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Conformational Equilibrium of Unsulphated Iduronate in Heparan Sulphate Tetrasaccharides

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Proton-proton coupling constants for terminal α-L-iduronate residues in tetrasaccharides obtained from heparan sulphates by complete nitrous acid deaminative cleavage were shown to vary with experimental conditions. It is proposed that the iduronate residue is in a conformational equilibrium between the $^1C_4$ chair and either the ^2S_O skew-boat or possibly the $^2H_3$ half-chair conformers. It was not possible to discriminate between the two non-chair forms empirically. The position of the equilibrium is sensitive to temperature, pH and sulphation of neighbouring residues. The likelihood of iduronate residues within glycosaminoglycans existing in the $^4C_1$ conformer in addition to the $^1C_4$ and ^2S_O forms is discussed.

The α-L-iduronate residue occurs in the glycosaminoglycans dermatan sulphate, heparan sulphate and heparin [1], and in fungal polysaccharides, such as protuberic acid [2]. In the glycosaminoglycans, iduronate is known to be formed at the polymer level by C-5 epimerisation of β-D-glucuronate [3]. The β-D-glucuronate residues adopt the $^4C_1$ chair conformation and are thus able to dispose all of their bulky substituent groups in equatorial positions. However, α-L-iduronate residues in either the $^4C_1$ or $^1C_4$ chair conformations would have one or several bulky substituent groups in the unfavourable axial disposition.

Empirical studies of iduronate conformation in the glycosaminoglycans have centred on three techniques: NMR spectroscopy, X-ray fibre diffraction and periodate oxidation. In NMR spectroscopy ^1H-^1H coupling constants can yield H-C-C-H dihedral angles around the ring and hence specify conformation. In X-ray diffraction, preliminary analysis gives an indication of likely residue length, i.e. 0(1) - 0(4) distance and further refinement of the data may uniquely specify the structure. Studies of the kinetics of periodate oxidation may indicate the dihedral angle 0(2)-C(2) - C(3)-0(3) in iduronate.

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For heparin, in which the iduronate residues are sulphated at the 2-position, initial analysis of the X-ray fibre diffraction data [4] eliminated the $^4C_1$ conformation but subsequent full structure factor analysis [5] was unable to distinguish between a $^1C_4$ and a skew-boat iduronate conformer. Proton NMR spectroscopic studies of heparin [6] yielded ^1H-^1H coupling constants around the iduronate ring which were broadly consistent with the $^1C_4$ conformation. Thus, both X-ray and NMR data are compatible with the $^1C_4$ conformer for sulphate iduronate.

For dermatan sulphate, both the initial X-ray studies [7, 8] and more recent full structure factor analysis [9] concluded that the unsulphated iduronate residue occurred in the $^4C_1$ conformation. Similarly, periodate studies [10, 11] demonstrated that the iduronate residues in dermatan sulphate were rapidly oxidised, a result that is inconsistent with the diaxially disposed glycol expected in the $^1C_4$ conformation but entirely consistent with the diequatorial glycol grouping in the $^4C_1$ conformer. However, NMR studies [12] showed that the ^1H-^1H coupling constants around the iduronate residue were similar to those identified as representing the $^1C_4$ conformation in heparin. Thus, the issue of iduronate conformation in dermatan sulphate remains unresolved.

In this investigation, evidence is presented for a conformational equilibirum of the non-reducing terminal iduronate residues within tetrasaccharides isolated from heparan sulphate chains.

Materials and Methods

Materials

All materials used were obtained as described previously [13, 14].

Preparation and Fractionation of Heparan Sulphate Tetrasaccharides

Heparan sulphates were prepared from bovine lung heparin by-products, and oligosaccharide derivatives obtained from a complete nitrous acid depolymerization were fractionated according to size by gel filtration as described previously [13].

Heparan sulphate tetrasaccharides were fractionated on a preparative scale by ion-exchange chromatography [14] into non-, mono- and di-sulphated species, designated fractions I, II and III respectively (see Fig. 2 of ref. 14). Full sequence characterization of the components of each fraction has been described [14]. This information is summarized in Table 1.

Sample Preparation for NMR

Samples for the variable temperature studies at pH 7 were prepared as described previously [14]. For solutions of pH values other than 7, samples of fraction I (approx. 7 mg) and 3-trimethylsilyl-[$^2H_4$] propionic acid sodium salt (TSP-d_4) as internal reference (approx. 0.1 mg), were dissolved in 0.5 ml of 100 mM sodium oxalate buffers. These solutions had measured pH values of 2.47, 3.25 and 3.98, and were exchanged several times with ^2H_2O (100.0 atom % ^2H).

Table 1. Saccharide sequences within the tetrasaccharide fractions.

Tetrasaccharide fraction	Saccharide sequence	
I	U — GlcNAc	— GlcU — aManOH
II	U — GlcNAc(6SO_3)	— GlcU — aManOH
III	U — GlcNAc(6SO_3)	— GlcU — aManOH(6SO_3)

U represents either β-D-glucuronate (GlcU) or α-L-iduronate (IdoU) in the approximate ratios (GlcU: IdoU) of 60:40, 36:64 and 33:67 for fractions I, II and III respectively (see ref. 14). GlcNAc represents 2-acetamido-2-deoxy-α-D-glucose, aManOH represents 2,5-anhydro-D-mannitol, and (6SO_3) represents an ester sulphate group at C-6 of the residue.

^{1}H-NMR Spectroscopy

Spectra were determined on a Bruker WH400 instrument with a 5 mm probe operating at 400.14 MHz for ^{1}H, at temperatures in the range 20-90°C. Connections between coupled nuclei were determined by a combination of decoupling difference spectroscopy and the two-dimensional COSY-45 experiment [15]. Accurate values for spin-spin coupling constants and chemical shifts were determined from spectra subjected to Gaussian resolution enhancement using the instrumental listing procedure. This approach routinely interpolated data, giving rise to line positions an order of magnitude greater in accuracy than would be implied from the 0.3 Hz separation between data points. Zero-filling from 32K to 256K data points prior to Fourier transformation produced no significant changes in observed coupling constants.

Two-dimensional J-resolved spectroscopy [16] was used to separate overlapped multiplets which were not mutually coupled. A two-dimensional C/H correlation experiment was also performed at 25 MHz on a JEOL FX100 spectrometer using the proton-decoupling modification of [17].

Results

The proton NMR spectrum for fraction I at 80°C and pH 3.98, under which conditions I-1, I-2 and I-5 are well resolved, is shown in Fig. 1. The signal at δ 4.844 in the proton NMR spectrum for fraction I at 60°C and pH 7 was assigned to the iduronic acid (IdoU) anomeric proton (I-1) by means of a two-dimensional C/H correlation experiment which showed this proton resonance to be correlated with the C-1 signal from IdoU assigned in ref. [14]. This IdoU anomeric proton resonance was a doublet with J(I-1, I-2) = 5.9 Hz. The site to which this proton was spin-coupled was not obvious because of the complexity of the main region of the spectrum. A signal of corresponding intensity due to the I-5 proton was observed at δ 4.510, together with overlapped resonances from the corresponding terminal GlcU and the internal GlcU anomeric protons (G-1 and G-1′). This I-5 proton signal correlated with a carbon resonance at ~ δ 72.6.

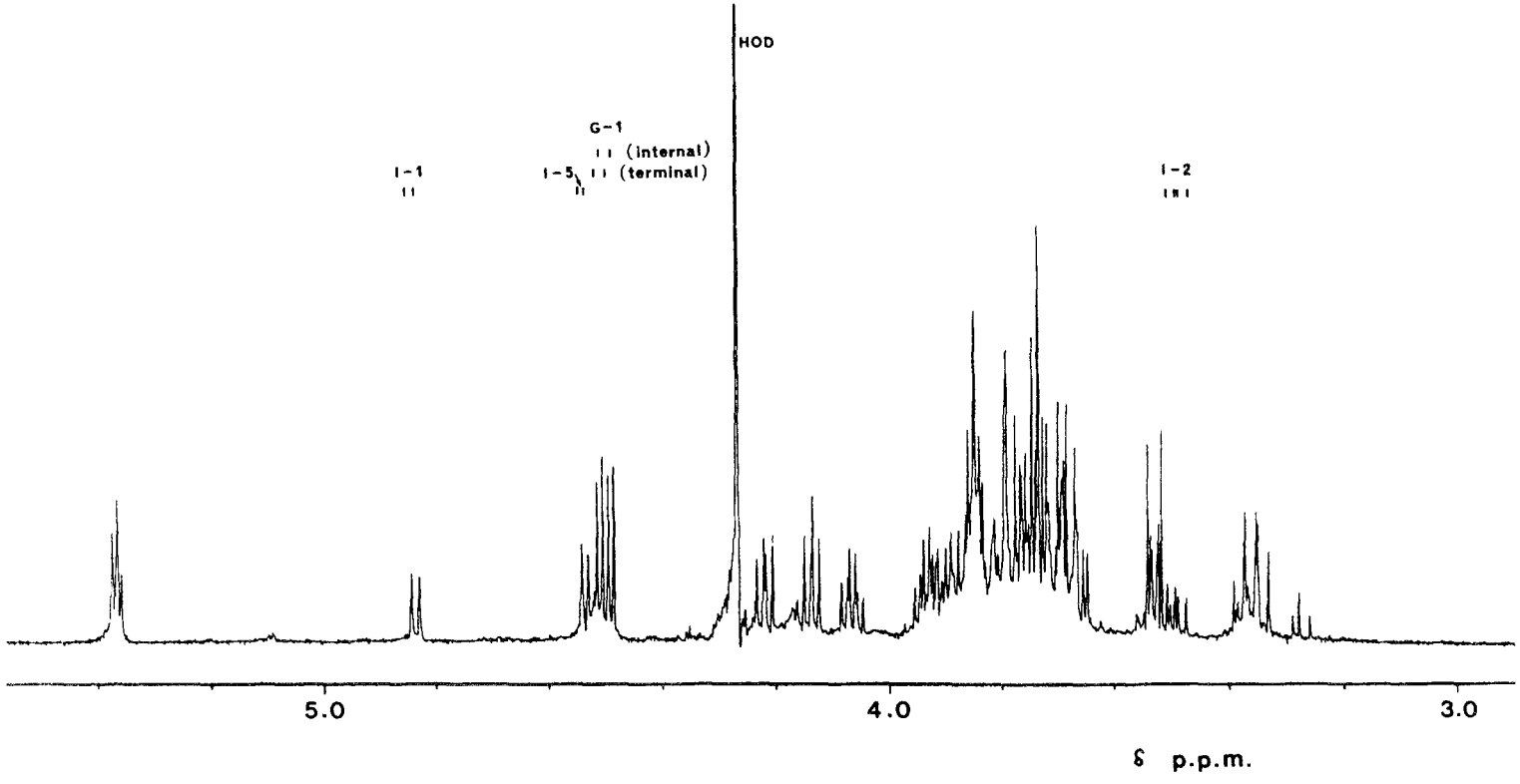

Figure 1. 400 MHz ^{1}H-NMR spectrum of heparan sulphate tetrasaccharide fraction I at 80°C and pH 3.98. Signals representing I-1, I-2, I-5 and G-1 protons are indicated.

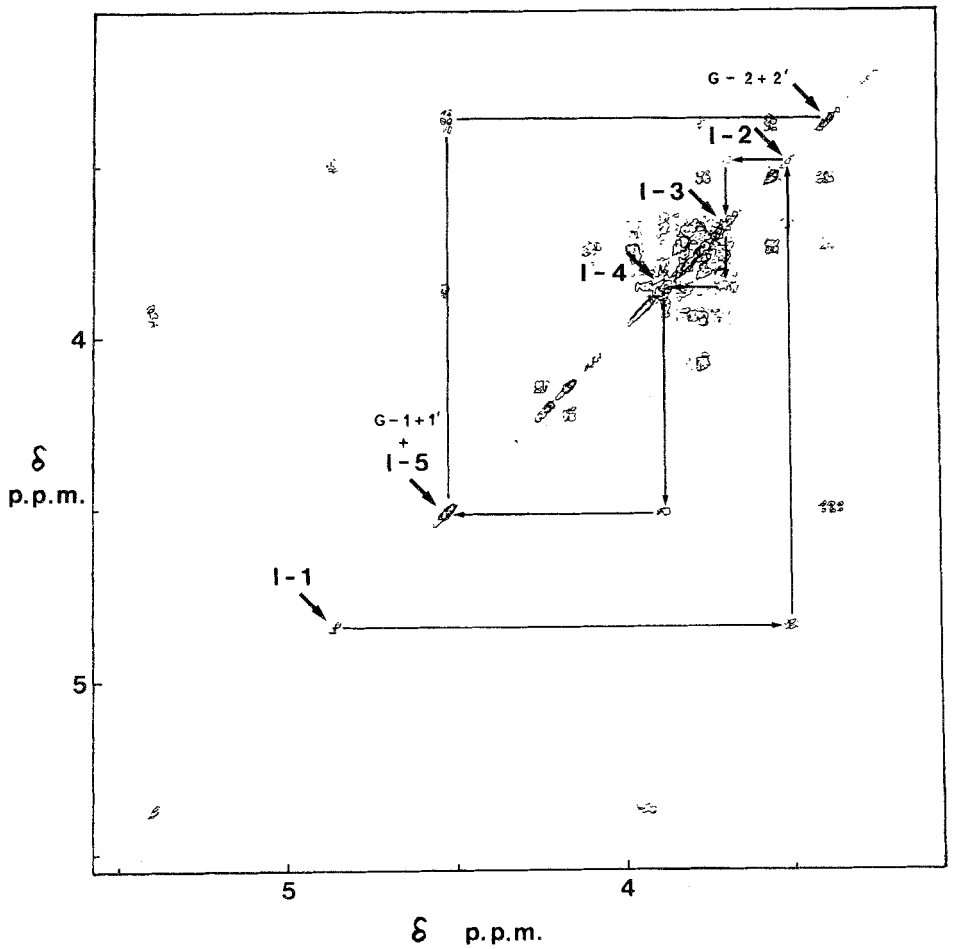

Figure 2. 400 MHz ^{1}H-NMR 2-D COSY-45 correlation plot for heparan sulphate tetrasaccharide fraction I at 60°C and pH 7. Connections between spin-coupled iduronate protons and glucuronate H-1 and H-2 are shown.

Using double-resonance difference spectroscopy it was shown that I-1 was spin-coupled to I-2 at δ 3.492 and the latter showed a further coupling to I-3 of 8.05 Hz. Irradiation at I-2 showed, *via* difference spectroscopy, an I-3 resonance at δ 3.675. A similar experiment involving I-5 disclosed I-4 at δ 3.87 with J(I-4, I-5) = 4.7 and J(I-3, I-4) = 7.3 Hz. It must be noted that coupling constants obtained from decoupling-difference measurements are approximate because resonances are artificially broadened before subtraction and "downwards" lines are superimposed on "upwards" lines. The assignments of IdoU chemical shifts at the positions indicated were confirmed by means of a two dimensional (2-D) COSY-45 experiment (Fig. 2). This procedure was necessary because the decoupling-difference experiments also produced many artifacts arising from Bloch-Siegert shifts. Comparison with the COSY experiment enabled identification of the true

Table 2. Proton-proton coupling constants for the terminal uronate residues in heparan sulphate tetrasaccharide fractions at 60°C.

Assignment	Coupling constants (in Hz)		
	Fraction I	Fraction II	Fraction III
J(I-1, I-2)	5.9	4.75	4.92
J(I-2, I-3)	8.05	7.0	7.14
J(I-3, I-4)	7.3[a]	—	6.20
J(I-4, I-5)	4.7[a]	3.95	3.87
J(G-1, G-2)	7.9	8.0	7.8
J(G-2, G-3)	9.05	—	—

[a] Signifies very approximate values obtained from decoupling-difference experiments in which signals are artificially broadened before subtraction. The IdoU couplings for fraction III have been determined from line listings obtained for individual f_1 slices through a 2-D J-resolved ^{1}H spectrum.

difference responses. In principle, the spin-spin couplings could be determined from the COSY experiment by the use of a sufficiently large data array. IdoU assignments for fractions II and III were made by comparison with those for fraction I and confirmed by decoupling-difference experiments.

The ^{1}H spin-spin coupling constants for the IdoU residues in all three heparan sulphate tetrasaccharide fractions at 60°C were markedly different from literature values for these residues in dermatan sulphate [12] and heparin [6]. In addition the values for fraction I differed from those for fractions II and III (see Table 2).

Further examination of spectra as a function of temperature revealed a continuous variation for both coupling constant and chemical shift parameters of the IdoU residue (Fig. 3). Similar parameters for both terminal or internal GlcU residues showed negligible changes with temperature. Proton NMR spectra for fraction I over the temperature range 20-90°C were repeated for samples buffered to pH values of 2.47, 3.25 and 3.98 and the chemical shift and spin-spin coupling parameters for the IdoU residue were determined in a similar manner. These data are summarised in Fig. 4.

Discussion

In all three tetrasaccharide fractions, and at different pH values for fraction I, there is a continuous variation of both chemical shifts and spin-spin coupling constants as a function of temperature for the protons in IdoU alone. The only plausible origin for this variation must be a change in the position of a conformational equilibrium. Because these parameters vary in a linear manner it is necessary and apparently sufficient to consider only two participating ring conformers. But which are they?

The two conformers usually considered in pyranose equilibrium studies are the $^4C_1$ and $^1C_4$ chairs. Published ^{1}H-^{1}H coupling constants for heparin [6], dermatan sulphate [12]

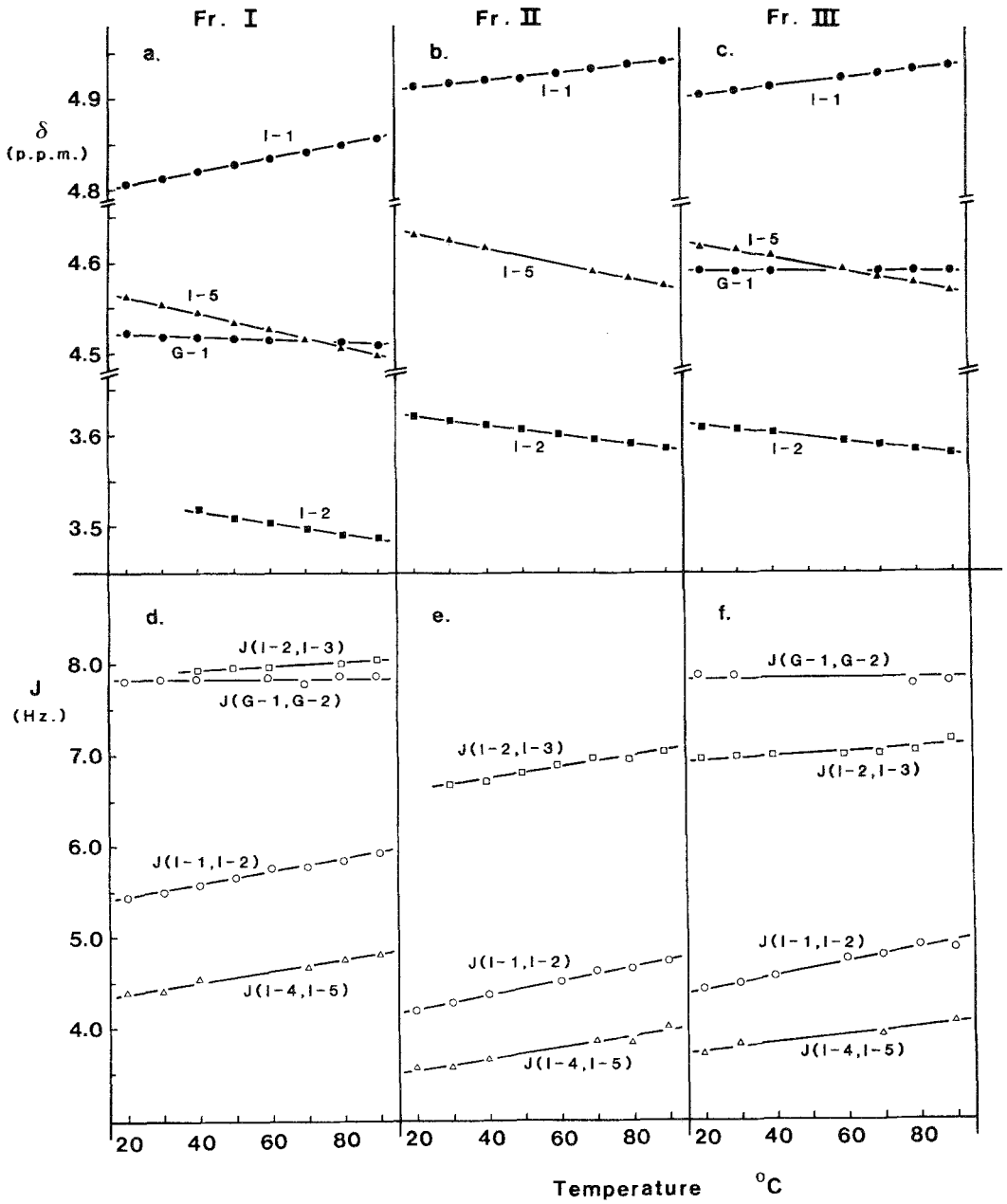

Figure 3. Variation with temperature of ^{1}H-NMR parameters at 400 MHz for the IdoU residue in heparan sulphate tetrasaccharide fractions. Variation of proton chemical shifts for (a) fraction I, (b) fraction II, (c) fraction III; and of proton-proton coupling constants for (d) fraction I, (e) fraction II, (f) fraction III are shown, together with values of H-1 chemical shifts and (H-1, H-2) coupling constants for the terminal GlcU residue in fractions I and III for comparison.

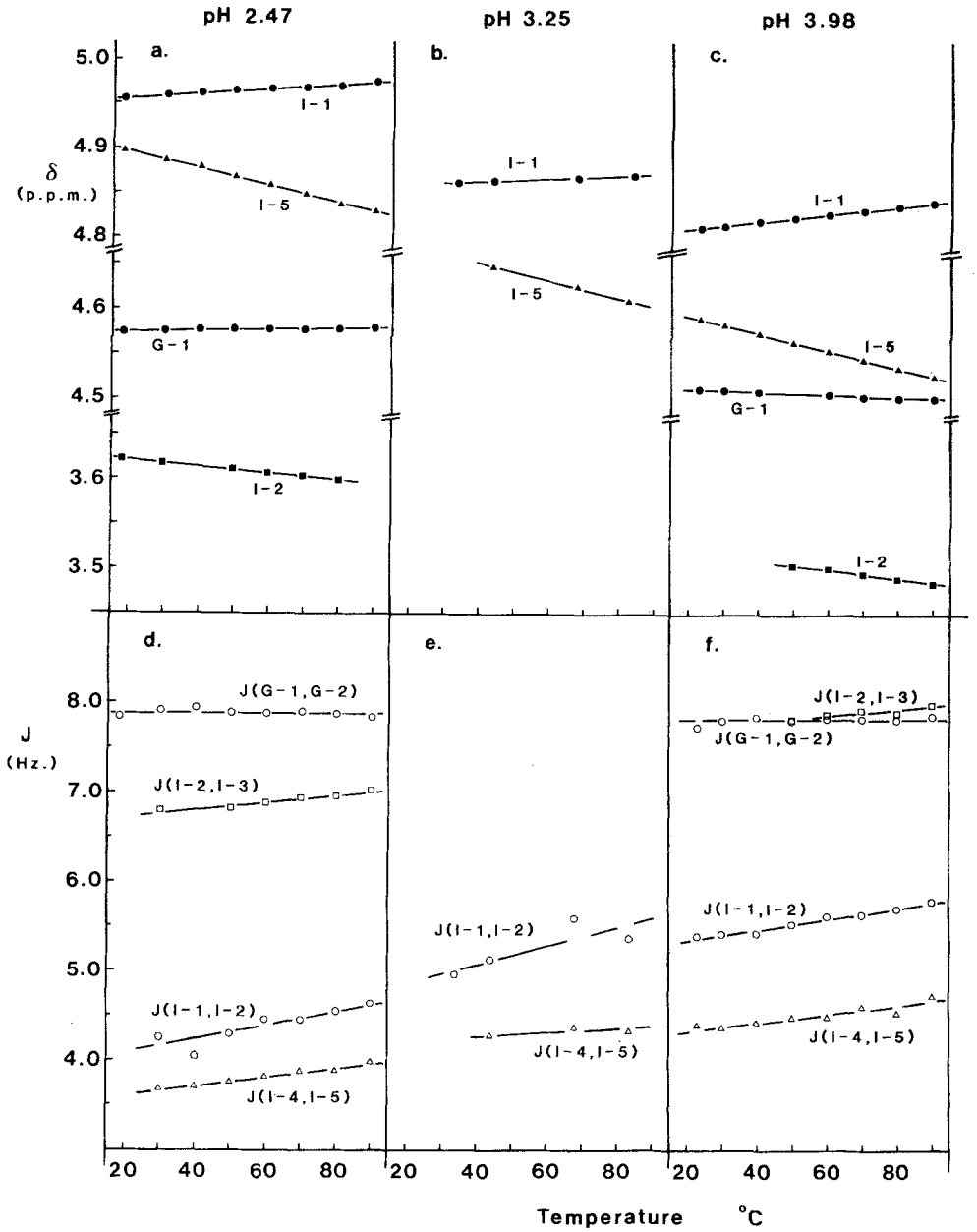

Figure 4. Variation with temperature of ^{1}H-NMR parameters at 400 MHz for the IdoU residue in tetrasaccharide fraction I at various pH values. Variation of proton chemical shifts at (a) pH 2.47, (b) pH 3.25, (c) pH 3.98; and of proton-proton coupling constants at (d) pH 2.47, (e) pH 3.25, (f) pH 3.98 are shown, together with values of H-1 chemical shifts and (H-1, H-2) coupling constants for the terminal GlcU residue at pH 2.47 and pH 3.98 for comparison.

Table 3. Proton-proton coupling constants for α-L-iduronate.

Location of iduronate residue	Temperature (°C)	Coupling constants (Hz)				
		J(I-1, I-2)	J(I-2, I-3)	J(I-3, I-4)	J(I-4, I-5)	Source
Bovine Lung Heparin IdoU($2SO_3$) in polymer	35 90	2.64 3.29	5.90 6.10	3.44 3.60	3.09 3.14	[6] [6]
Disulphated Disaccharide from Heparin IdoU($2SO_3$)-aManOH($6SO_3$)	60	2.62	4.42	4.19	2.67	[18]
Heparin Oligosaccharide Terminal IdoU-GlcNAc($6SO_3$)-	60	3.8-4.0	—	—	3.5-3.7	[25]
Dermatan Sulphate IdoU in polymer	70	3.0	6.0	3.5	3.3	[12]
Protuberic Acid IdoU in polymer	70	4.0	—	—	3.0	[2]
Heparan Sulphate Tetrasaccharide Terminal Low Range (pH 7, 30°C, Fraction II)	30	4.28	6.67	7.3[a]	3.56	This Work
High Range (pH 7, 90°C, Fraction I)	90	5.93	8.05		4.80	This Work
Predicted Values: Chair Conformers $^1C_4$ $^4C_1$	— —	2.4 7.85	3.6 9.3	3.6 9.3	2.4[b] 2.4[b]	[19] [19]
Non-Chair Conformers ^2S_O $^2H_3$	— —	~7 ~8	~9 ~9	~8 ~9	~7 ~7	see footnote[c] see footnote[c]

[a] Approximate value obtained from decoupling-difference experiments on fraction I at 60°C and pH 7.
[b] Predictive data for a carboxyl substituent are not available, but examination of experimental values for J(I-4, I-5) in IdoU($2SO_3$) suggests that values similar to the J(I-1, I-2) couplings are reasonable.
[c] Values for the non-chair conformers are predicted by examination of bond angles in molecular models and reference to the Karplus relationship, as rules for accurate prediction of these values are not available.

and for the disaccharide, iduronyl(2-sulphate)-anhydromannitol(6-sulphate) [18] amongst others, are given in Table 3. These values have been interpreted in terms of the IdoU residue adopting the $^1C_4$ conformation or a slightly distorted form thereof. In this study, for determinations at the lower temperatures and for fractions containing sulphate residues, the coupling constants observed are relatively close to the values expected for a $^1C_4$ conformer, and it is therefore proposed that this form contributes to the equilibrium. At higher temperatures, and notably for the unsulphated tetrasaccharide, the coupling constants show a considerable deviation from these values. In particular, the *J*(I-4, I-5) coupling increases to 4.8 Hz and this suggests that in the other contributing conformer there cannot be a 60° dihedral angle between this pair of protons. Therefore, the $^4C_1$ chair is not the other contributor in this equilibrium. Examination of all of the coupling constants around the ring using the predictive parameters of Altona and Haasnoot [19] together with application of the Karplus relationship [20] shows that of the 26 defined hexopyranose conformers [21] only the ^2S_O and $^2H_3$ forms would be predicted to have coupling constants higher than the values observed for fraction I at elevated

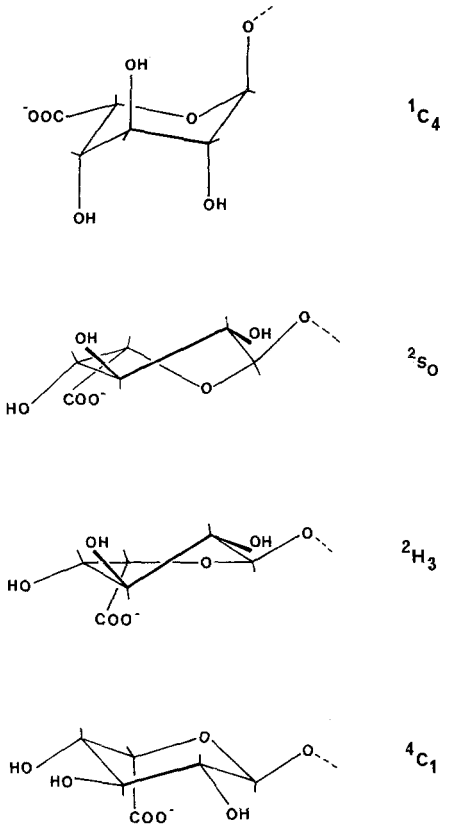

Figure 5. α-L-iduronate conformers.

temperature (see Table 3). Therefore, it is concluded that the equilibrium exists between the $^1C_4$ and one of either the ^2S_O or the $^2H_3$ IdoU conformers. The structures of these potential IdoU conformers are shown in Fig. 5.

It is difficult to specify the second contributing conformer because only four of the six ring torsional angles can be determined *via* the ^1H-^1H coupling constants as the conformational angles around the ring oxygen are not accessible. Therefore, the ^2S_O and $^2H_3$ cannot be distinguished on empirical grounds alone. Interestingly, on the basis of NMR studies and force field calculations, the ^2S_O conformer has recently been proposed as a possible contributor to a conformational equilibrium of the sulphated iduronate residue in a synthetic pentasaccharide corresponding to the binding sequence of heparin to antithrombin III [22]. The possibility of the existence of non-chair idose conformations has also recently been considered [23]. Furthermore, examination of both NMR spectroscopic and crystallographic literature on pyranoses reveals many skew-boats but half-chairs occur only where four contiguous atoms are constrained to be coplanar (e.g. C=C bonds). Studies of chair-to-chair transition states also reveal skew-boat forms

to be energetically more stable than half-chair conformers [24]. Hence the $^{2}S_{O}$ is more likely to be the second conformer in the equilibrium.

Under the experimental conditions studied, the position of the equilibrium is never very close to either of the two limiting conformers and it is influenced not only by extrinsic factors such as temperature and pH but also by the presence of sulphate groups on the adjacent *N*-acetylglucosamine. It must also be noted that the conformational versatility observed may somehow reflect the unconstrained terminal location of the IdoU residue, however, terminal glucuronate residues retain the $^{4}C_{1}$ conformation throughout the range of experimental conditions employed.

Do the $^{1}C_{4}$, $^{2}S_{O}$ and $^{4}C_{1}$ conformers of IdoU all exist within the glycosaminoglycans? The interpretation of X-ray and periodate oxidation data previously presented for the existence of the $^{4}C_{1}$ conformer can be questioned. Initial X-ray studies [7, 8] only considered the $^{1}C_{4}$ and $^{4}C_{1}$ chairs and, therefore, assigned the IdoU conformation within dermatan sulphate as $^{4}C_{1}$ on the basis of the axial rise per disaccharide which excluded the $^{1}C_{4}$ but did not specify the $^{4}C_{1}$. However the O(1) - O(4) distance in the $^{4}C_{1}$ is almost identical to that in the $^{2}S_{O}$ skew-boat (or the $^{2}H_{3}$ half-chair). Iduronate conformers other than the $^{4}C_{1}$ were not considered in the more detailed structure factor analysis of dermatan sulphates by Mitra *et al.* [9] and it would be interesting to know whether the incorporation of a $^{2}S_{O}$ conformer would improve the fit with the X-ray intensity data.

Controversy also arises between NMR spectroscopic and periodate oxidation studies of dermatan sulphate in solution. Proton-proton coupling constants have been interpreted [12] in terms of the $^{1}C_{4}$ IdoU conformer, however, oxidation by periodate [10, 11] would suggest a non-diaxial disposition of hydroxyl groups. Perhaps IdoU residues within dermatan sulphate chains in solution also exist in a state of equilibrium such that the position of the equilibrium is "sensed" by the coupling constants whereas the contribution from a $^{2}S_{O}$ form is indicated by periodate oxidation.

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