Detection of secondary structure in glycosaminoglycans via the ¹H n.m.r. signal of the acetamido NH group

John E. SCOTT* and Frank HEATLEY†
*Departments of *Biochemical Morphology and †Chemistry, University of Manchester, Chemistry Building, Brunswick Street, Manchester M13 9PL, U.K.*

Two simple methods for dissolving salts of acid glycosaminoglycans with inorganic cations (e.g. Li^+ and Na^+) in dry dimethyl sulphoxide are described. Complete n.m.r. spectra of, e.g., Na^+ and Li^+ salts of chondroitin sulphate and keratan sulphate were obtained on these solutions. In [$^2H_6$]dimethyl sulphoxide the NH resonance of 2-acetamido-2-deoxy hexoses is in the range 7.2–8.0 δ, but is downfield (8.3–9.3 δ) when the NH is H-bonded to $-CO_2^-$. Heparan sulphate shows two NH resonances, of which one (at 8.3 δ) is probably indicative of H-bonding. Space-filling models show that a very close approach of NH to $-CO_2^-$ across the α-glucosaminidic bond is possible, and a solution configuration for heparan sulphate is proposed. The n.m.r. results are entirely compatible with interpretations of periodate-oxidation kinetics, based on H-bonded secondary structures present in hyaluronate and chondroitin sulphates, but not in dermatan (or keratan) sulphate.

The differential distribution of the various acid glycosaminoglycans in animal connective tissues (Meyer *et al.*, 1956) is such that, despite their very similar chemical structures, they must fulfil quite dissimilar and specific roles. Until recently, there was little hint of a systematic basis for a structure–function relationship. A clue came from the observation that the C_2–C_3 glycol group of the uronic acid moiety in hyaluronate and chondroitin 4- and 6-sulphates was much less susceptible to cleavage by periodate than the analogous group in dermatan sulphate, heparan sulphate, teichuronate, alginate, pectate and relevant monomeric glycosides (Scott & Tigwell, 1978). The common feature shared by the resistant polymers was a 'co-planar' arrangement of acetamido, uronate carboxy and glycol groups, because of the equatorial disposition of these groups, and of the glycosidic bonds. Space-filling models showed that an H-bonded intramolecular secondary structure was possible, involving these groups, which could not form in the easily periodate-oxidizable glycosaminoglycuronans (Scott & Tigwell, 1978).

Experimental evidence for these structures came from n.m.r. investigations of the polymers in ^2H_2O (Scott & Heatley, 1979), and of hyaluronate oligomers in [^2H]dimethyl sulphoxide, in which the H-bonded NH of the acetamido group resonated downfield of the 'normal', non-H-bonded NH (Scott *et al.*, 1981). Thus the occurrence of the downfield NH resonance in the spectra of glycosaminoglycans

in dimethyl sulphoxide should provide an indication of secondary structure of more general application than the periodate-oxidation-kinetics method, for example, to compounds such as keratan sulphate, in which glycol groups do not occur regularly throughout the polymer.

In the present paper we survey the spectra of the group of connective-tissue acid glycosaminoglycans for the presence of the downfield NH resonance and compare the conclusions with those from periodate-oxidation and model-building studies. We also report two simple procedures for achieving quite concentrated (1–10%, w/v) solutions of inorganic-cation (e.g. Na^+, Li^+) salts of polymeric glycosaminoglycans in dry dimethyl sulphoxide, which greatly increases the possibilities for investigating the effect of the counterion on the solution configurations of the polymer.

Experimental

Materials

Chondroitin 4-sulphate (whale) and chondroitin 6-sulphate (shark) were from Sigma (London) Chemical Co. Both samples of keratan sulphate were gifts from Dr. H. W. Stuhlsatz and Dr. H. Greiling, Aachen, Germany. 'No. 70' contained 0.77 mol of sulphate/mol of glucosamine, less than 1% galactosamine, and had mol.wt. 7780. 'No. 82' contained 1.41 mol of sulphate/mol of glucosamine

and less than 1% galactosamine. The mol.wt. was 25 600. 'Nos. 72 and 82' contained 43 and 11 residues of amino acids/100 residues of glucosamine respectively. We are grateful to Dr. Stuhlsatz and Dr. Greiling for these results. Both samples (HS 0.9 and HS 1.2) of heparan sulphate were gifts from Dr. Alfred Linker, Salt Lake City, UT, U.S.A., prepared from bovine lung (Linker & Hovingh, 1973). Hyaluronate octasaccharide was the sample used by Scott *et al.* (1981). Dermatan sulphate was from pig skin, prepared by the method of Scott (1960). Heparin, from bovine mucosa, was a commercial sample (Paines and Byrne, Greenford, Middx., U.K.). Decyltrimethylammonium bromide was from Eastman Kodak and was used as an aq. 4% (w/v) solution.

Methods

Conversion of the glycosaminoglycan Na⁺ salts into Li⁺ salts. This was achieved by double-decomposition in ethanol/water solvents; e.g., to 50 mg of sodium chondroitin 4-sulphate in 2.5 ml of aq. 4.5 M-LiCi was added 2 vol. of ethanol. The precipitate was centrifuged, washed several times in ethanol, then in ethanol/diethyl ether (1:1, v/v), diethyl ether, and air-dried. Decyltrimethylammonium salts were precipitated from aqueous solution (Scott, 1960), centrifuged, washed in water and dried in air or *in vacuo* at room temperature.

Solutions in dry dimethyl sulphoxide of Li⁺ or Na⁺ salts of the polyanions. These were achieved in two ways.

(1) *Dialysis.* To a concentrated (>10%, w/v) aqueous solution of glycosaminoglycan Li⁺ or Na⁺ salt was added 10 vol. of dimethyl sulphoxide with thorough mixing. The clear solution was dialysed in a Visking dialysis tube against several changes of dimethyl sulphoxide, with stirring of the external solution, with or without molecular-sieve type 5A, over a period of 4–5 days.

(2) *Freeze-drying.* A concentrated solution of glycosaminoglycan Li⁺, Na⁺ or decyltrimethylammonium salt in dimethyl sulphoxide/water (9:1, v/v) was prepared, and freeze-dried with an Edwards Hi-vac pump at a pressure of 2–3 μmHg (about 30 Pa). A mercury pump, giving a vacuum of 0.1 Pa, was sometimes used, but was not essential. Care was taken that the solution remained frozen; this was done by placing a freezing mixture (−5 to 0°C) around the container. The friable residue dissolved easily in dry [²H₆]dimethyl sulphoxide, particularly rapidly in the case of decyltrimethylammonium salts. The polyanion could be recovered from the dimethyl sulphoxide solution as the sodium salt by adding 4 vol. of saturated ethanolic sodium acetate.

N.m.r. experiments. ¹H n.m.r. spectra were run on a Varian SC 300 spectrometer, operating at 300 MHz. Chemical shifts were expressed relative to tetramethylsilane and were measured relative to the resonance of [²H₅]dimethyl sulphoxide [taken as 2.50 p.p.m. (δ) from tetramethylsilane].

Results

As normally encountered, Na⁺ or Li⁺ salts of chondroitin sulphate etc. dissolve at negligible rates in dry dimethyl sulphoxide. After freeze-drying from solvent containing mainly dimethyl sulphoxide, the friable solid (similar in appearance to that obtained by freeze-drying from water) dissolved readily in dry dimethyl sulphoxide. The dialysis procedure did not remove the last traces of water with the same efficiency as freeze-drying, but ¹H n.m.r. spectra could be obtained in which NH and CH₃ resonances were visible.

Solutions (1–2%, v/v) in dimethyl sulphoxide of Li⁺ and Na⁺ salts of chondroitin 4- and 6-sulphates, heparin, heparan and keratan sulphates, and hyaluronate octasaccharide were all mobile, slightly viscous, clear colourless fluids. Those of hyaluronate and dermatan sulphate were very viscous, clear colourless fluids, from which spectra were not obtained. The addition of excess decyltrimethylammonium to these solutions produced no obvious effect. A spectrum was obtained of the Na⁺ salt of hyaluronate octasaccharide (mol.wt. 1600) without difficulty. Fig. 1 shows the complete spectrum of keratan sulphate in [²H₆]dimethyl sulphoxide compared with that of chondroitin 4-sulphate and heparan sulphate HS 0.9. Table 1 lists the chemical shifts of the NH signals of the glycosaminoglycans.

The chemical shifts of the hyaluronate–chondroitin–keratan sulphate series fall into two groups, one around 8.5–9.3 δ, the other at 7.5–7.9 δ, The spread of values in the latter group was smaller, and only one clear signal was observed for each polymer. The former group often showed more than the main downfield signal, with smaller peaks at lower δ values (see Fig. 1).

Each sample of heparan sulphate showed a very broad NH resonance (7.5–8.5 δ) as Li⁺ salt, but as decyltrimethylammonium salts both showed two resonances, at 7.9 and 8.3 δ (Fig. 1). The addition of decyltrimethylammonium to the Li⁺ heparan sulphate solutions did not affect the position or width of the NH resonance. The spectrum of heparin as Na⁺ salt contained no resonance downfield of 7.0

Discussion

Studies on oligomers from hyaluronate produced strong evidence for a secondary structure in dimethyl sulphoxide solution, involving H-bonded NH groups (Scott *et al.*, 1981). The n.m.r. resonance of these groups was well downfield (9.3 δ) of
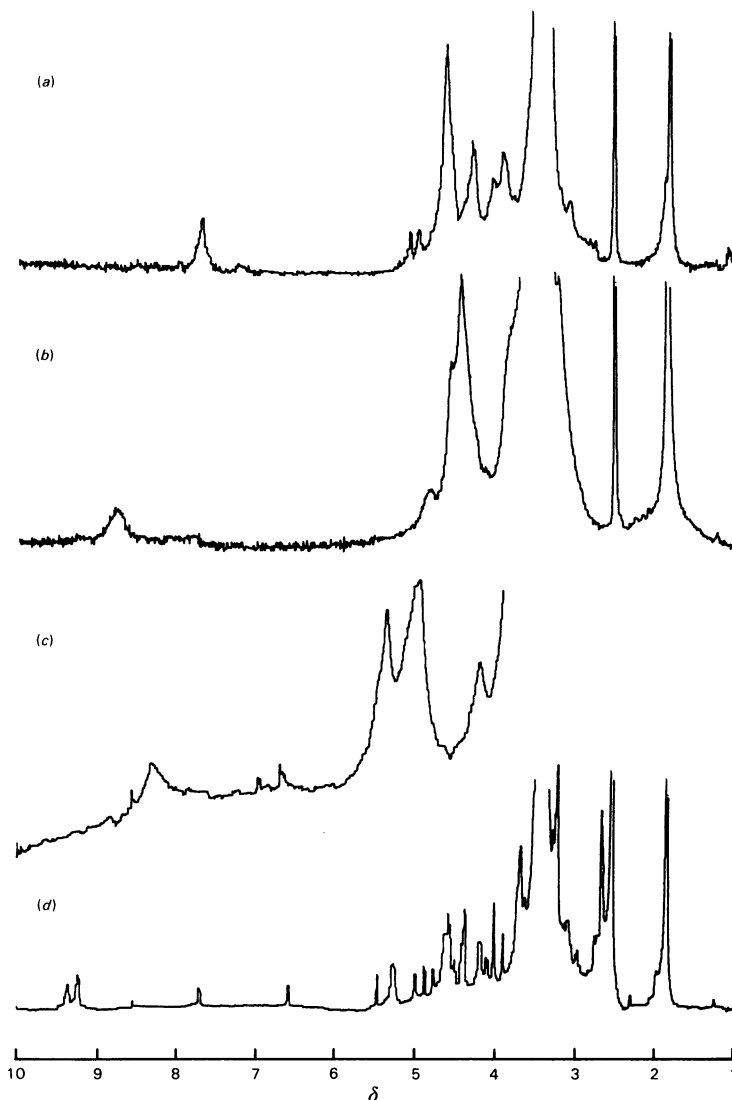
Fig. 1. *¹H n.m.r. spectra in [²H₆]dimethyl sulphoxide of (a) lithium keratan sulphate 'No. 70' (see under 'Materials'), (b) lithium chondroitin 4-sulphate, (c) decyltrimethylammonium heparan sulphate HS 0.9 (see under 'Materials') and (d) sodium hyaluronate octasaccharide*

Spectrum (d) shows clearly H-bonded and non-H-bonded NH resonances (9.3 and 7.8 δ) (Scott et al., 1981). The keratan sulphate spectrum (a) shows a non-H-bonded resonance (7.7 δ) and the other spectra show NH resonances indicative of various degrees of H-bonding. Only the downfield half of the spectrum of heparan sulphate is shown; the upfield spectrum is complicated by resonances from the quaternary ammonium ion. The signals at 2.0, 2.5 and 3.5 δ are acetamidomethyl, [²H₅]dimethyl sulphoxide and water resonances respectively. The smaller downfield signal in the group at 9.3 δ is the resonance of the single non-reducing terminal-disaccharide hydrogen-bonded NH, and the larger signal is from the hydrogen-bonded NH groups of the two 'internal' disaccharides of the octasaccharides (J. E. Scott & F. Heatley, unpublished work).

'normal', i.e. non-H-bonded acetamido NH. The NH resonance of 2-acetamido-2-deoxygalactose in five glycosphingolipids dissolved in [²H₆]dimethyl sulphoxide was in the range 7.1–7.79 δ (Gasa et al.,

1981), i.e. similar to, or lower than, the range of our 'normal' series. It would appear that the NH chemical shift in dimethyl sulphoxide of 2-acetamido-2-deoxy hexosides is less than 8.0 δ if the

Table 1. *Chemical shifts (δ) of NH resonances of acid glycosaminoglycans dissolved in [$^2H_6$]dimethyl sulphoxide at 21°C*

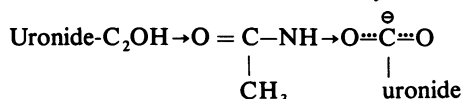|		δ		
Polymer Salt ...		Li$^+$	Na$^+$	Decyltrimethylammonium
Hyaluronate octasaccharide			9.3*, 7.8	
Chondroitin 4-sulphate		8.8*, 7.8	8.4*, 7.8	
Chondroitin 6-sulphate		8.8, 8.1*		
Dermatan sulphate				8.0†
Keratan sulphate 'No. 70'		7.7*, 7.2		
'No. 82'			7.8, 7.75‡	
Heparan sulphate HS 0.9				8.3*, 7.9
HS 1.2				8.3*, 7.9
Heparin			N.O.§	

* Major peak.
† Dodecyltrimethylammonium salt, data from Scott *et al.* (1981).
‡ Twin peak, not completely resolved.
§ N.O., no resonance observed.

NH is not H-bonded. This generalization would not necessarily hold in other solvents or for other types of compound (see, e.g., Llinas & Klein, 1975).

It is therefore noteworthy that the n.m.r. spectra of hyaluronate and chondroitin 4- and 6-sulphates, but not of keratan or dermatan sulphate, contain the downfield NH resonance, indicative of H-bonding. This suggests that, if there is regular intramolecular secondary structure in the keratan sulphates and dermatan sulphate, it does not involve H-bonded acetamido NH. This conclusion is entirely compatible with periodate-oxidation-kinetics data (Scott & Tigwell, 1978), which indicated that hyaluronate and the chondroitin sulphates differed systematically from dermatan sulphate. Models showed that the former were able to form H-bonded arrays

$$\text{Uronide-C}_2\text{OH}\rightarrow\text{O} = \text{C}-\overset{|}{\text{N}}\text{H}\rightarrow\text{O}\overset{\ominus}{\cdots}\overset{|}{\text{C}}\cdots\text{O}$$
$$\qquad\qquad\qquad\quad\ \text{CH}_3 \qquad\quad \text{uronide}$$

incorporating the acetamido group, whereas the latter was not, because the uronide carboxylate group was axial, resulting in severe distortion of the structure. The n.m.r. data strongly support the predictions from the models, in that H-bonded NH resonances are found only in the spectra of those polymers which can form the

$$\text{NH}\rightarrow\text{O}\overset{\ominus}{\cdots}\text{C}\cdots\text{O}$$
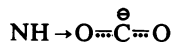
structure.

The absence of H-bonded NH resonance from the keratan sulphate spectrum is to be expected, since galactose replaces uronic acid, and hence there is no acceptor carboxylate.

As indicated by the two NH resonances, the acetamido NH of heparan sulphate is found in two different environments: one 'normal' and the other (8.3 δ) probably weakly H-bonded. Space-filling models (Fig. 2) (cf. Scott & Tigwell, 1978) show that

the uronate carboxylate can approach within H-bonding distance of the acetamido NH across the α1 →4 glucosaminidic (axial; Atkins & Laurent, 1973) link, provided the carboxylate is equatorial, as in D-glucuronic acid (C1 form) and L-iduronic acid (1C). Both are present in heparan sulphate (B. Casu, personal communication), but D-glucuronic acid predominates, and it seems reasonable to attribute the major resonance at 8.3 δ to the glucosamine–glucuronic acid pair, implying that the glucosamine–iduronic acid pair is not H-bonded. Heparan sulphate in solution may sustain the proposed arrangement, with carboxylate and acetamido groups in close proximity (Fig. 2) for significant periods of time, since this configuration allows a further H-bond between the C-3 OH group of glucosamine and the C-2 OH group of glucuronic acid. The heparan sulphate polymer backbone shows a pronounced zig-zag, in contrast with that of hyaluronate and chondroitin sulphate (Scott & Tigwell, 1978). The acetamido carbonyl group is not positioned to H-bond to uronate OH groups, unlike that in hyaluronate and chondroitin sulphate, which is presumably reflected in the much faster oxidation of the glycol of glucuronic acid in heparan sulphate by periodate (Scott & Tigwell, 1978).

The behaviour of the Li$^+$ salt of dermatan sulphate in producing very viscous solutions in dry dimethyl sulphoxide implies inter-polymer interactions not found in the other glycosaminoglycans. Aqueous solutions were not viscous, and the molecular weight of dermatan sulphate prepared by our methods (Scott, 1960) is similar to those of other sulphated glycosaminoglycans. Solutions of dodecyltrimethylammonium salts of dermatan sulphate in dimethyl sulphoxide were not highly viscous, and spectra were obtained at high concentrations (Scott *et al.*, 1981). Apparently the auto-aggregation of dermatan sulphate observed in aqueous solutions
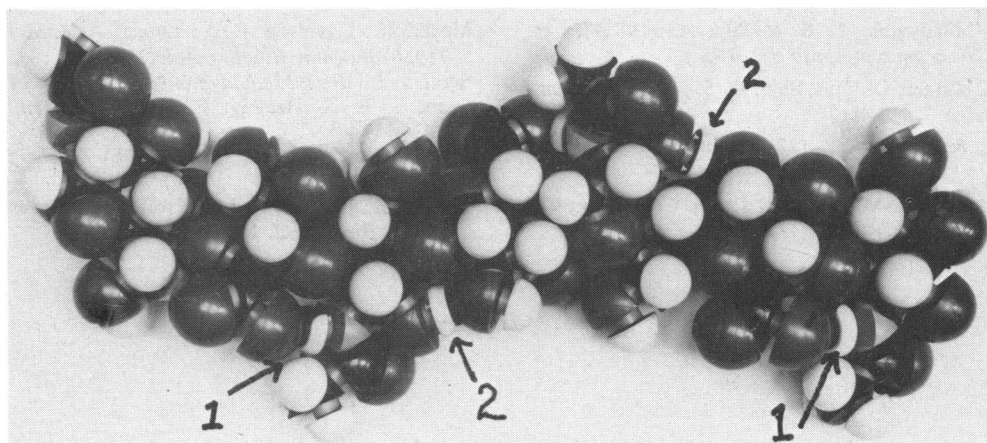
Fig. 2. *A model constructed from space-filling Courtauld units, of a heptasaccharide containing repeating disaccharides of 2-acetamido-2-deoxy-D-glucose and D-glucuronic acid*
The glucosaminidic link is α1 →4, and the glucuronide is β1 →4. The arrows indicate possible H-bonds, from (1) NH to CO_2^- and (2) hexosamine C-3 OH group to uronide C-2 OH group. The proposed configuration shows a pronounced zig-zag, in contrast with the linear, tape-like H-bonded structures of hyaluronate and chondroitin sulphate (Scott & Tigwell, 1978).

(Fransson, 1976) is more marked in dimethyl sulphoxide. The finding that aggregation in water was sensitive to the nature of the ambient anions (Fransson, 1976) is noteworthy in view of the difference we observed between Li⁺ and quaternary ammonium. However, heparan sulphates, which also self-associate (Fransson *et al.*, 1980), did not give viscous solutions as Li⁺ salts, although the very broad NH resonance may be evidence of aggregation.

The curious discrepancy between the *very* low rates of solubilization of inorganic-cation salts of acid glycosaminoglycans in dimethyl sulphoxide compared with the high concentrations finally achievable reflect the difference between kinetic and equilibrium effects. Presumably inter-molecular H-bonds and water bridges in the solid are split or replaced more rapidly by water than by dimethyl sulphoxide. Once in aqueous solution, dimethyl sulphoxide is able to replace water on the hydrated groups of the polymer, while maintaining a stable solution. The freeze-dried solid from [¹H]dimethyl sulphoxide contains appreciable amounts of dimethyl sulphoxide, detectable in the n.m.r. spectrum. Most of the OH groups etc. are probably H-bonded to dimethyl sulphoxide (see, e.g., Kim *et al.*, 1971), and hence cannot participate in inter-molecular H-bonds.

Since dimethyl sulphoxide, unlike water, is not bifunctional, it cannot bridge two molecules. Dimethyl sulphoxide-solvated polymers are thus freer to disperse into solvent than the corresponding hydrated polymers.

These findings, and the indication (see above) that dermatan sulphate aggregates more readily in dimethyl sulphoxide than in water, suggest that inter- or intra-molecular hydrogen bonds may form more readily in dimethyl sulphoxide. It is therefore relevant that there is much evidence that the secondary structures 'seen' in n.m.r. spectra in dimethyl sulphoxide are also present in ^1H_2O, ^2H_2O and salt solutions [see Scott *et al.* (1981) for further discussion].

There is spectroscopic advantage in using Li⁺ and Na⁺, rather than quaternary ammonium, since the latter contributes resonances which obscure part of the polymer spectrum. Dr. J. Boyd and Dr. F. B. Williamson (personal communication) informed us that they had obtained i.r. spectra from Li⁺ salts of the glycosaminoglycans dissolved in dimethyl sulphoxide. The methods we developed should be applicable to a range of cations and solvents with implications in the study of glycosaminoglycan physics and chemistry. The dialysis procedure requires only the simplest equipment, but is more prodigal of solvent, whereas the freeze-drying method produces solutions which are as 'dry' as the best available solvent.

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