# Comparison of Secondary Structures in Water of Chondroitin-4-sulfate and Dermatan sulfate: Implications in the Formation of Tertiary Structures

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ABSTRACT: Chondroitin-4-sulfate (CS4) and dermatan sulfate (DS) were examined in $D_2O$ solution, with or without NaCl, by NMR at 300 MHz, to investigate the physicochemical consequences of epimerization of glucuronate (GlcUA in CS4) to iduronate (IdoUA in DS). Nuclear Overhauser effects (NOEs) and spin−lattice relaxation times following selective and nonselective inversion were measured at up to 70 °C. (1) NOEs confirmed $^4C_1$ conformations of sugar rings in *N*-acetylgalactosamine and GlcUA, and $^1C_4$ or $^2S_0$ in IdoUA. Conflict between NMR data and periodate oxidation kinetics over IdoUA conformations is resolvable by postulating conversion of monodentate periodate−$^1C_4$ complexes to conformations in which periodate oxidation can procede. (2) Pairs of glycosidic protons in CS4 and DS showed strong NOEs, implying that stretches of 2-fold helix were present, with carboxylate and acetamido groups close to each other on the same side of tapelike molecules, extending previous work in dimethyl sulfoxide solution. CS4 and DS have large hydrophobic patches in this configuration, similar to those in keratan sulfate and hyaluronan. (3) Selective and nonselective inversion−recoveries implied similar segmental and backbone mobilities and hence flexibilities in CS4 and DS. This is discussed in terms of intrinsic flexibility of glycosidic conformations, modified by hydrogen-bonded arrays. (4) We postulate that hydrophobic and hydrogen bonding drives DS self-aggregation. Stronger self-aggregation of DS compared with CS4 is attributed to increased intermolecular hydrogen-bonding in DS, secondary to decreased intramolecular hydrogen-bonding. This is partly because the axial OH groups in $^1C_4$ IdoUA cannot hydrogen-bond to neighboring sugars as can the equatorial OH groups in GlcUA of CS4.

Chondroitin-4-sulfate (CS4),[1] consisting of alternating *N*-acetyl D-galactosamine $\beta$ 1:4 and D-glucuronate $\beta$ 1:3 residues (Figure 1), is one of a family of linear polymers based on a polylactose backbone (Scott, 1994). It is found in most, if not all, connective tissues (cartilage, skin, cornea, vessel walls, etc.), attached to proteins, in the form of proteoglycans. Some of these are associated with collagen fibrils, helping to maintain an elastically deformable interfibrillar space via which cells metabolize and communicate (Scott, 1992). Sulfation in the CS series is variable, depending on the tissue, its age, and the species. However, the potential to form a secondary structure including a regular multihydrogen-bond array thoughout the molecule, involving acetamido, carboxylate, hydroxyl, and ring oxygen is always present (Scott et al., 1992).

Dermatan sulfate [DS, alternatively dermochondan sulfate (Scott, 1993b)] is metabolically derived from CS by epimerization of D-glucuronate (GlcUA) to L-iduronate (IdoUA) (Figure 1). Almost all the GlcUA is epimerized to IdoUA in DS from some tissues, while in others less than 20% conversion occurs. Since sulfation is closely connected with epimerization, primary structures in DSs vary widely, even within the same chain [see Scott (1993a) for reviews]. Furthermore, in contrast to GlcUA, which has a stable $^4C_1$-conformation, IdoUA can occupy at least three conformations, $^4C_1$, $^1C_4$, and $^2S_0$ (Casu et al., 1988). There are thus many possible secondary structures within a DS chain, which might not include some features regularly present in the CS series (Scott, 1992). A most important question in this field is, what new potentialities and properties are opened up in DS as a result of GlcUA→IdoUA epimerisation?

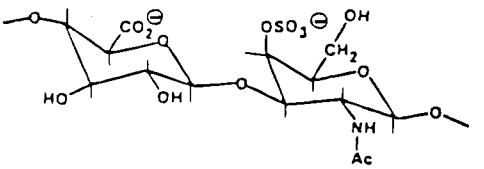


FIGURE 1: Structure of chondroitin-4-sulfate. Both sugar rings are $^4C_1$ and all major substituents are equatorial except the axial sulfate ester group on position 4 of GalNAc. *In vivo* epimerization of D-GlcUA in CS to L-IdoUA in DS, involving the C5 carboxylate group, produces new possibilities in which the L-IdoUA ring can take up at least three conformations, of which two ($^1C_4$ and $^2S_0$, see Scheme 1) predominate in DS (Results).

CS4 and DS differ markedly in their distributions in tissues. DS usually occurs at low concentrations compared with those of CS4, which comprises up to 10% dry weight in cartilage. "Small" (i.e. ~100 kDa) DS proteoglycans seem to organize and orient collagen fibrils—a vital role in all connective tissues—during development and in maturity (Scott, 1992). They are bifunctional molecules that crosslink collagen fibrils via anionic glycosaminoglycan (AGAG) ties and bridges, which are very probably inter-AGAG complexes. The detailed primary and secondary structures of DS chains determine whether these complexes can be formed [see, e.g., Scott (1993a) for reviews]. It is therefore relevant

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[1] Abbreviations: CS4; chondroitin-4-sulfate, DS; dermochondan (dermatan) sulfate, DMSO; dimethyl sulfoxide, GalNAc; *N*-acetylgalactosamine, GlcNAc; *N*-acetylglucosamine, NOE; nuclear Overhauser effect, GlcUA, glucuronate; IdoUA, iduronate.

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to ask, are the several IdoUA conformations (a) readily interconvertable, (b) stabilized in some way, or (c) do they take up alternative conformations depending on the molecules they interact with? NMR methods may elucidate these questions.

NMR studies of AGAGs have used spin−spin coupling constants (Perkin et al., 1970; Gatti et al., 1979) and chemical shifts (Welti et al., 1979) to determine sugar-ring conformations. Large negative nuclear Overhauser effects (NOEs) from slowly moving macromolecules provide information on interproton distances that can establish conformations of sugars as well as of glycosidic linkages, where the relevant protons are on different sugar residues. This aspect of polysaccharide structure is not accessible via $^1H$ coupling constants. Additional, though less direct, data on chain conformations are obtainable from spin−lattice relaxation times, which characterize the breadth of potential wells and the barriers between them.

This study examines $^1H$ relaxation and NOE data from CS4 and DS. A system of interresidue hydrogen bonds is formed in CS4 (Scott et al., 1983) that may not be identical in DS. These systems would be expected to affect chain dynamics. Longitudinal relaxation was studied using firstly, nonselective inversion in which all signals were inverted simultaneously and secondly, selective inversion in which only one signal or group of overlapping signals was inverted. Although the 300 MHz frequency we employed is modest, the spectral resolution was adequate for our purpose. The use of the selective inversion technique to study transient NOEs instead of the two-dimensional NOESY technique was justified by relatively simple accurate measurement of peak intensities and easy monitoring of the detailed time evolution of transient NOEs following selective inversion of one peak.

These results were reported preliminarily to the XIVth Federation of European Connective Tissue Societies, Lyon, Aug 31, 1994, in the workshop on "New approaches to extracellular matrix function".

## EXPERIMENTAL PROCEDURES

NMR data were acquired on a Varian Associates SC-300 spectrometer at 300 MHz. Longitudinal relaxation times were measured using the inversion−recovery sequence ($\pi-\tau-\pi/2$). Selective inversion (~70%) was achieved by pulsing the homonuclear decoupler to produce a $\pi$ pulse length of ~10 ms. Relaxation times were obtained from the slope of a straight line fitted by least-squares to a plot of time vs. log deviation from equilibrium, over a time extending to 75% recovery for nonselective inversion and 50% recovery for selective inversion. Points were weighted proportionally to the deviation from equilibrium. Uncertainties were ±5% for nonselective inversion relaxation times and ±10% for selective inversion experiments.

CS4 (bovine nasal septa) and DS (pig skin) were prepared according to Scott (1960). Both had ~1.0 sulfate group per disaccharide unit, and the DS contained more than 85% of its uronate as IdoUA. Typical molecular weights are ~20 kDa (Mathews, 1959), corresponding to ~40 repeating disaccharide units. The samples were used as sodium salts freeze-dried twice from "100%" $D_2O$ (Aldrich Chemical Co. Ltd., Gillingham, U.K.). Sodium chloride ("Analar" B. D. H. Ltd., Poole, U.K.) was dried overnight at 100 °C before use. Solutions in $D_2O$ contained 13 g/L (CS4) or 24 g/L (DS). NaCl was added to molarities of 0.22 (CS4) or 0.36 (DS), where relevant. Sample preparations were carried out in a dry box. Models were constructed from Courtauld space-filling atoms [see, e.g., Scott and Tigwell (1978)].

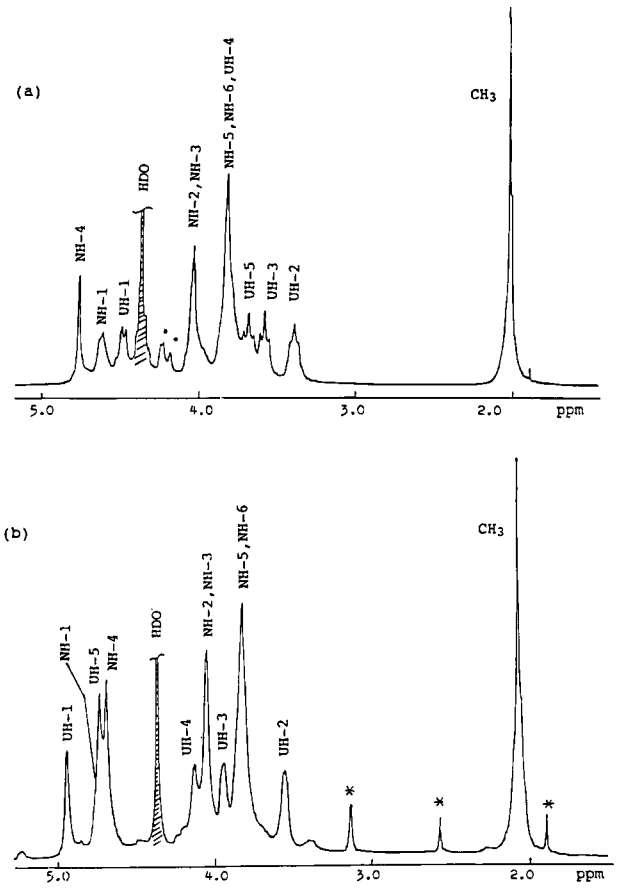



FIGURE 2: 300 MHz $^1H$ NMR spectra of CS4 (13 g/L) and DS (24 g/L) in $D_2O$ at 70 °C. (a) CS4; (b) DS. The asterisk (*) indicates minor components or impurities.

## RESULTS

Protons are numbered conventionally with prefixes U for uronate and N for hexosamine. $^1H$ spectra of both polymers have been assigned (Perkin et al., 1970; Gatti et al., 1979; Welti et al., 1979). At 70 °C, most protons were individually resolved (Figure 2); some showed fine structure due to trans diaxial spin−spin coupling of ~10 Hz. In CS4, NH-2 and NH-3 overlapped at ~4.0 ppm; NH-5, NH-6, and UH-4 overlapped at ~3.8 ppm. In DS, NH-1, NH-4, and UH-5 overlapped at ~4.7 ppm; NH-2 and NH-3 overlapped at ~4.1 ppm; NH-5 and NH-6 overlapped at ~3.8 ppm. Minor peaks from other structures were present. Significant line broadening was seen at lower temperatures (data not shown). Below 30 °C the spectra were poorly resolved. The spectra at 70 °C were very similar in $D_2O$, 0.22 or 0.36 M NaCl.

Table 1 gives nonselective inversion longitudinal relaxation times ($T_1$) of the resolved peaks. Recoveries were essentially exponential. $T_1$ values clustered around ~1 s, despite the wide range of structures, except for the peak incorporating the NH-6 protons. This was the only $T_1$ showing significant temperature dependence.

Table 2 lists selective inversion longitudinal relaxation times ($T_{1s}$) for solutions with or without NaCl at 70 °C. Recoveries were often nonexponential, showing a rapid initial

Table 1: $^1$H Nonselective Inversion Longitudinal Relaxation Times ($T_1$) in Seconds for Sodium Chondroitin-4-sulfate (CS4) and Sodium Dermochondan (Dermatan) Sulfate (DS) at 300 MHz

| | CS4 | | | DS | | |
|---|---|---|---|---|---|---|
| | NaCl-free | | 0.22 M NaCl | NaCl-free | | 0.36 M NaCl |
| peak | 32 °C | 70 °C | 70 °C | 32 °C | 70 °C | 70 °C |
| NH-2, NH-3 | 1.01 | 0.99 | 1.22 | 1.06 | 1.03 | 1.03 |
| NH-1 | 1.02 | 0.90 | 0.94 | a | a | a |
| NH-4 | 1.00 | 0.87 | 1.14 | 1.24[b] | 0.96[b] | 0.96[b] |
| UH-5 | 1.06 | 1.03 | 1.15 | | 1.05[b] | 1.06[b] |
| NH-5, NH-6, UH-4 | 0.90 | 0.75 | 0.84 | 0.90[c] | 0.73 | 0.68 |
| | | | | | 1.00 | 1.02 |
| UH-1 | 1.04 | 0.97 | 1.05 | 1.11 | 1.16 | 1.19 |
| UH-2 | 1.14 | 1.25 | 1.47 | 1.19 | 1.26 | 1.20 |
| UH-3 | 1.09 | 1.12 | 1.41 | 1.00[d] | 1.13 | 1.18 |

[a] Not resolved. [b] NH-1 underlies these signals. [c] Not sufficiently resolved at <50 °C. [d] At 44 °C; not resolved well enough at 32 °C.

Table 2: $^1$H Selective Inversion Longitudinal Relaxation Times ($T_{1s}$) in Seconds for Sodium Chondroitin-4-sulfate (CS4) and Sodium Dermochondan Sulfate (DS) at 300 MHz and 70 °C

| CS4 peak | DS NaCl-free | 0.22 M NaCl | NaCl-free | 0.36 M NaCl |
|---|---|---|---|---|
| NH-2, NH-3 | 0.32 | 0.40 | 0.36[a] | 0.36[a] |
| NH-1 | 0.12 | 0.18 | | |
| NH-4 | 0.34 | 0.38 | 0.30[b] | 0.34[b] |
| UH-5 | | | 0.26[b] | 0.31[b] |
| NH-5, NH-6, UH-4 | 0.38 | 0.38 | 0.43 | 0.41 |
| UH-1 | 0.17 | 0.27 | 0.35 | 0.37 |
| UH-2 | 0.39 | 0.57 | 0.32 | 0.38 |
| UH-3 | 0.31 | 0.43 | 0.37 | 0.41 |

[a] Because their peaks were very close, these values were measured with NH-2, NH-3, and UH-4 inverted simultaneously. [b] Values assigned to NH-4 and UH-5 must be interpreted cautiously since they were inverted simultaneously with the underlying NH-1 resonance.

decay followed by a slower return to equilibrium. $T_{1s}$ values were characteristic of the initial rapid phase, due to the method of calculation (see Experimental Procedures). $T_{1s}$ were usually considerably lower than $T_1$ and spanned a wider range. $T_{1s}$ decreased strongly with decreasing temperature. For UH-2 at 45 °C, $T_{1s}$ was 0.25 s in CS4 and 0.19 s in DS compared with 0.57 and 0.38 s, respectively, at 70 °C. DS relaxation times in NaCl solutions approximated those in salt-free solutions, whereas those of CS4 increased by ~20% in NaCl solution.

In selective inversion experiments, noninverted peaks showed negative transient NOEs resulting from initial rapid spin-diffusion processes involving the inverted peak, followed by slow return to thermal equilibrium. Examples are shown for CS4 and DS in Figures 3 and 4, respectively. In these figures, the NOE is defined as the fractional deviation from equilibrium.

$$\mathrm{NOEF} = \frac{S(\tau) - S^\circ}{S^\circ}$$

where $S(\tau)$ is the signal intensity at time $\tau$ after the selective inversion pulse, and $S^\circ$ is the thermal equilibrium intensity [i.e., $S(\infty)$]. When resonance peaks were broad and/or close together, perturbation by the decoupler pulse occurred. Peaks

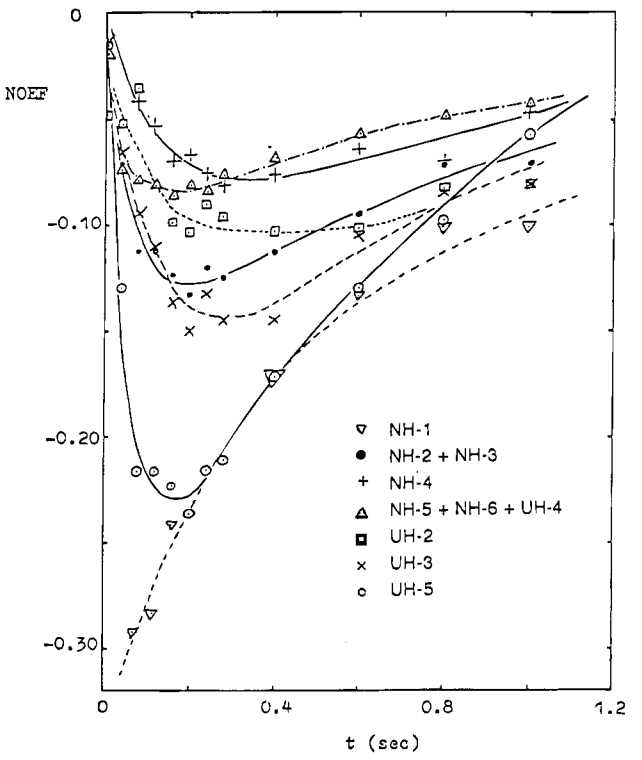



FIGURE 3: Transient nuclear Overhauser effects for protons in $D_2O$ solution of sodium chondroitin-4-sulfate (13 g/L) following selective inversion of UH-1 at 70 °C. See text for definition of NOEF.

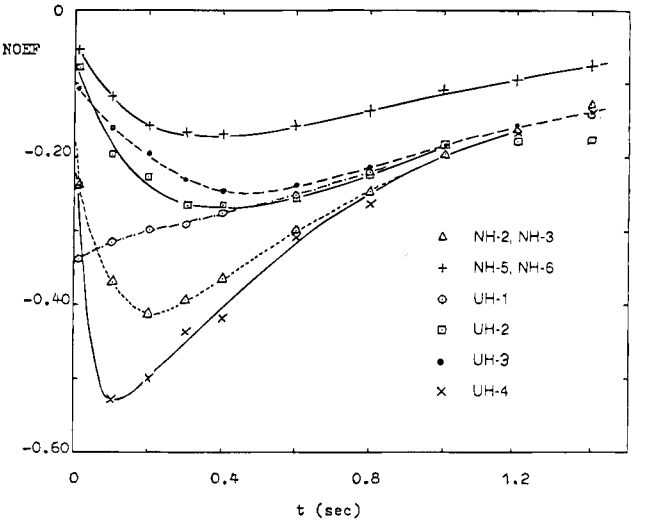



FIGURE 4: Transient nuclear Overhauser effects for protons in $D_2O$ solution of sodium dermochondan (dermatan) sulfate (24 g/L) following selective inversion of NH-1 + NH-4 + UH-5 at 70 °C. See text for definition of NOEF.

affected (e.g., NH-1 in Figure 3 and UH-1 in Figure 4) returned to equilibrium monotonically.

The depth of the transient NOE curve and the time at which the minimum occurs depend on the strength of the dipole−dipole interaction between the observed and inverted protons, i.e., on the interproton distance. The depths of the minima of uninverted peaks following selective inversion of various peaks in CS4 and DS are summarised in Tables 3 and 4, respectively. The structural significance of the NOE data is exemplified below on CS4, succeeded by summaries of other NOEs of particular relevance.

Following inversion of UH-1 in CS4, UH-3 and UH-5 showed large NOEs consistent with short distances expected

Table 3: Depths of Transient NOE Minima Following Selective Inversion of Protons in CS4 at 300 MHz and 70 °C

| | observed nucleus | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| inverted nucleus | NH-4 | NH-1 | UH-1 | NH-2 NH-3 | NH-5 NH-6 UH-4 | UH-5 | UH-3 | UH-2 |
| NH-4 | | −[a] | − | 0.17 | 0.09 | 0.05 | 0.03 | 0.08 |
| NH-1 | − | | − | 0.07 | 0.05 | − | 0.02 | 0.03 |
| UH-1 | 0.08 | − | | 0.13 | 0.06 | 0.22 | 0.15 | 0.08 |
| NH-2, NH-3 | 0.21 | 0.32 | 0.15 | | − | − | 0.05 | 0.07 |
| NH-5, NH-6, UH-4 | 0.32 | 0.44 | 0.20 | − | | − | 0.05 | 0.16 |
| UH-3 | 0.05 | 0.1 | 0.22 | 0.08 | − | − | | − |
| UH-2 | 0.07 | 0.09 | 0.12 | 0.1 | − | − | − | |

[a] Perturbed by selective inversion pulse.

Table 4: Depths of Transient NOE Minima Following Selective Inversion of Protons in DS at 300 MHz and 70 °C

| | observed nucleus | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| inverted nucleus | UH-1 | UH-5 NH-1 | NH-4 | UH-4 | NH-2 NH-3 | UH-3 | NH-5 NH-6 | UH-2 |
| UH-1 | | −[a] | − | 0.10 | 0.08[b] | 0.12 | 0.03 | 0.13 |
| UH-5, NH-1, NH-4 | − | | | 0.35 | 0.2 | 0.15 | 0.12 | 0.22 |
| UH-3 | 0.2 | 0.12 | 0.15 | 0.15 | − | | − | 0.2 |
| NH-5, NH-6 | 0.12 | 0.12 | 0.12 | 0.08 | 0.08 | − | | − |
| UH-2 | 0.14 | 0.11 | 0.06 | − | − | − | 0.13 | − |

[a] Perturbed by selective inversion pulse. [b] Minimum, "true" value >0.1.

for 1,3-diaxial interactions in $^4C_1$ GlcUA. UH-1→UH-5 and UH-1→UH-3 distances are 233 and 244 pm, respectively [quoted by Rees (1969)]. The larger UH-5 NOE is consistent with the smaller internuclear separation. UH-2 showed a smaller NOE than UH-3 (Figure 3) consistent with a greater UH-1→UH-2 distance of ~296 pm (Rees, 1969). The response of UH-4 to inversion was obscured by overlap with NH-5 and NH-6, but the UH-1→UH-4 distance is yet greater, ~384 pm (Rees, 1969), and no direct effect is expected. The combined (NH-5 + NH-6 + UH-4) peak showed a small NOE, developed in a time comparable to the UH-5 NOE, probably due to rapid cross-relaxation between UH-1, NH-3, NH-5, and NH-6 via the strong UH-1→NH-3 inter-ring interaction, NH-3→NH-5 1,3-diaxial interaction and NH-5→NH-6 vicinal interactions. The (NH-2 + NH-3) NOE (Figure 3) was only slightly less than that of UH-3 and developed more rapidly, almost certainly due to the UH-1/NH-3 interresidue interaction. If NH-2 was unaffected, the net UH-1→NH-3 NOE (~0.24) was as large as the UH-1→UH-5 NOE, indicating a comparably small internuclear distance.

The GalNAc rings of both CS4 and DS showed strong NOEs between NH-1, NH-3, and NH-5 but only weak interactions between NH-1, NH-2, and NH-4, consistent with a $^4C_1$ conformation.

In DS IdoUA, all vicinal interactions were significant, supporting the presence of $^1C_4$ conformation (Gatti et al., 1979). Interaction between UH-2 and UH-5 strongly suggests the presence of a 1,4-diaxial disposition as in the $^2S_0$ form.

The strong UH-1/UH-3 NOE could arise from either $^4C_1$ or $^2S_0$ conformations, but, in the absence of other evidence for significant amounts of $^4C_1$, it supports a $^2S_0$ form. Thus IdoUA in DS is in both $^1C_4$ and $^2S_0$ conformations [cf. Casu et al. (1988)].

## DISCUSSION

Comparison of CS4 with DS should help to answer fundamental questions arising from the epimerisation of GlcUA to IdoUA, viz., how does it alter the physical chemical properties of the CS chain? What are the new properties of the modified chain that give it special ultrastructural and biological potential?

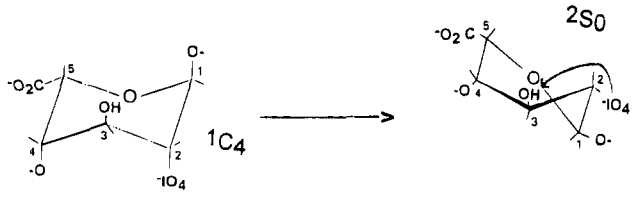
Several well established findings concerning the conformation of IdoUA apparently conflict. Periodate oxidation kinetics indicated a $^4C_1$ conformation, while ruling out $^1C_4$, which ought not be oxidized because all hydroxyl groups are trans-diaxial in this form (Scott & Tigwell, 1978). NMR data indicated, however, that IdoUA in a DS isolated from the urine of a Maroteaux-Lamy patient was $^1C_4$ [see Casu (1988) for review]. It was suggested that the two conformers could be in equilibrium and that, as periodate oxidized the $^4C_1$ form, it was replenished from the reservoir of $^1C_4$ (Rees et al., 1985). An alternative proposal is outlined below in *Secondary Structures*. Later, a third form was identified, the skew boat $^2S_0$ (Casu et al., 1988) (Scheme 1). Continuous interconversion of different shapes should result in a more flexible backbone, which is accessible to NMR investigation.

This discussion considers chain dynamics and chain conformations, followed by their implications to secondary and tertiary structures of DS in aqueous solution.

*Chain Dynamics.* The motions of possible significance in magnetic relaxation are (a) overall chain tumbling, (b) chain segmental motion, (c) $CH_2OD$ internal rotation, and (d) acetamido group internal rotation.

When the motions controlling magnetic relaxation are considerably slower than the observation frequency $\omega_o$, cross-relaxation is much more efficient than spin−lattice relaxation (Sykes et al., 1978), leading to a common value of $T_1$ for all protons in nonselective inversion experiments. The uniform

Scheme 1. The Two Forms ($^1C_4$ and $^2S_o$) of L-Iduronate found in DS[a]




[a] During oxidation, periodate probably reacts initially with *one* of the glycol hydroxyl groups, forming a monodentate ester. It is proposed that the bulky (axial) periodate ester destabilizes the $^1C_4$ ring toward the stable $^2S_o$ form in which it would be equatorial. It is relevant that the L-iduronate 2-*O*-sulfate ester, which strongly resembles this monodentate periodate ester, occurs in the $^2S_o$ form in heparin fragments (Casu et al., 1988). $^2S_o$ glycol hydroxyl groups are much closer to each other than in the $^1C_4$ ring and the monodentate periodate ester can then cyclize to the bidentate ester, which splits to give two aldehyde groups.

$T_1$ values for each sample in Table 1 thus indicate that correlation times $\tau_c$ for the operative processes meet the condition $\omega_o\tau_c \gg 1$.

In selective inversion experiments relaxation of the inverted peak proceeded in two stages, an initial rapid stage by cross-relaxation to adjacent unperturbed nuclei and a final slow stage with time constant $T_1$ when a common spin temperature was attained. The relaxation times $T_{1s}$ in Table 2 characterize the initial stage, and the fact that $T_{1s} < T_1$ also implies prevalent rapid cross-relaxation. $T_1$ of the NH-5 + NH-6 peak decreases with increasing temperature, confirming that $\omega_o\tau_c > 1$. However, since this $T_1$ is less than $T_1$ for the remaining peaks, the cross-relaxation rates are not rapid enough to produce complete $T_1$ averaging. $T_1$ passes through a minimum when the condition $\omega_o\tau_c \sim 1$ is fulfilled. At 300 MHz this corresponds to $\tau_c \sim 5 \times 10^{-10}$ s. From $T_{1s}$ and $T_1$, $\tau_c$ is $\sim 5 \times 10^{-9}$ s within a factor of 2−3, in CS4 and DS at 70 °C.

The correlation time for the overall motion calculated either for random-coil polymers (Matsuo et al., 1977) or for helical chains (Budd et al., 1981) was $>10^{-7}$ s for both CS4 and DS. This, considerably longer than the above estimate, would be associated with line widths so large as to obscure most chemical shifts. We therefore conclude that the major backbone motion is segmental.

The effect of internal rotation of the $CH_2OD$ group would be to reduce the correlation time for the NH-6 protons, thus decreasing $T_1$. This is consistent with the observation that the peak with lowest $T_1$ incorporates the NH-6 protons. In the presence of rapid cross-relaxation, $T_1$ is an average of individual relaxation rates, dominated by the fastest relaxation "sink" i.e., the NH-6 protons.

The near-identity of $T_1$ data for CS4 and DS implies comparable rates for their effective motions at 70 °C. $T_{1s}$ for a ring proton interacting only with other ring protons is determined by the rate of segmental motion and by the separation of the interacting nuclei. NH-2 and NH-4 are in similar environments in CS4 and DS, and the very similar $T_{1s}$ values imply that both chains undergo comparable segmental motions.

This result is relevant to the stiffening effect of the array of interresidue hydrogen bonds (-COO$^-$←HNCO←HO-) in CS4 (Scott et al., 1983) which may differ in DS, depending on which IdoUA form is involved (Scott & Tigwell, 1978). However, the conformational flexibility of glycosidic links in the absence of hydrogen bonds must also be taken into account. Rees and Smith (1975) calculated that 95% of accessible states of the α-1,4 (a−e) links in maltose lay within ±20° of the conformation ($\phi$=30°,$\psi$=0°), using the convention of Brant (1976) whereas the β-1,4 (e−e) links in cellobiose were less restricted; 77% of accessible states lying near the conformation (−30°, 30°) and 23% near the conformation (−30°, 180°). Whittington and Glover (1971, 1972), using a less precise approach on many 1,3 and 1,4 links, found that a−e or e−a have fewer allowed conformations than e−e. In CS4 the glycosidic links (β-1,3 and β-1,4) are both e−e, but DS links about IdoUA $^1C_4$ are a−e (α-1,3) and e−a (β-1,4). The $^2S_o$ conformer links are e−e and e−a. The intrinsic conformational freedom of many DS glycosidic links may therefore be less than those in CS4. Stronger interresidue hydrogen bonds and water bridges in CS4 would increase stiffness, perhaps resulting in a flexibility roughly equal to that of DS.

DS thus retains the intrinsic stiffness of CS4, at least during time scales accessible to these techniques ($\sim 10^{-8}$ s). The results were obtained at 70 °C, which might have reduced the secondary structures of both AGAGs to a common level. It would be expected that GlcUA would be more stable, conformationally, at this temperature than IdoUA. To this limited extent, our results do not support a simple hypothesis of rapid and continuing interconversion between IdoUA conformations in DS.

Similar chemical shifts, linewidths, and $T_1$ values in 0.0, 0.22, or 0.36 M NaCl indicate that screening of anionic groups caused no significant conformational changes at 70 °C.

*Chain Conformation.* Different $T_{1s}$ values from polymers with similar dynamics reflect differences in structure. The shortest $T_{1s}$ values (Table 2) were shown by NH-1 and UH-1 in CS4 and UH-4 in DS, as expected since these glycosidic protons experience strong inter- and intraresidue NOEs. In the $^4C_1$ CS4 GlcUA, UH-1 interacts with three close neighbors; UH-3 and UH-5 in 1,3-diaxial orientations and NH-3 *trans*-glycoside. In the $^1C_4$ form of DS IdoUA, UH-1 interacts with only two close neighbors, UH-2 (*gauche* vicinal) and NH-3 (*trans*-glycoside).

Strong interactions like that between UH-1 and NH-3 in CS4 (Results) were observed for DS *trans*-glycosidic protons, indicating similar average conformations.

Conformational energies calculated using various potential functions for β-D-1,3 links in AGAGs (Rees, 1969), homopyranoglycans (Rees & Scott, 1971), and a disaccharide model for the 1,3 link in CS4 (Gatti et al., 1979) agreed that most accessible states lie in a shallow potential located within the range $\phi = 0 \pm 45°$, $\psi = 0 \pm 30°$, giving UH-1→NH-3 distances of 200−270 pm. Thus the calculated β-1,3-glycoside conformations approximate those observed in $D_2O$ solution, with the *trans*-glycosidic protons eclipsed and very close (Figure 5).

*Secondary Structures.* Knowledge of (a) monosaccharide conformations combined with (b) that of the geometry of the glycosidic links is the basis for a secondary structure of the polymer backbone. Results on (a) and (b) together with chemical shift and spin-coupling data in DMSO solution (Scott et al., 1983) allow us to assign a secondary structure to CS4 in water and to consider that of DS. Molecular modeling of DS is thereby facilitated.

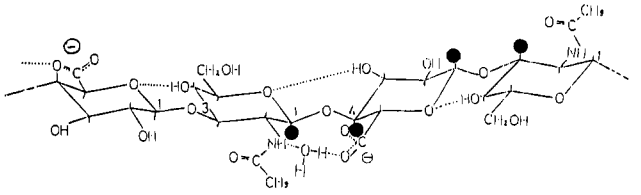


FIGURE 5: Tetrasaccharide fragment of typical $\beta$-1,4; $\beta$-1,3 linked anionic glycosaminoglycan (hyaluronan) showing potential hydrogen bonds (dotted lines) and water bridge which help to stabilise the 2-fold helix (Scott et al., 1984). The filled circles represent transglycosidic protons which show strong NOEs in the CS4 and DS analogues, thus establishing the geometry around the glycosidic links in these polymers and consequently that acetamido and carboxylate groups are close enough to hydrogen-bond to each other. Keratan sulfate also belongs to this group of compounds, which form 2-fold helices in water.

*CS4.* NOEs from GalNAc residues in both CS4 and DS confirmed the $^4C_1$ conformation previously established by chemical shifts and spin-coupling data (Results). This conformation may not be as stable as, e.g., that of GalNAc in CS6, or GlcNAc in hyaluronan, because of the presence of the bulky sulfate ester on the axial C4 hydroxyl group.

GlcUA NOEs in CS4 confirmed a $^4C_1$ conformation, which is unlikely to be affected by molecular and perimolecular environments. The stretches of CS4 in DS remaining after incomplete epimerization of GlcUA to IdoUA should contain the same $^4C_1$ GlcUA conformations.

The strong NOEs characteristic of pairs of protons on each side of the two glycosidic bonds (U→N and N→U) (Results) support the existence *in water* of the 2-fold helix previously proposed (Scott et al., 1983), on the basis of NMR studies of CS6 in DMSO. These NOEs imply that the sugar rings are positioned with the acetamido and carboxylate groups *cis* to the polymer chain, as required by the proposed (Scott et al., 1983, 1992) secondary structure (Figure 5). NOEs were obtained at 70 °C, but periodate oxidation kinetics indicated that the CS6 secondary structure persisted from <10 °C up to ~90 °C (Scott & Tigwell, 1978).

*DS.* NOEs demonstrated that DS IdoUA was present in both $^1C_4$ and $^2S_0$, but not $^4C_1$, forms, confirming earlier work (Casu et al., 1988). What determines the form a given residue of IdoUA adopts is then a key question.

$^2S_0$ was proposed as an intermediate between $^1C_4$ and $^4C_1$ forms. If so, the speeds of interconversion must be influenced by the molecular environment, since the rates of oxidation of GlcUA and IdoUA differ between heparans and DS (Scott & Tigwell, 1978). The abundance of each form must be influenced by the environment, with interresidue interactions stabilizing one or more of the alternatives, by analogy with secondary structures in hyaluronan and CS (Figure 5; Scott et al., 1984). If this were not so, every IdoUA residue would have the same probability of being in any of the three forms, and their relative abundancies in different DS/heparan preparations would be the same. In fact, there are large differences in the relative proportions of the three conformers in different AGAGs (Casu et al., 1988).

This argument applies to intermolecular as well as interresidue interactions, e.g., the substrate for DS or heparan interactions could determine which IdoUA form was present in the complex (Casu et al., 1988). This concept of "plasticity" has also been invoked in protein interactions (Woodward, 1993).

Similarly, interaction with periodate might *cause* a change in conformation. The conflict between periodate kinetics (which apparently exclude $^1C_4$) and NMR findings (indicating the predominance of $^1C_4$ and $^2S_0$) is only partly resolved by assuming that the $^2S_o$ C2-C3 glycol (with equatorial hydroxyl groups) would be oxidized rapidly, since essentially all the IdoUA in DS is rapidly oxidized, including the $^1C_4$ form that should be periodate-resistant.

Whatever the detailed mechanism of periodate oxidation, the first step must be monodentate complex formation with one of the glycol hydroxyl groups, followed by cyclization to the other hydroxyl (Scheme 1). In IdoUA $^1C_4$ all hydroxyls are axial. The very bulky (axial) monodentate periodate complex formed with any hydroxyl must decrease the stability of the $^1C_4$ form, encouraging a move toward an equatorial complex. When this reaches a point where it is sterically possible for the periodate ester to link to a vicinal hydroxyl, a cyclic complex is formed and oxidation proceeds. This proposal does not invoke continuous oscillation between the alternative forms of IdoUA and is thus compatible with the chain dynamics data.

For DS, as for CS4, the strong UH-1→NH-3 NOE implies that the $COO^-$ and acetamido groups are *trans* across the U→N glycosidic link, and the strong NH-1→UH-3 NOE accords with a *cis* arrangement of $COO^-$ and acetamido across the N→U bond, with IdoUA in the $^1C_4$ form (cf. Figure 5). If the IdoUA were $^2S_o$, there would be a similar NOE, but weaker.

The DS backbone thus strongly resembles that of CS4, which contains a 2-fold helix. Compatible with this, chemical shifts in DMSO (Scott & Heatley, 1982) indicated a hydrogen bond between acetamido NH and $COO^-$ similar to that in CS, but weaker, suggesting that the two groups are not as close as in CS.

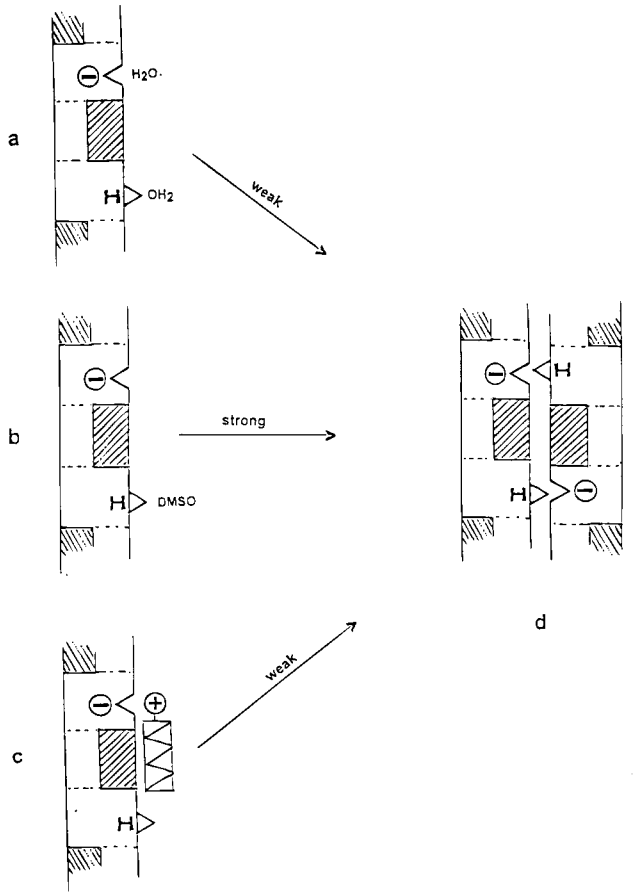
We do not imply that CS4 or DS are always and completely in 2-fold helices (at 70 °C) but that it is a strongly preferred configuration in both cases which must have a significant impact on tertiary structure formation (below).

*Tertiary Structures. In vitro* and *in vivo* evidence suggests that DS proteoglycans form duplexes and higher order aggregates. Aggregation of DS in tissues is probably antiparallel, and these structures appear to be fundamentally important in the organization of extracellular matrix collagen fibrils. In cornea, this determines its transparency (Scott, 1992).

Solution data implied that primary structure determines DS self-aggregation (Fransson et al., 1979). Rotary shadowing electron microscopy and computer dynamics strongly suggested that CS4 does not self-aggregate, probably because the sulfate groups are placed along the center line of the polymer backbone, giving a high negative charge density on the long axis of the molecule. CS6, in which the sulfate groups are at the periphery of the molecule, can self-aggregate (Scott et al., 1992). In CS4 and CS6 the carboxylate groups are peripheral equatorial.

Secondary structure is similarly important in determining whether tertiary structures are formed. It is assumed that the secondary structure is reasonably stable; if it were not the loss of configurational entropy in forming a duplex of definite structure would weaken the drive toward self-aggregation. The third alternative, that the chain configuration changes on interaction with its partner in the complex, is difficult to analyse *ab initio*.

Scheme 2. Mechanisms Accounting for Different Extents of Self-Aggregation of DS in $H_2O$ or DMSO[a]




[a] (a–c) Unaggregated forms of DS in $H_2O$ (a) or DMSO (b and c). Form d is the duplexed DS in which hydrophobic patches (cross hatched) come together with additional binding due to hydrogen bonds (H→O) (Scott, 1992). Our Results indicate that DS forms 2-fold helices, with hydrophobic patches on alternate sides of the tape-like molecule (seen sideways on in this diagram) as in the cases of the analogous ambidexterans hyaluronan, CS, and keratan sulfate (Scott, 1992). In form a (aqueous environment) $H_2O$ blocks the hydrogen-donating *and* -accepting groups, so that although hydrophobic interactions are probable, relatively weak aggregation does not significantly increase solution viscosity. In form b (DMSO solution, e.g., as $Li^+$ salt) only hydrogen-donor groups are blocked by DMSO molecules, so that the tendency to aggregate is marked and the solution viscosity was high (Scott & Heatley, 1982). In form c (DMSO solution, as dodecyltrimethylammonium salt) hydrophobic patches and hydrogen-acceptor sites are both blocked by the bulky amphipathic cation, so that aggregation is hindered and the viscosity of the solution was low (Scott & Heatley, 1982).

Aggregation in similar AGAGs, including hyaluronan, the chondroitins, and keratans, is probably driven by hydrophobic bonding and hydrogen bonding, opposed by electrostatic repulsion (Scott, 1992). In the 2-fold helix implied by our results there is a large hydrophobic patch in CS4, centered on the GalNAc residue, containing ~9 CH units stretched over three sugar rings. There is a similar patch (~9 CHs) in DS containing the $^2S_0$ and/or $^1C_4$ arrangement, assuming an approximately 2-fold helix in each case, and NH→$COO^-$ hydrogen bonds where possible. Thus, DS is of the class of interstitial tissue AGAGs possessing analogous hydrophobic patches. Epimerization of GlcUA to IdoUA does not delete the hydrophobic bonding potential.

On this basis, published experimental evidence (Scott & Heatley, 1982) can be reassessed, pointing to a role for hydrophobic bonding in DS tertiary structures (Scheme 2): (1) Marked self-aggregation of the $Li^+$DS salt (but not the $Li^+$ CS4 salt), evidenced by very high viscosity, took place in DMSO solutions. DMSO is a highly polar solvent in which nonpolar molecules are poorly soluble and in which "lyophobic" bonding is expected. Viscosity in $H_2O$ was much lower than in DMSO, indicating that polymer–polymer interactions were stronger in DMSO than in $H_2O$. DMSO is a proton acceptor, while $H_2O$ is both donor and acceptor. DMSO thus blocked *some* donor hydrogen-bonding capacity, while $H_2O$ partly blocked *both* acceptor and donor potential. Intermolecular interactions were therefore weaker in $H_2O$. (2) Dodecyltrimethylammonium salts of DS, on the contrary, did not give viscous solutions in DMSO. (3) Addition of decyltrimethylammonium salts to a $Li^+$ DS solution in DMSO did not reduce the high viscosity.

These findings (Scott & Heatley, 1982) can be incorporated into a scheme (Scheme 2) in which DS self-aggregates by a combination of hydrophobic and hydrogen bonding (case 1, above). An amphipathic cation such as dodecyltrimethyl ammonium is sufficiently strongly held to DS in DMSO, by electrostatic and lyophobic bonding (to the hydrophobic patches), to compete out the lyophobic-bonding contribution to the complex stability, and there is then insufficient intermolecular affinity left to maintain the aggregate (case 2). However, in the presence of other cations (e.g., $Li^+$) which compete with and partly displace the amphipathic cation, enough aggregate is formed to maintain high viscosity (case 3).

Since DS self-aggregates and CS4 does not, the drive to aggregation is stronger in DS than in CS4. The greater stability of DS complexes is proposed to be due to increased intermolecular hydrogen bonding, because intramolecular bonds which are strong in CS4 are weak or impossible in DS, freeing the relevant groups for intermolecular bonding. In the $^1C_4$ form, all the IdoUA -OH groups point away from potential ligands on the same polymer molecule, as compared with the opposite situation in GlcUA of CS4. It was pointed out (Scott, 1980) that HA and CS secondary valencies were self-satisfied intramolecularly, ensuring that AGAGs in the connective tissue spaces were unlikely to sequester and hinder the passage of molecules required in cell metabolism. In DS [and in KS, cf. Scott (1994)] some of the secondary valencies are more available for self-aggregation.

## REFERENCES

Brant, D. A. (1976) *Q. Rev. Biophys. 9*, 527–596.
Budd, P. M., Heatley, F., Holton, T. J., & Price, C. (1981) *J. Chem. Soc. Faraday Trans. 1, 77*, 759–771.
Casu, B., Petitou, M., Provasoli, M., & Sinay, P. (1988) *Trends Biochem. Sci. 13*, 221–225.
Fransson, L.-A., Nieduszynski, I. A., Phelps, C. F., & Sheehan, J. K. (1979) *Biochim. Biophys. Acta. 586*, 179–188.
Gatti, G., Casu, B., Torri, G., & Vercelloti, J. R. (1979) *Carbohydr. Res. 68*, C3–C7.
Mathews, M. B. (1959) *Biochim. Biophys. Acta 35*, 9–17.
Matsuo, K., Kuhlmann, K. F., Yang, H. W. H., Geny, F., Stockmayer, W. H., & Jones, A. A. (1977) *J. Polym. Sci. Polym. Phys. 15*, 1347–1361.
Perkin, A. S., Casu, B., Sanderson G. R., & Johnson, L. F. (1970) *Can. J. Chem. 48*, 2260–2268.
Rees, D. A. (1969) *J. Chem. Soc., Perkin Trans II*, 217–226.
Rees, D. A., & Scott, W. E. (1971) *J. Chem. Soc., Perkin Trans. II*, 469–479.
Rees, D. A., & Smith, P. J. C. (1975) *J. Chem. Soc., Perkin Trans. II*, 836–840.

Rees, D. A., Morris, E. R., Stoddart, J. F., & Stevens, E. S. (1985) *Nature 317*, 480.
Scott, J. E. (1960) *Methods Biochem. Anal. 8,* 145−197.
Scott, J. E. (1980) *Connect. Tissue (Japan) 11*, 111−120.
Scott, J. E. (1992) *FASEB J. 6*, 2639−2645.
Scott, J. E. (1993a) *Dermatan Sulphate Proteoglycans. Chemistry, Biology, Chemical Pathology,* Portland Press, London.
Scott, J. E. (1993b) *Glycoconjugate J. 10,* 319−421.
Scott, J. E. (1994) *Biochem. J. 298*, 221−222.
Scott, J. E., & Tigwell, M. J. (1978) *Biochem. J. 173*, 103−114.
Scott, J. E., & Heatley, F. (1982) *Biochem. J. 207*, 139−144.
Scott, J. E., Heatley, F., Jones, M. R. N., Wilkinson, A., & Olavesen, A. H. (1983) *Eur. J. Biochem. 130,* 491−495.
Scott, J. E., Heatley, F., & Hull W. E. (1984) *Biochem. J. 220*, 197−205.
Scott, J. E., Chen, Y., & Brass, A. (1992) *Eur. J. Biochem. 209*, 675−680.
Sykes, B. D., Hull, W. E., & Snyder, G. H. (1978) *Biophys. J. 21*, 137−146.
Welti, D., Rees, D. A., & Welsh, E. J. (1979) *Eur. J. Biochem. 94*, 505−515.
Whittington, S. G. (1971) *Macromolecules 4,* 569−571.
Whittington S. G., & Glover, R. M. (1972) *Macromolecules 5,* 55−58.
Woodward, C. (1993) *Trends Biochem. Sci. 18,* 359−360.

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