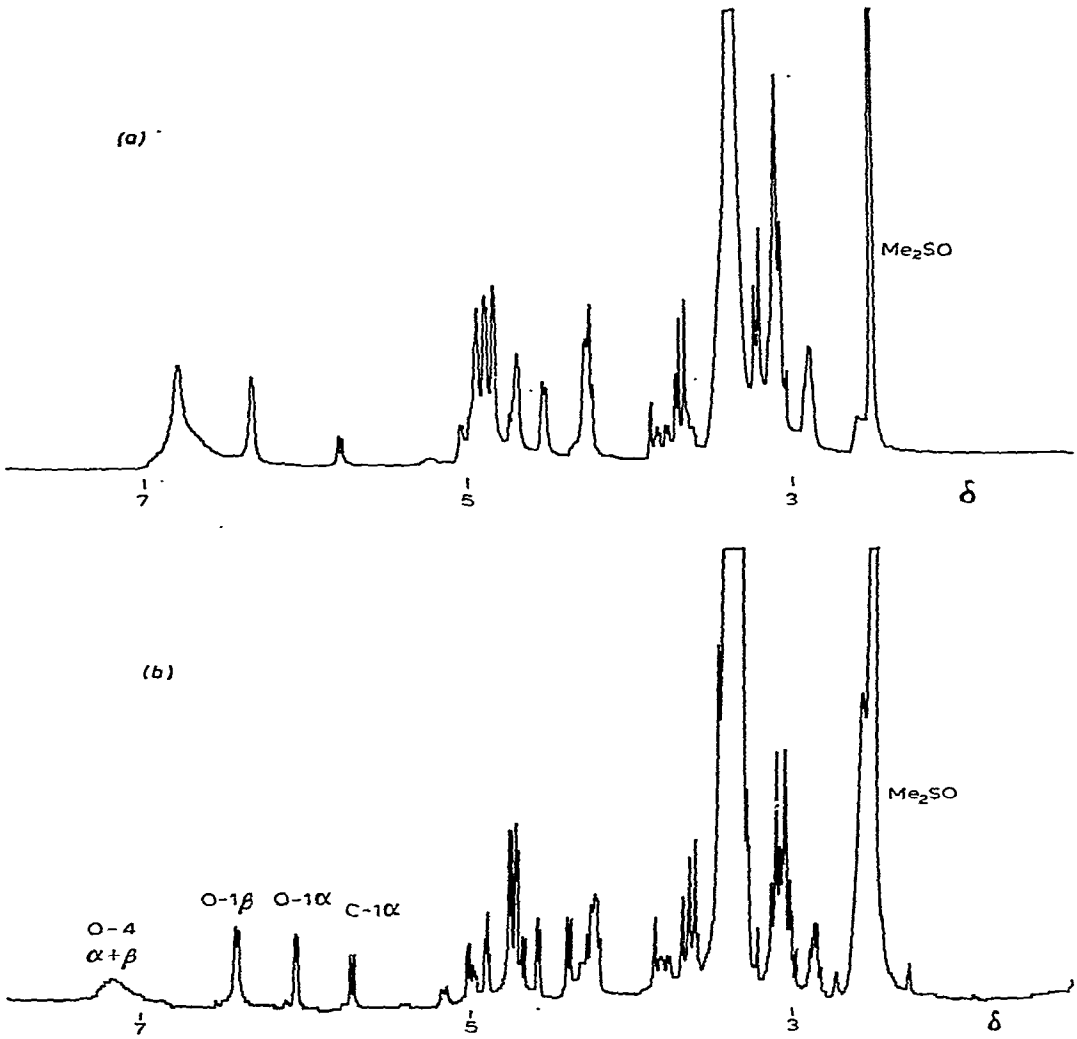

Fig. 1. ^{1}H-N.m.r. spectra (300 MHz) of solutions of sodium D-glucuronate in Me_2SO at 20°: (*a*) 1%, (*b*) 0.25%.

those generally found[10] for pyranoid structures. All the peaks for hydroxyl protons, except that for HO-4, show at least partially resolved ^3J-coupling, indicating slow proton-exchange. The resonance of HO-4 is broadened, even at the lowest concentration (0.01%).

The concentration and temperature dependences of the chemical shifts for hydroxyl protons are given in Tables II and III, and the concentration dependence is illustrated in Fig. 1. The methine-proton chemical shifts were almost independent of concentration and temperature. The large downfield shift of the signal for HO-4 on dilution is in marked contrast to the normal upfield shifts of the signals for the other hydroxyl groups. Moreover, the HO-4 peak shows no accompanying decrease

TABLE I

CHEMICAL SHIFTS AND COUPLING-CONSTANT DATA FOR A 1% SOLUTION OF SODIUM D-GLUCURONATE IN Me_2SO-d_6 AT 20°

	Chemical shifts			*Coupling constants (Hz)*		$J_{H,OH}$
α Anomer						
	H-1	5.01	HO-1 6.49	$J_{1,2}$	3	3
	H-2	~3.1	HO-2 4.61			6
	H-3	3.42	HO-3 4.77			4
	H-4	~3.1	HO-4 6.75	$J_{4,5}$	11	
	H-5	3.71				
β Anomer						
	H-1	4.31	HO-1 6.99	$J_{1,2}$	9	4
	H-2	2.91	HO-2 4.98	$J_{2,3}$	9	5
	H-3	~3.1	HO-3 4.92			3
	H-4	~3.1	HO-4 6.75	$J_{4,5}$	10	
	H-5	3.24				

TABLE II

VARIATION OF HYDROXYL CHEMICAL-SHIFTS (δ) WITH CONCENTRATION FOR SODIUM D-GLUCURONATE IN Me_2SO-d_6 AT 20°

Proton	*Concentration (% w/v)*			
	1	*0.5*	*0.25*	*0.125*
HO-1α	6.49	6.36	6.28	6.21
HO-1β	6.99	6.79	6.66	6.62
HO-2α	4.61	4.50	4.44	4.41
HO-2β	4.98	4.87	4.81	4.77
HO-3α	4.77	4.70	4.66	4.64
HO-3β	4.92	4.86	4.80	4.77
HO-4αβ	6.75	6.87	7.08	7.22

TABLE III

VARIATION OF HYDROXYL CHEMICAL-SHIFTS (δ) WITH TEMPERATURE FOR A 1% (W/V) SOLUTION OF SODIUM D-GLUCURONATE IN Me_2SO-d_6

Proton	*Temperature (K)*		
	20	*40*	*60*
HO-1α	6.49	6.33	6.15
HO-1β	6.99	6.80	6.61
HO-2α	4.61	4.45	4.26
HO-2β	4.98	4.83	4.64
HO-3α	4.77	4.61	4.42
HO-3β	4.92	4.76	4.57
HO-4αβ	6.75	6.72	6.68

TABLE IV

CHEMICAL SHIFTS AND COUPLING CONSTANTS FOR SOLUTIONS OF METHYLATED DERIVATIVES OF SODIUM D-GLUCURONATE IN Me_2SO-d_6 AT 20°

Chemical shifts				*Coupling constants (Hz)*			
Methyl (methyl 4-O-methyl-α-D-glucopyranosid)uronate							
H-1	4.61	MeO-1	3.29 or 3.35	$J_{1,2}$	4		
H-2	~3.3	HO-2	5.02	$J_{2,3}$	10	$J_{H-2,HO-2}$	7
H-3	3.48	HO-3	5.20	$J_{3,4}$	10	$J_{H-3,HO-3}$	6
H-4	3.16	MeO-4	3.29 or 3.35	$J_{4,5}$	10		
H-5	3.82						
		CO_2Me	3.71				
Sodium (methyl 4-O-methyl-α-D-glucopyranosid)uronate							
H-1	4.46	MeO-1	3.34 or 3.23	$J_{1,2}$	4		
H-2	[a]	HO-2	4.87	$J_{2,3}$	[a]	$J_{H-2,HO-2}$	7
H-3	[a]	HO-3	4.97	$J_{3,4}$	10	$J_{H-3,HO-3}$	5
H-4	3.09	MeO-4	3.34 or 3.23	$J_{4,5}$	10		
H-5	3.46						

[a]Not resolved.

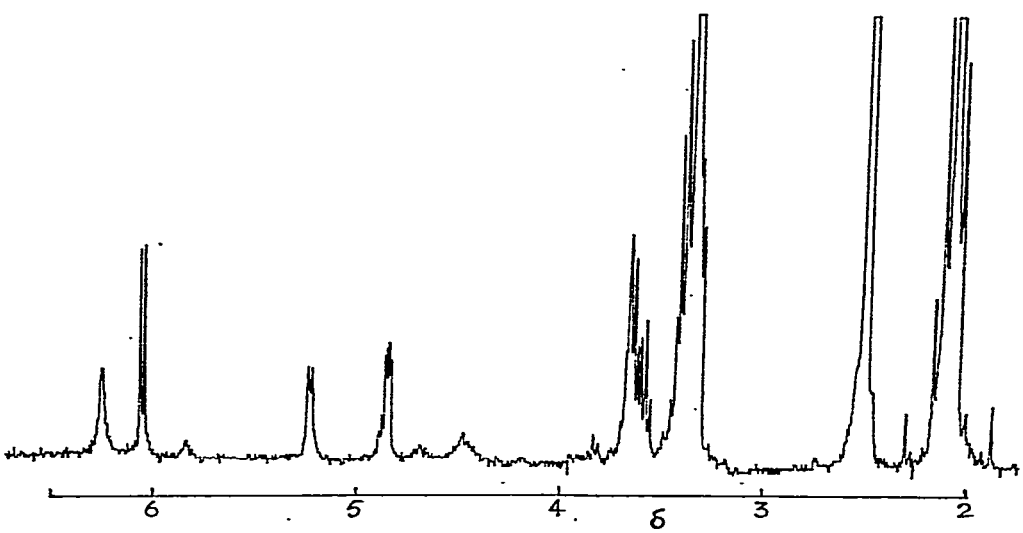

Fig. 2. ^{1}H-N.m.r. spectrum (300 MHz) of a 0.2% solution of sodium D-galacturonate in Me_2SO at 20°.

in line-width. With increasing temperature, all hydroxyl signals, except that for HO-4, move upfield by amounts comparable to those typically observed for alcohols experiencing intermolecular hydrogen bonding. The HO-4 shift is relatively insensitive to temperature.

TABLE V

CHEMICAL SHIFTS AND COUPLING CONSTANTS FOR A 1% SOLUTION OF SODIUM D-GALACTURONATE IN Me_2SO-d_6 AT 20°

Chemical shifts			*Coupling constants (Hz)*		
H-1 4.87	HO-1 6.09		$J_{1,2}$ 3	$J_{H-1,HO-1}$ 5	
H-2 3.65	HO-2 6.29	[a]		$^3J_{H,HO}$ 5, <3	[b]
H-3 3.65	HO-3 5.25				
H-4 3.58	HO-4 4.49				
H-5 3.41					

[a]Assignment not made; see text. [b]Only two peaks show spin-coupling. Assignment not made; see text.

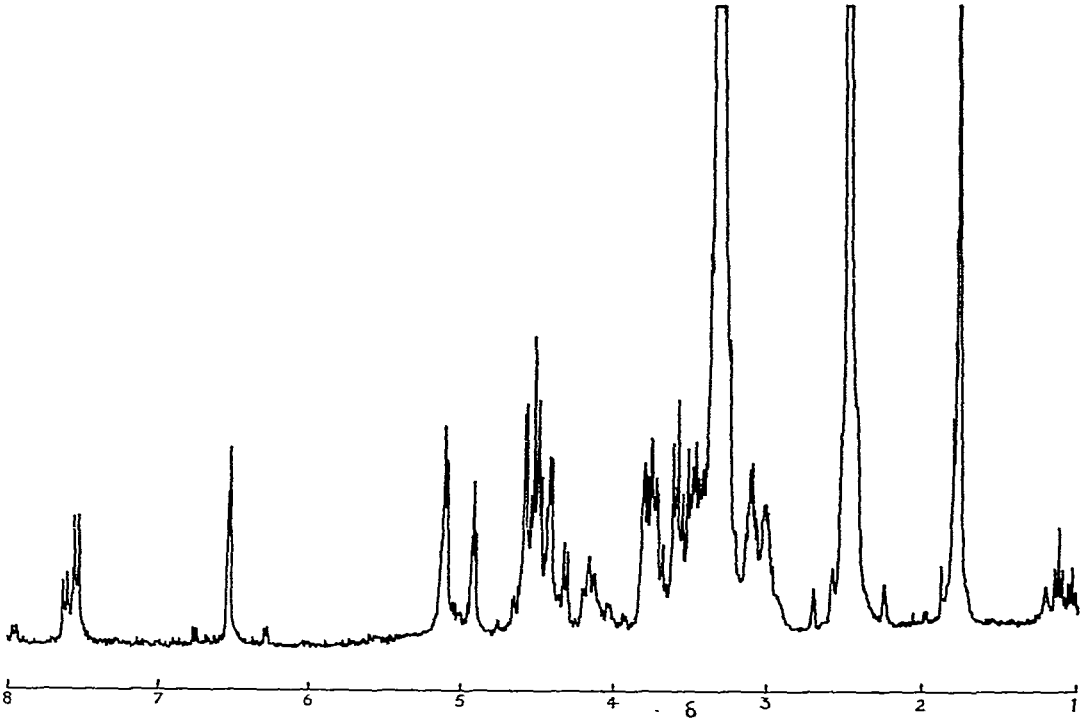

Fig. 3. ^{1}H-N.m.r. spectrum (300 MHz) of a 0.15% solution of *N*-acetylchondrosinate (sodium salt) in Me_2SO at 20°.

Methyl (methyl 4-*O*-methyl-α-D-glucopyranosid)uronate and sodium (methyl 4-*O*-methyl-α-D-glucopyranosid)uronate gave clearly resolved, essentially first-order spectra. The chemical shifts and coupling constants are reported in Table IV. The difference in positions of the hydroxyl peaks is significant. At comparable concentrations, the sodium salt shows more shielded, broader peaks than the methyl ester, and the hydroxyl peaks move ~0.4 p.p.m. upfield on 16-fold dilution, as do the shifts of HO-1, HO-2, and HO-3 in sodium D-glucuronate. This dilution sharpens the

peaks, but even then the coupling is never as clear as in the concentrated solution of the methyl ester.

Sodium D-*galacturonate.* — Fig. 2 shows the spectrum of a 0.2% solution of sodium D-galacturonate in Me_2SO-d_6 at 20°. In contrast to the glucuronate, only the α anomer was observed and identified from the $J_{1,2}$ value of 3 Hz; the signals for H-2,3,4 were almost coincident and it was not possible to identify any of the hydroxyl resonances unequivocally by spin decoupling, except that for HO-1.

Too few definite assignments in related compounds are available to warrant even tentative assignments by analogy. Table V lists the chemical shifts, coupling constants, and assignments.

Unlike glucuronate, galacturonate hydroxyl- and methine-proton chemical shifts are almost insensitive to concentration, at least within the concentration range studied (Table VI). The largest shift observed for a 20-fold dilution was 0.25 p.p.m. upfield for the hydroxyl peak at lowest field, and the average shift for all four hydroxyls was only 0.07 p.p.m. However, like the glucuronate, the hydroxyl peaks differ considerably in the degree of exchange-broadening, the peak at δ 4.54 in Fig. 2 being particularly affected.

N-*Acetylchondrosinate.* — The spectrum of a 0.13% solution of *N*-acetylchondrosinate in Me_2SO-d_6 is shown in Fig. 3. The occurrence of two NH doublets at δ 7.6 clearly shows that two forms are present; the $J_{1,2}$ values of 3.5 and 8 Hz for the H-1 signals at δ 4.93 and 4.49 are characteristic of *gauche* and *trans*-diaxial dispositions of H-1,2.

Because of its complexity, a full assignment of the *N*-acetylchondrosinate spectrum is not yet possible. As described above, the NH, H-1, and H-2 resonances were located, and decoupling of each H-1 peak located the corresponding HO-1 peaks. Decoupling of the H-2 peak clearly revealed that for H-3, but the H-3 peak was obscured by a number of others in the region of δ 3.5–3.60. A $J_{H\text{-}2,NH}$ value of 9 Hz was observed for the α and β forms, together with the values $J_{1,2\alpha}$ 3.5, $J_{1,2\beta}$ 8, $J_{H\text{-}1,HO\text{-}1\alpha}$ 4.5, $J_{H\text{-}1,HO\text{-}1\beta}$ 7, and $J_{2,3\alpha}$ 10 Hz. The chemical shifts, and their variation with concentration, are given in Table VII. Like sodium D-galacturonate, the con-

TABLE VI

VARIATION OF HYDROXYL CHEMICAL-SHIFTS WITH CONCENTRATION FOR SODIUM D-GALACTURONATE IN Me_2SO-d_6 AT 20°

Proton	*Concentration (% w/v)*		
	1	*0.2*	*0.05*
HO-1α	6.09	6.03	6.03
HO-2α	6.29	6.48	6.54
HO-3α	5.25	5.25	5.26
HO-4α	4.49	4.54	4.57

TABLE VII

VARIATION OF CHEMICAL SHIFTS (δ) WITH CONCENTRATION FOR SOLUTIONS OF *N*-ACETYLCHONDROSINATE IN Me_2SO-d_6 AT 20°

Proton	*Concentration (% w/v)*		
	1.8	*0.9*	*0.15*
NH-α	7.62	7.57	7.49
NH-β	7.68	7.63	7.55
HO-1α	6.89	6.84	6.76
HO-1β	6.90	6.85	6.77
H-1α	4.93	4.93	4.93
H-1β	4.49	4.49	4.49
H-2α	4.18	4.18	4.18
H-2β	3.70	3.70	3.70

centration dependence is small compared to that of sodium D-glucuronate. Other signals, although unassigned, show similar behaviour.

^{1}H-N.m.r. spectra for aqueous solutions. — Although hydroxyl protons usually exchange rapidly at room temperature in aqueous solvents, this rate can be so reduced by working at sub-zero temperatures[11] as to allow observation of distinct (though broad) signals for the hydroxyl groups of glucose and other neutral monosaccharides in aqueous solution. Recording such spectra is difficult in the Fourier mode, due to overloading of the computer by the water peak, but results were obtained at 90 MHz in the continuous-wave mode.

Although the ionic character of sodium glucuronate leads to a greater depression of the freezing point than for equivalent molarities of neutral sugars, thus permitting experiments at lower temperatures, the exchange of the hydroxyl protons was faster than for glucose, as shown by the broader hydroxyl peaks. The peak-broadening did not allow identification of the signals as was possible for solutions in methyl sulphoxide. Temperature-dependence experiments did not disclose any temperature-insensitive hydroxyl signals such as those observed for solutions in methyl sulphoxide.

Although somewhat broadened by proton exchange, the N-H resonances can be observed in aqueous solutions at room temperature, as shown in studies on polypeptides[12]. The spectra of 2-acetamido-2-deoxy-D-glucose, -D-galactose, and -D-mannose, and *N*-acetylchondrosinate in aqueous solution showed clearly distinguishable doublets at 8.12–8.15 p.p.m. from internal DSS (or TSP).

The signals for 2-acetamido-2-deoxy-D-glucose persisted up to 60°. At lower temperatures, the signals sharpen, and the hydroxyl signals become discernible. The hydroxyl signals of the amino sugars are much less broadened than those of sodium D-glucuronate.

DISCUSSION

The evidence from solution studies is compatible with X-ray data for solids and suggests that glycosaminoglycuronans have a secondary structure in aqueous solution, with the acetamido group acting as both donor and acceptor of hydrogen bonds which organise the polysaccharide structures[8,13]. ^{1}H-N.m.r. data for solutions in methyl sulphoxide allow such protons to be studied.

Comparison of the spectra of sodium D-glucuronate and (methyl 4-*O*-methyl-α-D-glucosid)uronate (the prototype uronate "monomer" in the glycosaminoglycuronan series), and *N*-acetylchondrosinate (the next stage toward the polymer), reveals the effects of the carboxylate group, as opposed to those of methoxycarbonyl or hydroxymethyl groups, on the broadening of OH peaks. Some striking concentration-dependent chemical shifts and broadening of signals were observed only for sodium D-glucuronate, which demonstrates the necessity for caution in interpreting single spectra, without a background of temperature- and concentration-dependence studies.

The variation of chemical shift with concentration is probably due to self-association. Insight into this process may be derived from a simple dimerisation model $2A \rightleftharpoons A_2$; it is unlikely that larger aggregates are formed in such dilute solutions.

If a_0 is the total concentration of A, a is the concentration of monomeric A, and K is the equilibrium constant, then from the definition[14] of K,

$$a = [-1 + (1 + 8Ka_0)^{\frac{1}{2}}]/4K.$$

The observed chemical shift is an average over the monomer and dimer[14]:

$$\bar{\nu} = \rho_A \nu_A + \rho_{A_2} \nu_{A_2},$$

where ρ_A and ρ_{A_2} are the probabilities of finding A as the monomer or dimer, and ν_A, ν_{A_2} are the respective chemical shifts. Since $\rho_A = a/a_0$, then

$$\bar{\nu} = \nu_{A_2} + (\nu_A - \nu_{A_2}) \left(\frac{-1 + (1 + 8Ka_0)^{\frac{1}{2}}}{4Ka_0} \right).$$

When $8Ka_0 \ll 1$ (weak association), this equation simplifies to

$$\bar{\nu} = \nu_{A_2} + (\nu_A - \nu_{A_2})(1 - 2Ka_0),$$

and a plot of $\bar{\nu}$ vs. a_0 will be linear.

When $8Ka_0 \gg 1$ (strong association), the equation becomes

$$\bar{\nu} = \nu_{A_2} + (\nu_A - \nu_{A_2})/(2Ka_0)^{\frac{1}{2}},$$

and a plot of $\bar{\nu}$ vs. $a_0^{-\frac{1}{2}}$ will be linear. Sodium galacturonate fits the weak association condition and sodium glucuronate the strong association condition, as shown by the plots in Fig. 4 for the lowest-field hydroxyl signal of sodium D-galacturonate (HO-3) and HO-4 of sodium D-glucuronate.* The relative temperature independence of the chemical shifts of the latter (noted earlier) is explicable in terms of two competing effects. On raising the temperature, both ν_A and ν_{A_2} will move upfield, and so will ν if K is constant. However, for strong association, K should decrease with temperature, thus moving $\bar{\nu}$ to lower field. The two opposing effects fortuitously balance.

*The signal for HO-4 was not identified for sodium D-galacturonate.

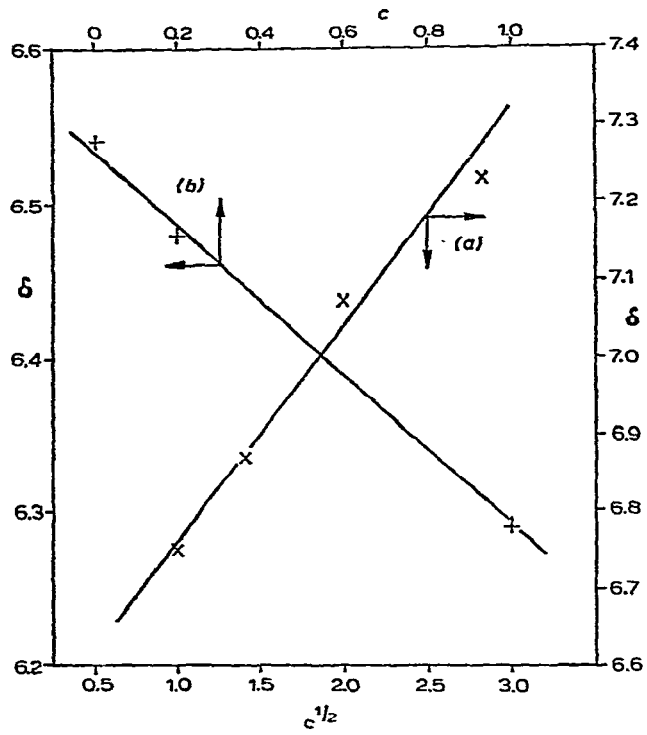

Fig. 4. (*a*) Chemical shift (δ) of HO-4 of sodium D-glucuronate *vs.* $c^{\frac{1}{2}}$. (*b*) Chemical shift (δ) of HO-3 of sodium D-galacturonate *vs.* *c* (*c* in %).

Possibly the self-association of sodium D-glucuronate (and probably also of the *galacto* analogue) in solution in methyl sulphoxide involves HO-4 and the carboxylate group. A similar association might be expected for *N*-acetylchondrosinate. However, the spectrum of this disaccharide does not show low-field or temperature-independent hydroxyl signals, thus excluding substantial self-association.

The present work provides reference data for studies of oligomers and polymers made up of uronate and *N*-acetylated amino-sugar residues, which are in progress.

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