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Conformational equilibria of α-L-iduronate residues in disaccharides derived from heparin

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The disaccharides IdoA($2SO_3$)-anManOH($6SO_3$) and IdoA-anManOH (where IdoA represents α-L-iduronate, anManOH represents 2,5-anhydro-D-mannitol and SO_3 represents sulphate ester) were prepared from bovine lung heparin using HNO_2 depolymerization, borohydride reduction and desulphation, and were examined by 400 MHz ^{1}H-n.m.r spectroscopy. Three-bond proton–proton coupling constants around the IdoA ring were determined under a range of experimental conditions. For unsulphated IdoA all four proton–proton coupling constants varied markedly as a function of temperature, pH and solvent, providing clear evidence for a rapid conformational equilibrium. These data were analysed in terms of the three most energetically stable IdoA conformers: $^1C_4$, $^4C_1$, and $^2S_0$. Predicted coupling constants for these conformers were determined using a modified Karplus-type relationship. For unsulphated IdoA in dimethyl sulphoxide the equilibrium was provoked strongly in favour of a slightly distorted $^4C_1$ 'chair' IdoA conformer for which coupling constants have not previously been reported. For sulphated IdoA in aqueous conditions and at low pH the equilibrium is strongly in favour of the alternative $^1C_4$ chair conformer. Under many conditions, however, significant contributions from all three conformers occur for the non-reducing terminal IdoA in these disaccharides.

INTRODUCTION

The α-L-iduronate (IdoA) residue occurs in dermatan sulphates (Lindahl & Höök, 1978), heparan sulphates (Gallagher *et al.*, 1986; Höök *et al.*, 1984) and in the blood anticoagulant heparin (Comper, 1981), where it is predominantly sulphated at C-2. The favoured IdoA residue conformations (Ragazzi *et al.*, 1986) are shown in Fig. 1.

X-ray diffraction (Nieduszynski *et al.*, 1977) and ^{1}H n.m.r. (Gatti *et al.*, 1978) studies assigned a $^1C_4$, or a slightly distorted $^1C_4$, conformation to the sulphated IdoA residues in heparin. However, a 'conformational peculiarity' of the sulphated IdoA in a synthetic pentasaccharide corresponding to the binding sequence of heparin to antithrombin has recently been reported (Torri *et al.*, 1985).

In dermatan sulphates the unsulphated IdoA residues were suggested to adopt the $^1C_4$ conformation by ^{1}H n.m.r. studies (Gatti *et al.*, 1979), but X-ray diffraction (Atkins & Isaac, 1973; Mitra *et al.*, 1983) and periodate oxidation (Scott & Tigwell, 1978) investigations favoured the $^4C_1$ form. This controversy is not explained by the IdoA residues adopting a different conformation in solution to that in the solid state (Winter *et al.*, 1986; Cziner *et al.*, 1986). However, the demonstration of a conformational equilibrium for IdoA residues in tetrasaccharides derived from heparan sulphates (Sanderson *et al.*, 1985) and in various heparin and dermatan sulphate sequences (Casu *et al.*, 1986), provides an explanation for these apparently contradictory results.

A third IdoA conformer, the $^2S_0$ 'skew boat', has recently been calculated to have a stability comparable with that of the two 'chairs' (Ragazzi *et al.*, 1986). This conformer has been proposed as a contributor to conformational equilibria of iduronate in heparan sulphate-derived tetrasaccharides (Sanderson *et al.*, 1985), heparin and dermatan sulphate polysaccharides (Casu *et al.*, 1986) and in synthetic oligosaccharides (Torri *et al.*, 1985; Casu *et al.*, 1986).

Proton–proton coupling constants from n.m.r. spectra are directly dependent on dihedral angles and consequently provide the most specific technique for the determination of ring geometry. In the present study complete sets of three-bond proton–proton coupling constants have been obtained under different temperature, pH, countercation and solvent conditions for both sulphated and unsulphated IdoA residues in heparin-derived disaccharides in order to study the factors which determine the position of IdoA conformational equilibria.

EXPERIMENTAL

Materials

Heparin from bovine lung was supplied by Dr. W. E. Lewis (formerly of Glaxo Operations, Runcorn, Cheshire, U.K.) Sephadex G-15 was purchased from Sigma Chemical Co. (Poole, Dorset, U.K.), Dowex 50W-X8 cation-exchange resin from Bio-Rad Laboratories (Watford, Herts., U.K.) and DMSO from BDH (Poole, Dorset, U.K.). ^2H_2O (> 99.8 atom % ^{2}H) for routine n.m.r. use was from Nuclear Magnetic Resonance Ltd. (High Wycombe, Bucks., U.K.). ^2H_2O (100.0 atom % ^{2}H) for proton n.m.r. studies

Abbreviations used: IdoA, α-L-iduronate; anManOH, 2,5-anhydro-D-mannitol; SO_3, sulphate ester; DMSO, dimethyl sulphoxide; TSP, sodium 3-trimethylsilylpropionate; ϕ, dihedral angle; J, coupling constant.

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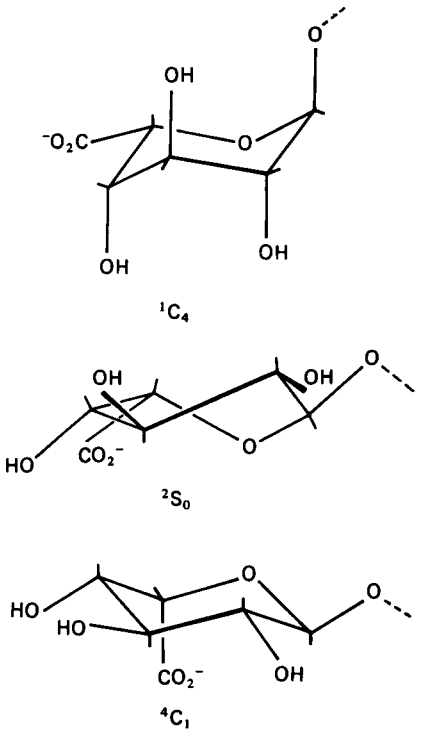

Fig. 1. The three most energetically favoured IdoA conformers

([$^2H_6$]DMSO, 99.9 atom % ^2H), and [$^2H_4$]methanol (100.0 atom % ^2H) were from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). Sodium 3-trimethylsilyl-[$^2H_4$]propionate ([$^2H_4$]TSP) was from Lancaster Synthesis (Morecambe, Lancs., U.K.).

Preparation of disaccharides

The preparation of the disulphated disaccharide after deamination of bovine lung heparin with HNO_2 has been described previously (Huckerby *et al.*, 1985). This disaccharide was desulphated using the method of Nagasawa *et al.* (1977) by heating its pyridinium salt in DMSO/water (9:1, v/v) at 105 °C for 3 h in sealed, evacuated, hydrolysis tubes. The products were concentrated and then desalted on a Sephadex G-15 column (size 84 cm × 1.4 cm; eluent, distilled water; flow rate, 13 ml/h). Complete desulphation of the disaccharide was achieved under these conditions and was confirmed by means of a 25 MHz ^{13}C-n.m.r. spectrum.

To prepare disaccharides with countercations other than sodium, samples were passed through a 1 ml Dowex 50W-X8 (H^+ form) column eluted with distilled water and titrated to pH ~ 6 with aqueous solutions of the appropriate cation hydroxide. For low-pH studies, samples were buffered with sodium oxalate buffers (Sanderson *et al.*, 1985).

N.m.r. spectroscopy

For proton n.m.r., samples of disaccharide (5–10 mg) together with [$^2H_4$]TSP (~ 0.5 mg) as internal standard were exchanged several times with ^2H_2O and finally dissolved in 100.0 atom % ^2H_2O (0.4–0.5 ml). For mixed solvent studies aliquots of one solvent were added directly to the n.m.r. tube containing a solution of the disaccharide in the other solvent.

Preliminary ^{13}C-n.m.r. spectra were obtained with a JEOL FX-100 spectrometer operating at 25.05 MHz. Proton-n.m.r. spectra were determined on Bruker WH 400 instruments operating at 400.13 MHz using 5 mm variable temperature probes, with high-field ^{13}C spectra and ^{13}C–^1H correlations being determined at 100.61 MHz, with [$^2H_4$]TSP as internal standard (Huckerby, 1983).

Proton resonances were assigned by a combination of decoupling difference spectroscopy and two-dimensional COSY (COrrelation SpectroscopY) (Bax & Freeman, 1981). Line positions were determined from spectra subjected to Gaussian resolution enhancement by using the instrumental listing procedure (Sanderson *et al.*, 1985), and accurate values for proton–proton spin–spin coupling constants were then derived by means of iterative spectral simulations using the computer program LAME (LAocoon with Magnetic Equivalence).

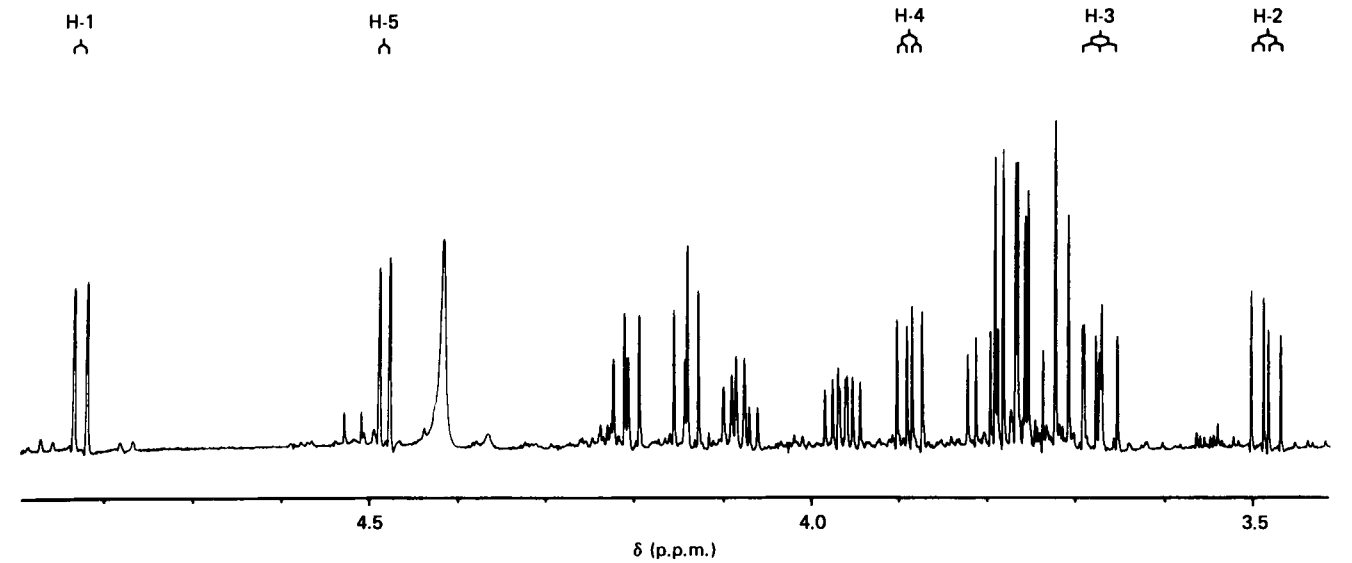

Fig. 2. 400 MHz ^1H-n.m.r. spectrum of the disaccharide IdoA-anManOH

The resonances due to the IdoA protons are indicated.

RESULTS

A complete analysis of the 400 MHz ^{1}H-n.m.r. spectrum of the disulphated disaccharide in ^2H_2O has been reported previously (Huckerby *et al.*, 1985).

The 400 MHz ^{1}H-n.m.r. spectrum of the desulphated disaccharide in ^2H_2O at 60 °C and pH 7 is shown in Fig. 2. All of the ^{1}H and ^{13}C chemical shifts for the desulphated disaccharide were assigned by means of decoupling difference and ^{13}C–^{1}H correlation experiments and are presented in Table 1. The assignment for H(4) in anManOH was confirmed by observation of a nuclear Overhauser enhancement when H(1) in IdoA was irradiated.

Complete sets of all four three-bond proton–proton coupling constants for the IdoA residues were obtained and these show considerable variation with experimental conditions (see Fig. 3 and Tables 2 and 3). These data are consistent with rapid conformational equilibria of the IdoA residues involving at least two conformers (Sanderson *et al.*, 1985).

Predicted coupling constants (Table 4) for the three most energetically stable IdoA conformers were calculated using the equation of Haasnoot *et al.* (1980). The

Table 1. ^{1}H and ^{13}C chemical-shift (δ) values for IdoA-anManOH in ^2H_2O at pH 7 and 60 °C

^{1}H and ^{13}C chemical shifts are given in p.p.m. downfield from internal [$^2H_4$]TSP. The assignments [a] and [b] may be reversed.

Sugar	Ring position	δ (p.p.m.) ^{1}H	^{13}C
IdoA	1	4.825	103.70
	2	3.485	74.15
	3	3.671	75.44
	4	3.886	73.80
	5	4.482	74.06
	6	–	~ 179
anManOH	1	3.775[a]/3.744[b]	63.98
	2	3.963	85.55
	3	4.207	78.62
	4	4.140	88.43
	5	4.079	84.43
	6	3.800[a]/3.699[b]	64.27

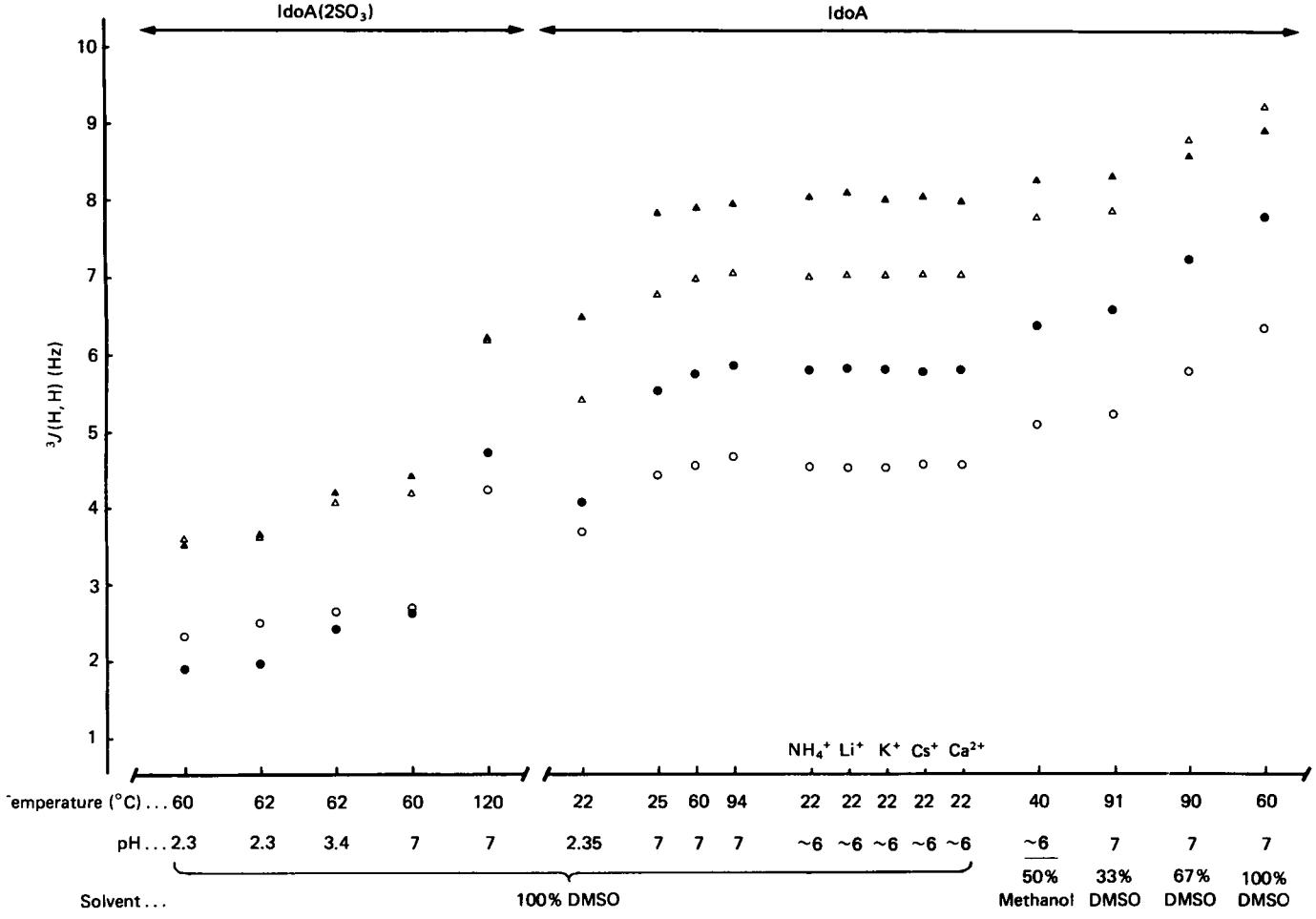

Fig. 3. Proton–proton coupling constants (J) for IdoA in heparin-derived disaccharides

The effects of experimental conditions on IdoA ^3J(H,H) coupling constants: ●, J(1,2); ▲, J(2,3); △, J(3,4); ○, J(4,5). The solvent was ^2H_2O unless otherwise indicated.

Table 2. Observed proton–proton coupling constants for IdoA in IdoA-anManOH

Solvent	pH	Cation	Temperature (°C)	Coupling constant (Hz)							
				J(1,2)	*J*(2,3)	*J*(3,4)	*J*(4,5)	*J*(1,5)	*J*(2,4)	*J*(2,5)	*J*(3,5)
^2H_2O	7	Na^+	25	5.53	7.83	6.78	4.42				
	7	Na^+	40	5.58	7.85	6.82	4.45				
	7	Na^+	54	5.67	7.88	6.90	4.51				
	7	Na^+	66	5.74	7.92	6.97	4.58	0.35*			
	7	Na^+	80	5.80	7.94	7.01	4.63	†			†
	7	Na^+	94	5.85	7.95	7.05	4.67	0.52*			†
	~ 6	NH_4^+	22	5.80	8.03	6.99	4.54				
	~ 6	NH_4^+	40	5.83	8.05	7.06	4.60				
	~ 6	Li^+	22	5.81	8.10	7.02	4.52				
	~ 6	Li^+	60	5.94	8.09	7.26	4.73	0.42			†
	~ 6	K^+	22	5.81	8.00	7.01	4.54				
	~ 6	K^+	60	5.89	8.12	7.10	4.70	†			†
	~ 6	Cs^+	22	5.79	8.05	7.03	4.57				
	~ 6	Cs^+	40	5.87	8.10	7.10	4.63	†			†
	~ 6	Ca^{2+}	22	5.80	7.98	7.03	4.56				
	~ 6	Ca^{2+}	40	5.82	8.02	7.09	4.60				
	~ 6	Ca^{2+}	60	5.87	8.04	7.10	4.66				
	2.35	H^+	22	4.06	6.49	5.40	3.68		0.48		
	2.35	H^+	40	4.16	6.54	5.41	3.61		0.52		
	2.35	H^+	60	4.27	6.50	5.75	3.70		0.49	0.51	
^2H_2O/[$^2H_6$]DMSO (v/v):											
91:9	7	Na^+	91	6.14	8.13	7.38	4.89	0.60			0.35
83:17	7	Na^+	91	6.25	8.21	7.49	4.97	0.59			0.35
74:26	7	Na^+	91	6.44	8.26	7.70	5.13	0.48			
67:33	7	Na^+	91	6.58	8.32	7.85	5.22				
33:67	7	Na^+	90	7.23	8.57	8.78	5.79				
17:83	7	Na^+	90	7.49	8.76	8.97	6.07				
9:91	7	Na^+	90	7.61	8.85	9.02	6.19				
[$^2H_6$]DMSO	7	Na^+	60	7.79	8.93	9.21	6.35				
	7	Na^+	90	7.69	8.85	9.08	6.27				
	7	Na^+	117	7.56	8.77	8.93	6.20				
^2H_2O/[$^2H_4$]methanol (1:1, v/v)	7	Na^+	22	6.28	8.22	7.70	5.01				
	7	Na^+	40	6.39	8.26	7.78	5.08	0.53			0.37

* Indicates approximate value not included in the iterations.
† Indicates poorly resolved small long-range coupling observed.

Table 3. Observed proton–proton coupling constants for sulphated IdoA in IdoA(2SO_3)-anManOH(6SO_3)

Solvent	pH	Temperature (°C)	Coupling constant (Hz)							
			J(1,2)	J(2,3)	J(3,4)	J(4,5)	J(1,3)	J(1,4)	J(1,5)	J(2,4)
^2H_2O	7	60	2.62	4.42	4.19	2.67	0.75	0.62	0.62	0.49
	3.4	62	2.40	4.19	4.06	2.64	0.91	0.49	0.58	0.80
	3.4	90	2.63	4.43	4.21	2.70	0.87	0.46	0.58	0.76
	2.3	62	1.95	3.67	3.65	2.49	1.01	0.59	0.47	0.84
	2.3	90	2.14	3.83	3.80	2.59	0.93	0.53	0.46	0.85
^2H_2O/[$^2H_6$]DMSO (v/v):										
33:67	7	91	3.53	4.98	4.99	3.37	0.77		0.45	0.62
26:74	7	91	3.66	5.18	5.31	3.52	0.65		0.4*	0.64
17:83	7	92	4.10	5.64	5.65	3.76	0.55			0.55
9:91	7	92	4.30	5.75	5.86	3.92	†			0.51
[$^2H_6$]DMSO	7	60	4.61	6.17	6.08	4.21				
	7	120	4.71	6.21	6.20	4.23				0.45
	2.3	60	1.88	3.52	3.59	2.32	0.95	†		0.93
	2.3	90	2.11	3.72	3.74	2.45	0.81	†		0.83

* Indicates approximate value not included in iterations.
† Indicates poorly resolved small long-range coupling observed.

dihedral angles used in these calculations (Table 4) were obtained from the crystallographic data of Nieduszynski *et al.* (1977) for the $^1C_4$, Mitra *et al.* (1983) for the $^4C_1$ and Chamberlain *et al.* (1981) for the $^2S_0$ ring systems with substituent groups fixed using the internal co-ordinates of Arnott & Scott (1972).

In the desulphated disaccharide, three participating IdoA conformers need to be invoked because the coupling constant data cannot be explained by an equilibrium involving *only* the two 'chair' forms. In particular, the J(2,3) coupling constant is predicted to have values approximately equal in magnitude to those of J(3,4) in each of the two 'chair' forms (Table 4). Experimentally, however, J(2,3) progressively exceeds J(3,4) as all four values decrease (i.e. as the $^4C_1$ conformer population diminishes) (see Fig. 3). For the $^2S_0$ conformer, J(2,3) is predicted to be much greater than J(3,4) (see Table 4) and this, together with its energetic stability (Ragazzi *et al.*, 1986), suggests the $^2S_0$ 'skew boat' as the third contributing conformer to the equilibrium.

The highest coupling constants are observed in [$^2H_6$]DMSO at pH 7 and 60 °C. Under these conditions J(1,2), J(2,3) and J(3,4) are very close to those predicted for the $^4C_1$ chair (Table 4). However, the observed J(4,5) value of 6.35 Hz far exceeds the predicted value of 3.85 Hz. The modified Karplus equation of Haasnoot *et al.* (1980) defines ^3J(H,H) values in terms of substituent electronegativity as well as dihedral angle. The electronegativity of the carboxylate group is ill-defined, but an approximate value can be obtained from the $^4C_1$ galacturonate conformer for which both crystallographic (Gould *et al.*, 1975) and observed computer-refined best-fit coupling-constant (Jacques *et al.*, 1979) data are available. Substituting the J(4,5) value of 1.26 Hz and the ϕ(4,5) value of 304° into the equation of Haasnoot *et al.* (1980) yields an 'electronegativity factor' of 0.93 for the carboxylate group. This value lies between that of a non-glycosidic ring carbon (0.08) and a hydroxy (1.3) substituent. If the carboxylate group is considered as an α-carbon with two β-oxygen substituents, the 'electronegativity factor' would be −0.09 (identical with that of a glycosidically linked anomeric carbon substituent!). Calculated values of J(4,5), using limiting values of −0.1 and 1.4 for the carboxylate 'electronegativity factor', are plotted in Fig. 4 for the complete 0–360 ° range of ϕ(4,5) for IdoA. Also shown in Fig. 4 is the curve obtained for a value of 0.09, which is assumed to represent the electronegativity of the carboxylate group for the purposes of calculating J(3,4) and J(4,5) in Table 4. As can be seen from Fig. 4, only an increase of around 25° for the ϕ(4,5) dihedral angle will reconcile the highest J(4,5) value observed with that predicted for the 'ideal' $^4C_1$ conformer. The data observed with [$^2H_6$]DMSO are therefore consistent with a slightly distorted $^4C_1$ IdoA conformer.

In the disulphated disaccharide the coupling constants are significantly lower than those obtained in the desulphated disaccharide under comparable experimental conditions (see Tables 2 and 3), indicating a much

Table 4. Dihedral angles (ϕ) and predicted proton–proton coupling constants (J) for the three most energetically stable conformers of a glycosidically linked IdoA residue

Notes: (1) Dihedral angles (H–C–C–H) are obtained from crystallographic data (see the text) and are presented in the $0° < \phi < 360°$ form required for the equation of Haasnoot *et al.* (1980). (2) An 'electronegativity factor' of 0.9 is assumed for the carboxylate group. (3) The data set used to obtain the parameters for the equation of Haasnoot *et al.* (1980) was necessarily biased towards 'chair' conformers, hence the predicted values for the $^2S_0$ conformer may be subject to greater error.

Proton pair coupled	$^1C_4$		$^4C_1$		$^2S_0$	
	ϕ (°)	J (Hz)	ϕ (°)	J (Hz)	ϕ (°)	J (Hz)
H(1)–H(2)	62.3	2.74	176.8	7.83	151.1	5.59
H(2)–H(3)	293.5	3.10	188.0	9.29	178.9	10.10
H(3)–H(4)	67.5	2.97	171.2	9.08	143.8	4.89
H(4)–H(5)	55.9	1.25	304.0	3.85	37.2	3.04

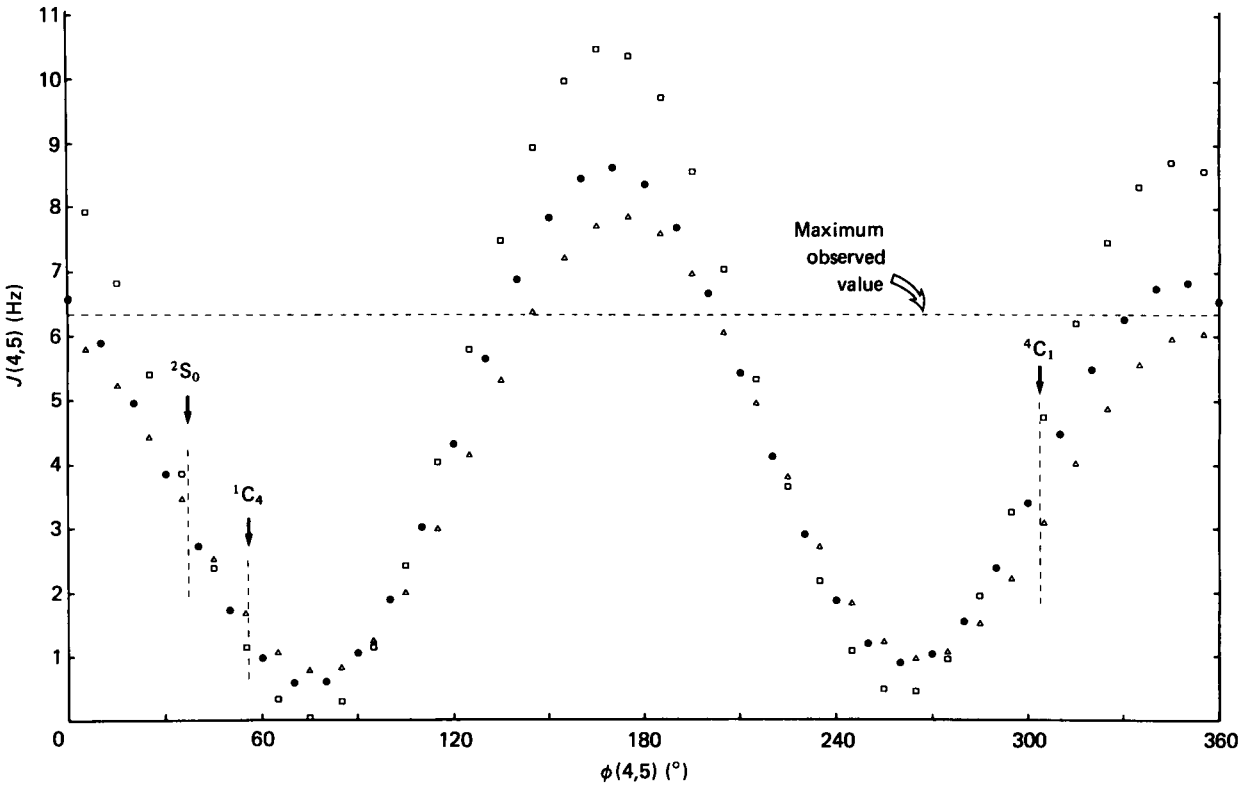

Fig. 4. Variation of calculated $J(4,5)$ coupling constant with ϕ

Values of $J(4,5)$ as a function of the torsional angle H(4)–C(4)–C(5)–H(5) were calculated by using the equation described by Haasnoot *et al.* (1980) using 'electronegativity factors' of −0.1 (□), 0.9 (●) and 1.4 (△). The range of the predicted $J(4,5)$ values for each of the conformers is indicated, as is the maximum observed value.

larger contribution from the $^1C_4$ conformer for sulphated IdoA.

All sets of IdoA coupling constants were analysed by least-squares methods in order to ascertain the relative contributions of the three participating conformers at equilibrium under each condition (Fig. 5). The predicted coupling constants (Table 4), representing fixed conformational states, were employed in all cases with the exception of $J(4,5)$ for the $^4C_1$ conformer (discussed above), for which the largest experimentally observed value (6.35 Hz) was used.

DISCUSSION

The position of conformational equilibrium for an IdoA residue at the non-reducing terminus of oligosaccharides was sensitive both to the experimental conditions and to the local structural environment. Sulphated IdoA in the disulphated disaccharide in aqueous solution exhibited a high proportion of the $^1C_4$ conformer (Fig. 5). Additional evidence was provided by long-range proton–proton coupling constants, which are characteristic of the planar 'W' arrangement of bonds (Booth, 1960). Such co-planarities occur in the $^1C_4$ conformer and, indeed, long-range couplings were observed whenever this form predominated. There was no evidence of long-range couplings from the distorted $^4C_1$ or the $^2S_0$ conformers.

The IdoA $^1C_4$ conformer population generally decreased as the proportion of the solvent [$^2H_6$]DMSO increased, such that unsulphated IdoA in 100% [$^2H_6$]DMSO adopted almost exclusively the $^4C_1$ conformation. A decrease in pH was observed to perturb the position of the equilibrium away from the $^4C_1$ conformer for both disaccharides. Interestingly, the shift in the position of the IdoA conformational equilibrium characteristically induced by [$^2H_6$]DMSO was *not* observed for IdoA(2SO_3) in [$^2H_6$]DMSO at pH 2.3, and this indicates that the effect of [$^2H_6$]DMSO on IdoA conformation may operate via the charged carboxylate group. Over the temperature range examined, the proportion of the $^1C_4$ conformer generally decreased with an increase in temperature, as previously observed for IdoA in heparan sulphate tetrasaccharides (Sanderson *et al.*, 1985). The very small influence of cations on the position of the equilibrium was probably due to an insufficiently large co-ordination environment of the disaccharides. In these studies, therefore, the position of the IdoA conformational equilibrium was influenced chiefly by solvent and pH.

For unsulphated IdoA in [$^2H_6$]DMSO the position of the conformational equilibrium lay strongly in favour of the $^4C_1$ 'chair' conformer, and the observed coupling constants are higher than any previously reported for IdoA. These represent a distortion from the 'ideal' $^4C_1$ conformation in which the carboxylate group is displaced away from the axial position. This distortion of the $^4C_1$ conformer is not unreasonable, as it would lead to a reduction of the 'steric clashes' of the bulky carboxylate group.

These studies have addressed the conformation of IdoA at the non-reducing terminus and indicate that

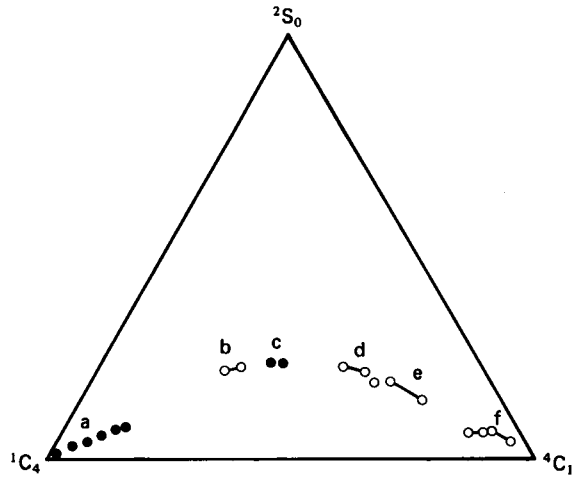

Fig. 5. Calculated proportions of the three iduronate conformer populations

Circles indicate the proportion of each of the conformers calculated to be present under various experimental conditions. ●, Sulphated IdoA in IdoA($2SO_3$)-anManOH($6SO_3$); ○, IdoA in IdoA-anManOH. Groups of results are coded alphabetically as follows: a, IdoA($2SO_3$)-anManOH($6SO_3$) in ^2H_2O at various pH values and at low pH in [$^2H_6$]DMSO; b, IdoA-anManOH in ^2H_2O at pH 2.35; c, IdoA($2SO_3$)-anManOH($6SO_3$) in 100% [$^2H_6$]DMSO at pH 7; d, IdoA-anManOH in ^2H_2O at various temperatures; e, IdoA-anManOH in mixed ^2H_2O (67–91%)/[$^2H_6$]DMSO (9–33%) solutions; f, IdoA-anManOH in mixed ^2H_2O (9–33%)/[$^2H_6$]DMSO (67–91%) solutions and in 100% [$^2H_6$]DMSO at various temperatures.

three conformers are in equilibrium. However, results from studies of oligosaccharides and polysaccharides containing iduronate flanked on both sides by sugar residues appear to indicate equilibria involving only $^1C_4$ and $^2S_0$ conformers (Casu *et al.*, 1986; P. N. Sanderson, T. N. Huckerby & I. A. Nieduszynski, unpublished work).

As significant proportions of all three conformers are observed in equilibria, it is evident that the differences in free energy between these forms are very small [$\sim$4.2 kJ·mol^{-1} ($\sim$1kcal·mol^{-1})], and consequently even small contributions of energy from interacting molecules may provoke a change in favour of a single conformation.

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