Secondary structure of hyaluronate in solution

A ^1H -n.m.r. investigation at 300 and 500 MHz in $[\text{}^2\text{H}_6]$ dimethyl sulphoxide solution

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1. The ^1H -n.m.r. spectra of solutions in $[\text{}^2\text{H}_6]$ dimethyl sulphoxide of the sodium salts of tetra-, hexa- and octa-saccharides prepared from hyaluronate by testicular-hyaluronidase digestion were examined at 300 and 500 MHz. 2. The signals from hydroxy groups at positions 2 and 3 in the glucuronic acid moiety were assigned. Their chemical shifts and associated temperature-dependencies, as well as their coupling constants, depended on whether or not the uronic acid was at the non-reducing end. Deviations from the 'normal' pattern of hydroxy-group proton n.m.r. behaviour were attributable to participation in hydrogen bonds, either to the acetamido carbonyl oxygen atom or the pyranose ring oxygen atom of neighbouring *N*-acetylhexosamine moieties. 3. A secondary structure, containing four different hydrogen bonds per tri-saccharide unit of glucuronosyl-hexosaminyl-glucuronic acid, was demonstrated. This is the first complete and detailed secondary structure to be established for hyaluronate in any solvent. 4. Hyaluronate is compared with chondroitin sulphate, dermatan sulphate, heparan sulphate and keratan sulphate in their potential to form secondary structures with features in common. 5. The significance of the details of the structure to its overall stability, and the probability of their persistence into aqueous environments, are discussed. 6. The presence of all or most of the secondary structure in glycosaminoglycuronans is correlated with a space-filling function in the tissue, and with a high carbohydrate content in the parent proteoglycan in the case of the chondroitin sulphates.

The significance of secondary structures in glycosaminoglycans has been assessed at several levels of complexity. The simplest, which ignored interactions with solvents, ions and macromolecules, was model-building, with the use of space-filling atoms (Scott & Tigwell, 1978) or computer simulation (Atkins *et al.*, 1980). X-ray-diffraction fibre data and periodate oxidation kinetics correlated the model with the real molecule. The application of n.m.r. to solutions of polymers can be very informative about polymer configurations, secondary structure and intermolecular interactions, and thus, in principle, about situations of physiological interest. Unfortunately, glycosaminoglycans are insoluble in most solvents except water, and water is a solvent that presents dynamic-range problems for ^1H n.m.r. At present, by using special n.m.r. techniques slowly exchanging groups with chemical shifts at least

0.5 p.p.m. from the H_2O resonance can be investigated. $^2\text{H}_2\text{O}$ is a useful alternative, since it does not have the 100 M ^1H resonance of H_2O . However, all exchangeable protons, i.e. from NH and OH, are quickly lost into the solvent, and the resulting N^2H and O^2H are not seen in the spectrum. Consequently it is difficult to diagnose hydrogen-bonded systems unless exchange is slow and the resonances are shifted well away from water (e.g. nucleic acids). We found, following work on polyglucoses by Casu *et al.* (1966), that oligomeric and polymeric glycosaminoglycans are soluble in deuterated dimethyl sulphoxide (Heatley *et al.*, 1979; Scott & Heatley, 1982), which does not have a strong ^1H signal, and in which NH and OH do not exchange protons rapidly. We therefore sought for evidence of glycosaminoglycan secondary structure in solution in dimethyl sulphoxide. Ultimately, the results must be extended to those situations,

aqueous and *in vivo*, in which the glycosaminoglycans function physiologically.

Intramolecular secondary structure in hyaluronate and in chondroitin 4- and 6-sulphates was first inferred from periodate oxidation kinetics (Scott & Tigwell, 1978). Computer calculations (Atkins *et al.*, 1980) indicated four different hydrogen bonds within a hyaluronate trisaccharide repeating unit (Fig. 3). N.m.r. spectra of disaccharides in $[^2\text{H}_6]$ -dimethyl sulphoxide showed clear evidence of one of these, from glucosamine $\text{C}_{(4)}\text{OH}$ to the glucuronate ring oxygen atom (Heatley *et al.*, 1982). A hydrogen-bonded NH was shown to exist in the tetrasaccharide and higher oligomers prepared from hyaluronate by hyaluronidase digestion (Scott *et al.*, 1981). The two other hydrogen bonds, from the acetamido $>\text{C}=\text{O}$ to HO-2 of glucuronate, and from HO-3 of glucuronate to the hexosamine ring oxygen atom, could not be shown at 300 MHz, because the relevant proton signals were not resolvable in the crowded region of δ 4–6 p.p.m. From the use of 500 MHz ^1H n.m.r. we now report the necessary decoupling data for specific assignments of all the relevant signals in tetra-, hexa- and octa-saccharides. Our findings show that all the secondary-structural features that are demonstrable '*in vacuo*', in models and computer simulations, persist into dimethyl sulphoxide solution. They correlate well with data of limited specificity from aqueous solution, with the use of periodate oxidation kinetics (Scott & Tigwell, 1978) and c.d. (Cowman *et al.*, 1981), and from X-ray-diffraction fibre work (Atkins *et al.*, 1980).

Some of this work has been preliminarily communicated (Scott *et al.*, 1983a).

Experimental

Materials

The tetra-, hexa- and octa-saccharides, prepared from hyaluronate by hyaluronidase digestion, were the samples used previously (Scott *et al.*, 1981).

N.m.r. methods

^1H -n.m.r. spectra were obtained at 300 MHz with a Varian Associates SC-300 spectrometer, and at 500 MHz with a Bruker WM-500 spectrometer. Spectra were recorded on solutions of approx. 10 mg/ml in $[^2\text{H}_6]$ -dimethyl sulphoxide. Resolution enhancement by means of a Lorentz–Gaussian lineshape transformation (Ferrige & Lindon, 1978) was applied to the spectra at 500 MHz.

Results

^1H -n.m.r. spectra of hyaluronate oligomers

Fig. 1 shows part of the 500 MHz ^1H -n.m.r. spectrum of the sodium salt of the tetrasaccharide oligomer of hyaluronate in $[^2\text{H}_6]$ -dimethyl sulphoxide solution. The spectrum is very well resolved, and a large number of resonances were assigned (Table 1). Initially, HO and HN resonances were identified by deuterium exchange, after which several unequivocal assignments were made by comparison with chemical shifts established for related mono- and di-saccharides (Heatley *et al.*, 1979, 1982). These assignments were: (a) the HN resonances appear as low-field doublet signals at δ 7.70, 7.81, 9.21 and 9.28 p.p.m., (b) the $\text{N}_{(1)}$ –HO-1 resonances give the doublets at δ 6.58 and

Fig. 1. Part of the ^1H -n.m.r. spectrum at 500 MHz in $[^2\text{H}_6]$ -dimethyl sulphoxide of the tetrasaccharide (Fig. 3) from hyaluronate, showing signals from hydroxy groups that participate in hydrogen-bonding (arrowed). Some assignments are shown, together with relevant coupling constants.

Table 1. Chemical-shift assignment (δ) for hyaluronate tetrasaccharide in dimethyl sulphoxide ($\delta = 2.4\text{--}9\text{ p.p.m.}$) at 27°C

δ (p.p.m.)			
H		OH and NH	
N₍₁₎ residue			
H-1	4.98 (α), ~ 4.57 (β)	HO-1	6.58 (α), 6.65 (β)
H-2	3.67 (α), ~ 3.47 (β)	HN-2	7.70 (α), 7.81 (β)
H-3	*		
H-4	*	HO-4	5.46 ($\alpha + \beta$)
H-5	*		
H-6	~ 3.47 , ~ 3.67	HO-6	4.46 (α), ~ 4.57 (β)
COCH ₃	1.81 (α), 1.79 (β)		
G₍₁₎ residue			
H-1	4.35 (α), 4.19 (β)		
H-2	~ 3.11	HO-2	4.75 (α), ~ 4.57 (β)
H-3	~ 3.20	HO-3	4.36
H-4	*		
H-5	*		
N₍₂₎ residue			
H-1	~ 4.57		
H-2	~ 3.47	HN-2	9.28 (α), 9.21 (β)
H-3	*		
H-4	*	HO-4	5.24
H-5	*		
H-6	~ 3.47 , ~ 3.67	HO-6	~ 4.57
COCH ₃	1.82 (α), 1.81 (β)		
G₍₂₎ residue			
H-1	4.09		
H-2	2.94	HO-2	3.87
H-3	~ 3.11	HO-3	4.82
H-4	3.07	HO-4	7.09
H-5	*		

* Unassigned overlapping peaks in δ 3.1–3.7 p.p.m. region.

6.65 p.p.m., (c) the N₍₁₎-H-1 and N₍₂₎-H-1 resonances show doublets at $\delta \sim 4.57$ and 4.98 p.p.m. and (d) the G₍₁₎-H-1 and G₍₂₎-H-1 resonances appear as doublets at δ 4.09, 4.19 and 4.35 p.p.m. (N and G indicate hexosamine and glucuronate residues respectively.)

The assignments of the H-2 protons were established by spin-decoupling the H-1 protons, leading by further decoupling experiments to the assignments of the HO-2, H-3 and HO-3 protons in the G residues. The HO-6 signals were assigned on the basis of their triplet structure and their disappearance on treatment with ²H₂O. The G₍₂₎-HO-4 peak was assigned on the basis of a comparison of its chemical shift with related mono- and di-saccharides (Heatley *et al.*, 1979, 1982). The N₍₁₎-HO-4 and N₍₂₎-HO-4 peaks were assigned on the basis of the variation in relative intensity in the tetra-, hexa- and octa-saccharide oligomers (see below).

As indicated in Table 1, several protons gave two resonances (notably N₍₁₎-H-1, N₍₁₎-HO-1, N₍₂₎-H-2, N₍₂₎-HN-2, G₍₁₎-H-1 and G₍₁₎-HO-2)

attributable to α/β anomerism in residue N₍₁₎. The anomer assignments were based on the fact (Heatley *et al.*, 1979, 1982) that in glucose rings with a ⁴C₁ conformation the H₍₁₎- α proton (equatorial) resonates approx. 0.5 p.p.m. to lower field than the H₍₁₎- β proton (axial). Furthermore, in the α -anomer the H-1–H-2 coupling constant was 3 Hz, consistent with the equatorial–axial disposition, compared with a value of 9 Hz in the β -anomer, consistent with the axial–axial disposition. The α/β ratio was approx. 4:1. The coupling constants observed between other vicinal CH proton pairs were consistent with ⁴C₁ conformations of all rings, being approx. 8–9 Hz. Vicinal coupling constants involving HO and HN protons are given in Table 2.

The chemical shifts of peaks in the spectra of the hexa- and octa-saccharide oligomers were almost identical with those of the tetrasaccharide, but the relative intensities altered in proportion to the number of repeating disaccharide units. The intensity of the total NH signal at δ 7.70 and 7.81 p.p.m. ($\alpha + \beta$ -anomers) to those at δ approx.

Table 2. *Vicinal coupling constants and chemical-shift temperature coefficients for hyaluronate tetrasaccharide in dimethyl sulphoxide at 27°C*

Residue	Position (type)	J (Hz)*	Temperature coefficient (p.p.b./K)†
N ₍₁₎	1 (OH)	4.7 (α), 6.4 (β)	-4.3 (α), -5.3 (β)
	2 (NH)	7.2 (α), 8.2 (β)	-4.3 (α), -2.0 (β)
	4 (OH)	≤ 2	-1.4
	6 (OH)	6.0	-5.7 (α), ~ -5 (β)
G ₍₁₎	2 (OH)	4.9	-6.7
	3 (OH)	3.5	-4.3
N ₍₂₎	2 (NH)	6.2	-4.3
	4 (OH)	≤ 2	-1.4
	6 (OH)	6.0	-5.0
G ₍₂₎	2 (OH)	1.6	-0.97
	3 (OH)	4.9	-7.0

* ± 0.3 Hz.† $\pm 10\%$. Negative shift is to higher field with increasing temperature. Fig. 2. Part of the spectra at 300 MHz in $[^2H_6]$ dimethyl sulphoxide of the (a) tetra-, (b) hexa- and (c) octa-saccharides from hyaluronate, showing the HN resonance region, δ approx. 7.8 and 9.2 p.p.m.

Note that the ratio of the signal at δ 9.2 p.p.m. to that of δ 7.8 p.p.m. increases in the proportions 1:2:3 from (a) to (b) to (c), as required by the hydrogen-bonded structures based on Fig. 3.

9.2p.p.m. was in the ratio 1:1, 1:2 and 1:3 on passing from the tetra- to the hexa- and octa-saccharides respectively (Fig. 2). In the HO region, the signals at δ 6.58 and 6.65p.p.m., assigned to $N_{(1)}$ -HO-1 in the tetrasaccharide, combined to give unit intensity in all oligomers, as did the signals at δ 3.87p.p.m. ($G_{(2)}$ -HO-2), δ $\sim$ 4.6 and 4.75p.p.m. (together; $G_{(1)}$ -HO-2, α and β), δ 4.82p.p.m. ($G_{(2)}$ -HO-3), δ 5.46p.p.m. ($N_{(1)}$ -HO-4) and δ 7.09p.p.m. ($G_{(2)}$ -HO-4). The HO signals at δ 4.36p.p.m. ($G_{(1)}$ -HO-3) and δ 5.24p.p.m. ($N_{(2)}$ -HO-4) increased in intensity in the proportions

1:2:3 on going from tetra- to hexa- to octa-saccharide. An HO signal not present in the tetra-saccharide appeared at δ 3.98p.p.m. with unit intensity in the hexasaccharide and twice-unit intensity in the octasaccharide. Spin-decoupling experiments assigned this to a G-HO-2 proton. Of the CH protons, only the anomeric H-1 signals were resolved in all oligomers. For the G residues, those assigned in the tetrasaccharide to $G_{(1)}$ -H-1 (δ 4.19 and 4.35p.p.m. together; α - and β -anomers) and to $G_{(2)}$ -H-1 (δ 4.09p.p.m.) remained at unit intensity in the higher oligomers, and an additional

Table 3. Chemical shifts of $H_{(1)}$, OH and NH protons of hyaluronate hexa- and octa-saccharides, with temperature coefficient of OH and NH protons in the hexasaccharide, in dimethyl sulphoxide solution at 25°C

Residue	Proton	Hexasaccharide		Octasaccharide δ (p.p.m.)
		δ (p.p.m.)	Temperature coefficient (p.p.b./K)*	
$N_{(1)}$	H-1	4.99 (α) $\sim$ 4.58 (β)		4.98 (α) $\sim$ 4.6 (β)
	HO-1	6.57 (α) 6.64 (β)	-3.8 -3.8	6.62 (α) 6.68 (β)
	HN-2	7.69 (α) 7.80 (β)	-3.8 -4.8	7.74 (α) 7.83 (β)
	HO-4	5.47	-1.4	5.46
	HO-6	4.49 (α) $\sim$ 4.6 (β)	-3.7	$\sim$ 4.6
$G_{(1)}$	H-1	4.38 (α) 4.19 (β)		4.39 (α) 4.17 (β)
	HO-2	4.75 (α) $\sim$ 4.6 (β)	-5.7	4.76 (α) 4.6 (β)
	HO-3	4.38	-4.3	4.36
$N_{(2)}$	H-2	$\sim$ 4.58		$\sim$ 4.6
	HN-2	9.21 (α) 9.13 (β)	-4.3 -4.3	9.23
	HO-4	5.26	-1.4	5.26
	HO-6	$\sim$ 4.6	-5.0	$\sim$ 4.6
$G_{(2)}$	H-1	4.17		4.17
	HO-2	3.99	-1.4	3.98
	HO-3	$\sim$ 4.38	-4.3	4.36
$N_{(3)}$	H-1	$\sim$ 4.6		$\sim$ 4.6
	HN-2	9.33	-3.8	9.23
	HO-4	5.26	-1.4	5.26
	HO-6	$\sim$ 4.6	-5.0	$\sim$ 4.6
$G_{(3)}$	H-1	4.08		4.17
	HO-2	3.81	-0.97	3.98
	HO-3	4.86	-6.7	4.36
	HO-4	7.10	-0.47	
$N_{(4)}$	H-1			$\sim$ 4.6
	HN-2			9.36
	HO-4			5.26
	HO-6			$\sim$ 4.6
$G_{(4)}$	H-1			4.08
	HO-2			3.87
	HO-3			4.86
	HO-4			7.11

* $\pm 10\%$. Negative shift is to higher field with increasing temperature.

G-H-1 signal appeared at δ 4.17 p.p.m. with unit intensity in the hexasaccharide and twice-unit intensity in the octasaccharide. For the N residues, the $N_{(1)}$ -H-1 resonance at δ 4.98 p.p.m. remained at an intensity representing slightly less than one proton in the α -anomer of the $N_{(1)}$ residue, the remaining $N_{(1)}$ -H-1 resonances occurring in a complex multiplet at δ approx. 4.6 p.p.m. together with the HO-6 signals. Comparison of these relationships between chemical shifts, relative intensities and oligomer structure, together with spin-decoupling experiments, led to the assignment of HN, HO and CH-1 signals for the hexa- and octa-saccharide given in Table 3.

In order to obtain information on hydrogen-bonding of HN and HO groups, the temperature coefficients of these chemical shifts were measured. Data for the tetra- and hexa-saccharide are given in Tables 2 and 3 respectively.

During observations of the tetrasaccharide spectra at 500 MHz, changes were noted in the HN region over a period of 2 h after dissolving the sample in dimethyl sulphoxide. Initially the peaks were rather broad, but gradually they sharpened, eventually giving well-resolved doublets.

Discussion

The tetrasaccharide (Fig. 3) is crucial to our understanding of the secondary structure in hyaluronate. It is the smallest oligomer in which two different resonances can be observed (hydrogen-bonded and non-hydrogen bonded), and in which the features of the polymer and of the simple monosaccharides and disaccharides can be discerned.

The resolving power at 500 MHz has enabled us to isolate, estimate the coupling constants of, and

selectively decouple most of the proton resonances in the hyaluronate tetrasaccharide (including α -/ β -anomers). Consequently, the resonances from two protons (HO-2 and HO-3) concerned in the proposed secondary structure, previously unassigned, have been identified, and the temperature coefficients of their chemical shifts determined.

N.m.r. evidence for hyaluronate secondary structure

The pattern of HN signals (Fig. 2) is similar to that observed in the corresponding oligomers of chondroitin 4-sulphate (Scott *et al.*, 1983b), namely a high-field resonance of unit intensity and a low-field resonance increasing in intensity in proportion to the number of repeating units. The high-field resonance is characteristic of the single environment found in the monosaccharide 2-acetamido-2-deoxy- α -D-glucose (Schamper, 1974) and in the model disaccharides of hyaluronate, methyl and allyl 2-acetamido-2-deoxy-3-O-(β -D-glucopyranosyluronic acid)- α -D-glucopyranoside (Heatley *et al.*, 1982). The low-field resonance is characteristic of the single HN environment found in the dodecyltrimethylammonium salt of hyaluronic acid (Scott *et al.*, 1981). The variation in relative intensity of the HN signals with oligomer size is consistent with the assignment of the high-field resonance to the reducing terminal residue and of the low-field resonance to internal disaccharides. The considerable downfield shift of the internal resonance compared with the terminal resonance is strongly suggestive of hydrogen-bonding in the polymer. A structure previously suggested to account for periodate oxidation kinetics (Scott & Tigwell, 1978) and X-ray-diffraction data (Atkins *et al.*, 1980) can also

Fig. 3. Structure of a tetrasaccharide segment of hyaluronate showing four different hydrogen bonds (.....), based on the work of Atkins *et al.* (1980)

Key: $N_{(1)}$, *N*-acetylglucosamine in reducing terminal position with no hydrogen bond at the acetamido NH; $G_{(1)}$, glucuronate moiety in penultimate sugar residue position; $N_{(2)}$ etc., *N*-acetylglucosamine in position of third sugar residue etc. In the hexa- and octa-saccharides the labelling system is similar, starting from the reducing terminal.

accommodate the HN resonance pattern both quantitatively and qualitatively (Fig. 3). The 'internal' acetamido group acts as donor and acceptor in a hydrogen-bonded array involving three sugars, whereas the terminal acetamido group may act only as an acceptor.

Further evidence for a difference between terminal and internal acetamido groups is obtained from the HC–NH coupling constants, which are significantly lower for the internal than for the terminal groups. Bystrov *et al.* (1973) reported that the HC–NH coupling constant in peptides depends on the dihedral angle according to:

$$J_{\text{HC-NH}} = 9.4 \cos^2 \phi - 1.1 \cos \phi + 0.4 \quad (1)$$

The relatively high values of $J_{\text{HC-NH}}$ for 'free' acetamido groups is consistent with either a *cis* or *trans* disposition of the CH and NH protons. Space-filling molecular models suggest that the *trans* arrangement is preferred. The lower value 6.2 Hz for $J_{\text{HC-NH}}$ in internal groups indicates a greater deviation of the H–C–N–H fragment from planarity consistent with the rotation required to form the hydrogen bonds indicated in Fig. 3. However, the coupling constant is much larger than the value of 0.4 Hz predicted by eqn. (1) for the dihedral angle of approx. 90° occurring in the proposed structure (Atkins *et al.*, 1980). Furthermore, the chemical-shift temperature coefficients of both terminal and internal HN signals are similar (–4.3 p.p.b./K), compared with –7.0 p.p.b./K for 2-acetamido-2-deoxy-D-glucose (Heatley *et al.*, 1982). It would be expected that a strongly intramolecularly hydrogen-bonded proton would have a much lower temperature coefficient than a similar proton forming hydrogen bonds only with the solvent. The chemical-shift, coupling-constant and temperature-coefficient data therefore suggest that the internal HN protons exist in equilibrium between an intramolecularly hydrogen-bonded state and a solvent hydrogen-bonded state, with the former predominating.

The suggested structure (Fig. 3) also incorporates three hydrogen bonds involving the HO groups N–HO-4, G–HO-2 and G–HO-3, for which the n.m.r. data provide evidence of the same type as that for the HN protons. The low-field shift and smaller temperature coefficient in the oligomers are consistent with intramolecular hydrogen-bonding of all N–HO-4 groups. H–C–O–H coupling constants vary with dihedral angle according to:

$$J_{\text{HC-OH}} = 10.4 \cos^2 \phi - 1.5 \cos \phi + 0.2 \quad (2)$$

(Fraser *et al.*, 1969). From this relationship, the monosaccharide coupling constant corresponds to free rotation, whereas the oligomer coupling constant indicates a substantial preference for dihedral angles in the range 60–120°. This range is

consistent with the proposed structure (Atkins *et al.*, 1980).

For the G–HO-2 protons, the $G_{(1)}$ penultimate residue signal is distinguished from the remaining signals (designated $G_{(2)+}$) by lower shielding, a higher temperature coefficient and a higher coupling constant. The parameters of the $G_{(1)}$ signal are similar to those of sodium glucuronate (Heatley *et al.*, 1979). The low temperature coefficient and coupling constant for the $G_{(2)+}$ resonances suggest a restricted conformation consistent with the proposed hydrogen bond to the adjacent acetamido carbonyl group. $G_{(1)}$ is not restricted in this way. However, the upfield shift of $G_{(2)+}$ relative to $G_{(1)}$ suggests that the intramolecular hydrogen bond of $C_{(2)+}$ to carbonyl is weaker than an intermolecular hydrogen bond of $G_{(1)}$ to dimethyl sulphoxide. The higher shielding of $G_{(2)+}$ cannot be explained by the magnetic anisotropy of the carbonyl bond, since a proton lying on or near the C=O axis, such as $G_{(2)+}$ in the proposed structure, is deshielded (Lyndon-Bell & Harris, 1969).

The pattern of the G–HO-3 resonances is similar to that of G–HO-2, though less marked, and with the difference that the unique proton is that in the non-reducing terminal ($G_{(n)}$) residue. The $G_{(n)}$ –HO-3 proton resonates to lower field than the internal protons and has a higher temperature coefficient and coupling constant. The chemical shift and temperature coefficient (though not the coupling constant) are similar to those of HO-3 in sodium glucuronate (Heatley *et al.*, 1979). The lower temperature coefficients and coupling constants of the internal groups are consistent with the suggested structure, and the greater shielding suggests a relatively weak intramolecular bond.

The spectral parameters for dimethyl sulphoxide solutions of hyaluronate model disaccharides (Heatley *et al.*, 1982) correspond closely to those of the unique reducing and non-reducing terminal resonances reported here.

With two exceptions the hyaluronate oligomer chemical shifts are within 0.2 p.p.m. of the corresponding chondroitin 4-sulphate resonances (Scott *et al.*, 1983b). The first exception is the N–H-4 resonance, which occurs approx. 0.8 p.p.m. to lower field in the chondroitins as a consequence of the replacement of N–HO-4 in the hyaluronate oligomers by the much more electronegative sulphate ester group. The second exception is the $G_{(2)+}$ –HO-2 resonance, which occurs at approx. 0.6 p.p.m. to lower field in the chondroitin 4-sulphate. This shift implies stronger hydrogen bonding of this proton in chondroitin 4-sulphate than in hyaluronate. In hyaluronate, two hydrogen bonds can be formed across the G→N junction, one from G–HO-2 to the *N*-acetamido carbonyl and the other from N–HO-4 to G–O-6. From the

discussion above, the latter bond is preferred to the former. In the chondroitin series the $N_{(m)}\text{-HO-4}$ bond cannot form because the HO-4 group is axial (or sulphated), thus permitting some adjustment of glycoside torsion angles leading to a more favourable $G\text{-HO-2}\rightarrow\text{carbonyl}$ bond. The same conclusion was reached from a comparison of hyaluronate disaccharides and sodium *N*-acetylchondrosinate (Heatley *et al.*, 1982).

Implications of the observed structure

Our result, that hyaluronate possesses an extensive secondary structure in dimethyl sulphoxide, is the first unequivocal and detailed demonstration in any solution. The questions raised are: (1) how important to its stability are the details of the structure, (2) does the structure exist in other solvents, particularly water, (3) does this structure throw light on the configurations and functions of similar glycosaminoglycans, and (4) does the polymer interact in this configuration in any or all of its physiological roles?

How important is each contributing hydrogen bond? The strength and permanence of each of the four hydrogen bonds in the 'repeating trisaccharide' can be assessed by comparing the observed coupling constants of the H atoms involved with those expected from the molecular models. The very small values associated with $G_{(2)}\text{-HO-2}$ and $N\text{-HO-4}$ imply that the O-H bond is oriented as required by the structure in Fig. 3, and that they are oriented in this way most of the time. If there was a rapid equilibration between an internal hydrogen-bonded configuration and normal solvation, the observed J values would be the weighted average of the normal value (i.e. 6–7 Hz) and an internal hydrogen-bonded value of approx. 0. The J values for the NH hydrogen atom established that the group is on average oriented differently from the normal solvated state. The J values are sufficiently high, however, to imply that a significant population of NH in the normal solvated configuration exists in equilibrium with the hydrogen-bonded form. Alternatively, the geometry of the sugar rings and the glycosidic bonds could differ significantly from those assumed for model-building. No evidence for this can be seen in the coupling constants we have observed for other groups in the molecule. The $G\text{-HO-3}$ couplings and temperature coefficients suggest a somewhat weaker hydrogen bond than that to $G\text{-HO-2}$. There is a considerable element of co-operation in the proposed structure. If the $G\text{-HO-2}\rightarrow\text{acetamido}\rightarrow\text{carboxylate}$ bonds form, the two $\text{OH}\rightarrow\text{ring-oxygen}$ bonds would inevitably follow, and vice versa, since the orientation of all four sets of groups is not independent, and the juxtaposition of one pair requires the coming together of the rest,

assuming that ring geometries do not alter significantly. This co-operation does not extend beyond the trisaccharide.

Does the structure exist in H_2O ? The c.d. behaviour of the lower oligomers of hyaluronate in H_2O parallels that of the NH resonance in dimethyl sulphoxide, implying that, in dimethyl sulphoxide and H_2O , the acetamido group environment undergoes a change on passing from disaccharide to tetrasaccharide, and this is compatible with the first opportunity to form the structure shown in Fig. 3 (Scott *et al.*, 1981). B. Casu (personal communication), interpreting the results reported by Welti *et al.* (1979), suggested that the changes in the n.m.r. spectrum on going from 2H_2O to $^2H_2O/NaOH$ solution were compatible with some aspects of the structure in Fig. 3. Periodate oxidation of hyaluronate is much slower than would be expected, compared with model compounds and other glycosaminoglycuronans in aqueous solution with and without added salt (Scott & Tigwell, 1978). It is therefore relevant that the glycol group ($G\text{-HO-2}$ and $G\text{-HO-3}$) is hydrogen-bonded via both hydroxy groups in the structure shown in Fig. 3. Presumably both hydrogen bonds must be disrupted before the periodate-ion-glycol complex forms and oxidation takes place.

Preliminary results have shown that the rate of hydrogen exchange in H_2O of the potentially hydrogen-bonded NH in the tetrasaccharide (i.e. $N_{(2)}\text{-HN}$) is slower than that of the non-hydrogen-bonded NH (i.e. $N_{(1)}\text{-HN}$), thus supporting the idea that some hydrogen-bonded character is associated with the former but not with the latter (J. E. Scott, F. Heatley & W. E. Hull, unpublished work).

Comparison with other glycosaminoglycans. The present work, and that presented previously (Scott & Tigwell, 1978; Scott *et al.*, 1981, 1983b), shows that hyaluronate and the chondroitin 4- and 6-sulphates are the only members of the glycosaminoglycuronans in which this type of structure (Fig. 3) could occur. All the hydrogen-bonding groups, as well as the glycosidic bonds, must be equatorial. The bond from the axial galactosamine $N\text{-HO-4}$ to the G ring oxygen atom cannot form in the chondroitin sulphates, leaving a maximum of three different hydrogen bonds, as compared with four in hyaluronate. In dermatan sulphate, regardless of whether the iduronate confirmation is 1C_4 or 4C_1 , the $\text{NH}\rightarrow\text{CO}_2^-$ bond cannot form, and probably not the $G_{(2)}\text{-OH}\rightarrow\text{O}=\text{C}<$. At most, dermatan sulphate may have one of the hydrogen bonds shown in Fig. 3 (i.e. $G\text{-HO-3}\rightarrow\text{N ring O}$), but only if the iduronate is the 4C_1 form. A slightly different arrangement, involving only two hydrogen bonds, is possible in heparan sulphate (Scott &

Heatley, 1982), and no evidence of secondary structure in keratan sulphate has emerged.

The potential to form the hydrogen-bonded array of Fig. 3 is correlated with several functionally significant facts. Those polymers (i.e. chondroitin 4-sulphate, chondroitin 6-sulphate and hyaluronate) that can form most or all of the hydrogen-bonded array in Fig. 3 are present in tissues in distributions suggesting that their function is to fill space, i.e. occupy large domains of solution. The space that they fill is defined by the protein fibrils, particularly collagen, with the result that the fibrils are held apart. The tissue is able to oppose forces that would bring the fibrils together. Thus in cartilage and cornea, Wharton's jelly etc. the macroscopic property of the tissue is to oppose elastically those forces that tend to change its size and shape. The stiffer the polymer chain, the greater the resistance to opposing forces that would diminish the polymer domain. The hydrogen-bonded array (Fig. 3) would be expected to contribute significantly to the stiffness of the polymer. Significantly, those polymers that do not possess the ability to form the array do not occur in large accumulations, as does chondroitin sulphate in cartilage, but in small amounts, specifically located, as e.g. dermatan sulphate in tendon, heparan sulphate in cell membranes etc. In this context, the space-filling chondroitin sulphates constitute a large proportion (80–90%) of their parent proteoglycan, whereas dermatan sulphate and heparan sulphate constitute less than 50% of their proteoglycans. This is compatible with the idea that the chondroitin sulphate proteoglycan is an attempt to pack as much space-filling and deformation-resistant polymer into one molecule

as possible, whereas the dermatan and heparan proteoglycans interact more specifically with their 'receptors', probably via their protein moieties.

References

- Atkins, E. D. T., Meader, D. & Scott, J. E. (1980) *Int. J. Biol. Macromol.* **2**, 318–319
- Bystrov, V. F., Portnova, S. L., Balashova, T. A., Koz'min, S. A., Gavrilov, Yu. D. & Afanasev, V. A. (1973) *Tetrahedron* **29**, 873–877
- Casu, B., Reggiani, M., Gallo, G. G. & Vigevani, A. (1966) *Tetrahedron* **22**, 3061–3083
- Cowman, M. K., Balazs, E. A., Bergman, C. W. & Meyer, K. (1981) *Biochemistry* **20**, 1379–1385
- Ferrige, A. G. & Lindon, J. C. (1978) *J. Magn. Reson.* **31**, 337–340
- Fraser, R. R., Kaufman, M., Morand, P. & Govil, G. (1969) *Can. J. Chem.* **47**, 403–409
- Heatley, F., Scott, J. E. & Casu, B. (1979) *Carbohydr. Res.* **72**, 13–23
- Heatley, F., Scott, J. E., Jeanloz, R. & Walker-Nasir, E. (1982) *Carbohydr. Res.* **99**, 1–11
- Lyndon-Bell, R. M. & Harris, R. K. (1969) *Nuclear Magnetic Resonance Spectroscopy*, pp. 86–91, Thomas Nelson and Sons, London
- Schamper, T. J. (1974) *Carbohydr. Res.* **36**, 233–237
- Scott, J. E. & Heatley, F. (1982) *Biochem. J.* **207**, 139–144
- Scott, J. E. & Tigwell, M. J. (1978) *Biochem. J.* **173**, 103–114
- Scott, J. E., Heatley, F., Moorcroft, D. & Olavesen, A. H. (1981) *Biochem. J.* **199**, 829–832
- Scott, J. E., Heatley, F. & Hull, W. (1983a) *Proc. Int. Symp. Glycoconjugates 7th* 59–60
- Scott, J. E., Heatley, F., Jones, M. R. N., Wilkinson, A. & Olavesen, A. H. (1983b) *Eur. J. Biochem.* **130**, 491–495
- Walti, D., Rees, D. A. & Welsh, E. J. (1979) *Eur. J. Biochem.* **94**, 505–514