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The effect of variation of substitution on the solution conformation of heparin: a spectroscopic and molecular modelling study

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ABSTRACT

The effect of variations in substitution on the conformation of iduronate-containing sequences in heparin and heparan sulphate has been studied using a series of chemically-modified heparins in which substitution with *O*- and *N*-sulphate and *N*-acetyl substituents has been systematically altered. Monosaccharides corresponding to residues in these modified heparins have also been investigated. The conformations of the glycosidic linkages in *O*- and *N*-desulphated re-*N*-acetylated heparin, *O*-desulphated re-*N*-sulphated heparin, and 6-*O*-desulphated re-*N*-sulphated heparin have been compared with those of *N*-desulphated re-*N*-acetylated heparin and of heparin itself, which have previously been reported [B. Mulloy, M.J. Forster, C. Jones, and D.B. Davies, *Biochem. J.*, 293 (1993) 849–858]. The overall conformation of all the polysaccharides is shown to be similar, regardless of substitution pattern. The conformational equilibrium of the pyranose ring of iduronic acid residues in the polysaccharides has been monitored by the use of ^{13}C NMR chemical shift temperature coefficients, and shown to be similar for all the modified heparins with the exception of *N*-desulphated re-*N*-acetylated heparin. Circular dichroism spectra of all the polysaccharides are reported, and their variations attributed to differences in the proportions of pyranose ring forms in the iduronate conformational equilibrium.

INTRODUCTION **

The conformation of the major repeating unit in heparin, 4)-α-L-IdoA2SO_3^--(1 → 4)-α-D-GlcNSO_3^-(1 →, has been determined both in the solid state[1] and in

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** Abbreviations and definitions used in this paper: A; α-D-glucosamine, *N*-acetylated or *N*-sulphated (and its 6-*O*-sulphate): I, IdoA; α-L-idurornic acid (and its 2-*O*-sulphate): α-D-IdoA-OMe; methyl α-D-idopyranosiduronic acid: NOE; nuclear Overhauser effect: TSP-d_4; 3-(trimethylsilyl)propionic acid 2,2,3,3-d_4 sodium salt

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solution[2]. All samples of heparin, and of the closely related compound heparan sulphate, are heterogeneous to some degree, containing 4)-β-D-GlcA-(1 → 4)-α-D-GlcNAc-(1 → sequences, and iduronate-containing sequences in which substitution with *O*- and *N*-sulphate varies. Specific heterogeneous sequences of both heparin and heparan sulphate are linked with particular biological activities of these compounds, the prime example being the unusual pentasaccharide sequence which confers high affinity for antithrombin on heparin[3]; also a heparan sulphate sequence consisting of alternate 2-*O*-sulphated iduronate and *N*-sulphated (but not 6-*O*-sulphated) glucosamine residues has high affinity for basic fibroblast growth factor[4] and is associated with high antiproliferative activity[5].

The ability to determine the solution conformations of these sequences enables their interactions with ligands to be studied at a detailed level, in order to elucidate the molecular basis for their biological function and develop therapeutically-useful properties of these glycosaminoglycans.

Sulphate groups are charged and bulky, and might be expected to play a part in determining the overall conformation of highly sulphated polysaccharides such as heparin. Chemical modification of heparin can be used to produce polysaccharides with systematically altered patterns of *O*-sulphation, *N*-sulphation, and *N*-acetylation. In this study, four chemically modified heparins are compared with heparin itself, using spectroscopic and molecular modelling techniques, with respect to both conformation of the glycosidic linkages and ring mobility of the iduronate residues. Of these compounds, heparin and *N*-desulphated, re-*N*-acetylated heparin have been the subject of a previous study in which the methodology for the determination of the glycosidic linkage in these compounds has been discussed[2]. In the present study, three further compounds are added: *N*- and *O*-desulphated, re-*N*-acetylated heparin, *N*- and *O*-desulphated, re-*N*-sulphated heparin, and *N*- and 6-*O*-desulphated, re-*N*-sulphated heparin. From these compounds insights into the effect of sulphate and *N*-acetyl substitution on the conformation of both the glycosidic linkages and iduronate pyranose ring can be obtained, allowing the conformational properties of heterogeneous parts of the heparin (or heparan sulphate) polysaccharide chain to be deduced. The two re-*N*-acetylated modified heparins contain the sequence GlcNAc-IdoA, which is not found in native heparin. Methods for the determination of glycosidic linkage conformation follow Mulloy et al.[2], using $^{1}H-^{1}H$ NOE data interpreted with the aid of full multi-spin NOE simulation[6] using molecular models derived from conformational energy calculations. This methodology is unusual in that, in order to explain the relative magnitudes of interproton NOEs, the normal assumption that NMR relaxation phenomena are determined by a single reorientational correlation time has to be replaced by a symmetric top motional model, in which NMR relaxation phenomena are considered to be dependent on the overall reorientation in solution of molecules approximated as rigid cylinders. Two correlation times, one for motion parallel to the main axis of the cylinder ($\tau_{\parallel}$) and one for motion perpendicular to it ($\tau_{\perp}$) are now needed; in addition to which both proton–proton NOEs and ^{13}C

relaxation times become dependent on the angle between the H–H or C–H vector and the main axis.

The conformational mobility of the iduronate ring is probed using ^{13}C NMR spectroscopy and CD spectra of the five compounds are presented and discussed. Comparison of the ^{1}H and ^{13}C NMR spectra of these five polysaccharides with monosaccharides corresponding to their constituent residues enables the effects of polymerization and the effects of sulphate substitution to be distinguished.

EXPERIMENTAL

Materials.—*N*-Acetylglucosamine (**1**), 6-*O*-sulphated *N*-acetylglucosamine (**2**), *N*-sulphated glucosamine (**3**), and 6-*O*-sulphated, *N*-sulphated glucosamine (**4**) were obtained from Sigma Chemical Co. (Poole, UK). The sample of α-D-IdoA-OMe sodium salt (**5**) was the gift of Professor F. Diamantini, Opocrin, Milan.

Lung heparin (**6**) was material remaining from the Second International Standard[7].

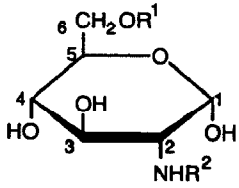
N-Desulphated re-*N*-acetylated heparin (**7**) was prepared from (**6**) by *N*-desulphation[8], followed by re-*N*-acetylation[9]. *N*- And *O*-desulphated re-*N*-acetylated heparin (**8**) was prepared by solvolytic *O*- and *N*-desulphation[10], followed by re-*N*-acetylation. The partially *O*-desulphated (at O-6 of glucosamine) re-*N*-sulphated heparin (**10**) was prepared by *O*- and *N*-desulphation[10], followed by re-*N*-sulphation[11]; totally *O*- and *N*-desulphated re-*N*-sulphated heparin (**9**) required an adapted method.

Compound **8** was produced by a second solvolytic step after the re-*N*-acetylation[10]. If the conditions used for this second solvolysis are applied to heparin directly, some *O*-desulphation occurs but a large proportion of the 2-*O*-sulphated iduronate survives as such, leading to partially *O*-desulphated re-*N*-sulphated heparin (**10**) after re-*N*-sulphation. If partially-solvolysed heparin is acylated with a 9-fluorenylmethoxycarbonyl (FMOC) reagent group and the solvolysis is repeated, the desulphation proceeds to completion. The FMOC is removed by increasing the pH to above 11 with NaOH, and fluorene derivatives removed by extraction with CH_2Cl_2. The product may then be re-*N*-sulphated to give totally *O*- and *N*-desulphated, re-*N*-sulphated heparin (**9**)

All reagents used were of analytical grade.

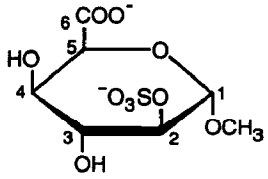
Molecular mass measurements.—Determination of the molecular-mass distribution of the samples by gel permeation chromatography was performed as previously described[2], on a 60-cm column of TSK SX 3000 (Anachem, Luton, UK) with 0.1 M ammonium acetate as eluant at 0.5 mL/min using refractometric detection.

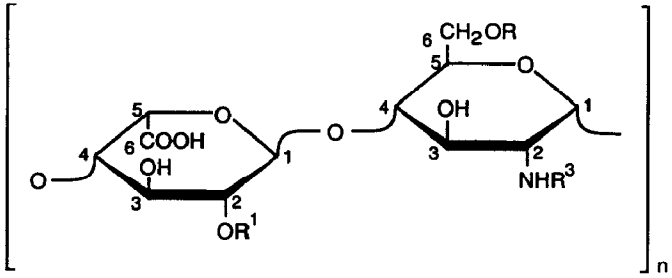
Preparation of samples for NMR studies.—The polysaccharides (**6–10**) were subjected to ion-exchange chromatography to remove paramagnetic impurities. A column (1 × 10 cm) of AG 50W-X8 (Bio-Rad, Hemel Hempstead, UK) was converted to the Na^+ form by treatment with 5 mL 0.1 M NaOH and washed with H^2O for at least 24 h before use. Samples for NMR were applied to the column,



	R^1	R^2
1	H	$COCH_3$
2	SO_3^-	$COCH_3$
3	H	SO_3^-
4	SO_3^-	SO_3^-

5





	R^1	R^2	R^3
6	SO_3^-	SO_3^-	SO_3^-
7	SO_3^-	SO_3^-	$COCH_3$
8	H	H	$COCH_3$
9	H	H	SO_3^-
10	SO_3^-	H	SO_3^-

eluted with ~ 30 mL water, and dried by evaporation under reduced pressure. For ^1H NMR, 20–30 mg of each polysaccharide was lyophilised three times from 99.8% D_2O (Goss Scientific Instruments Ltd., Ingatestone, UK), then dissolved in 0.6 mL 100% D_2O (Aldrich Chemical Co., Poole, UK) for NMR spectroscopy in a 5-mm tube. For ^{13}C NMR, ~ 100 mg of each polysaccharide was dissolved in 2.5 mL D_2O for spectroscopy in a 10-mm NMR tube.

NMR spectroscopy—Spectra at 500 MHz for ^1H and 125 MHz for ^{13}C were recorded on Bruker AM 500 and Jeol GSX 500 spectrometers. Spectra at 400 MHz

for ^1H and 100 MHz for ^{13}C were recorded on a Bruker AM 400 spectrometer. Spectra at 270 MHz for ^1H and 67.5 MHz for ^{13}C were recorded on a Bruker WH 270, and 200 MHz for ^1H and 50 MHz for ^{13}C spectra on a Bruker WM 200 spectrometer.

^1H NMR spectra in 80% H_2O–20% D_2O were recorded at 400 MHz using the $1.\bar{3}.3.\bar{1}$ sequence for water suppression[12]. Magnitude mode COSY spectra of the monosaccharides were acquired at 200 MHz, and of the polysaccharides at 500 MHz. The instrument manufacturer's standard pulse sequences were used in all cases. Typically, 256 spectra of 1024 points each were used, with narrow spectral width (3–4 ppm) giving digital resolution in F2 of ~ 2 Hz/point. Zero-filling to 512 points was used in F1, with no zero-filling in F2.

Truncated driven NOEs were measured at 500 MHz from difference spectra by integration, and are quoted as a percentage of the area under the irradiated resonance.

^1H–^{13}C heteronuclear correlated spectra of the monosaccharides **1–3** were carried out at 200 MHz; similar spectra of **4** and **5** were carried out at 400 and 500 MHz, respectively. ^1H–^{13}C heteronuclear correlated spectra of **6–10** were recorded at 400 MHz using standard pulse sequences supplied by the instrument manufacturers. Typically, 256 spectra of 4K points each were accumulated and transformed after zero-filling in F1.

^{13}C Spin-lattice relaxation rates (R_1) were measured at 50 MHz by the inversion recovery method. Ten spectra were obtained with a variable delay of 0.001–7.0 s; a relaxation delay of 7.0 s was sufficient to allow full relaxation of the ring carbons, though probably insufficient for the methyl carbons. Relaxation rates were calculated from the intensities in the spectra using the Marquardt algorithm for non-linear curve-fitting.

Circular dichroism.—Circular dichroism (CD) spectra were recorded using a Jasco J-600 spectropolarimeter. Data obtained below 190 nm were noisy and unreliable, and accordingly rejected. The polysaccharides were dissolved in 0.05 M phosphate buffer at pH 7.0 and the spectra recorded in cells of path length 0.05 cm (for 4 mg/mL solutions) or 0.2 cm (for 1 mg/mL solutions). Spectra are reported in terms of (M^{-1} cm^{-1}), based on the molecular mass of the repeating unit in the case of the polysaccharides **6–10**.

Computational methods.—Molecular models were built using ChemX software (designed and distributed by Chemical Design Ltd., Oxford, UK) running on a MicroVax II computer. Energy calculations and modelling of the polysaccharides were performed as previously described[2]. The structures shown in Fig. 4 were produced using InsightII (Biosym, San Diego, CA) running on a Silicon Graphics Iris 4D/25. NOE simulations from molecular models were carried out using the program NOEMOL[6] (QCPE 636, Quantum Chemistry Program Exchange, Indiana University) running on Sun 3/160 or Silicon Graphics Iris 4D/310 computers

RESULTS

Molecular mass measurements.—The molecular mass distributions of the polysaccharides determined by gel permeation chromatography were similar to those of the parent heparin, and to **7** (ref 2), showing that the chemical modification processes had not resulted in depolymerization of **8–10**.

^1H and ^{13}C NMR spectroscopy of monosaccharides.—The 200 MHz ^1H NMR spectra of compounds **1–4** were assigned by means of COSY spectra and chemical shifts and coupling constants are summarized in Table I. Spin systems arising from the α anomers were traced from the downfield anomeric doublet ($^3J_{H,H}$ 4.7 Hz).

TABLE I

NMR data for monosaccharides 1–5 in aqueous solution

Atom(s)	^1H chemical shifts (ppm)				
	1 [a]	**2** [a]	**3** [a]	**4** [a]	**5** [b]
H-1	5.21	5.24	5.46	5.25	4.65
H-2	3.89	3.91	3.22	3.25	3.43
H-3	3.77	3.77	3.64	3.65	3.68
H-4	3.49	3.55	3.48	3.54	3.82
H-5	3.86	4.07	3.83	4.05	4.37
H-6 pro-*S*	3.83	4.30	3.80	4.27	
H-6 pro-*R*	3.83	4.27	3.80	4.27	
NH	8.13	8.13			
OCH_3					3.46
Acetyl	2.06	2.07			
	$^3J_{H,H}$ (Hz)				
	1 [a]	**2** [a]	**3** [a]	**4** [a]	**5** [b]
H-1,H-2	3.5	3.5	3.5	3.5	4.95 [c]
H-2,H-3	10.8	10.6	10.1	10.1	6.93 [c]
H-3,H-4	8.6	8.7	8.7	9.3	6.43[c]
H-4,H-5	9.4	10.0	9.7	9.4	4.20 [c]
H-5,H-6pro-*S*		3.0		3.5	
H-5,H-6pro-*R*		3.5		3.5	
H-2,NH	8.6	8.6			
	^{13}C chemical shifts (ppm) [d]				
	1	**2**	**3**	**4**	**5**
C-1	93.70	93.80	94.10	94.30	104.50
C-2	56.90	56.80	60.80	60.70	73.80
C-3	72.90	73.50	74.00	73.90	75.00
C-4	73.50	72.70	73.00	72.70	73.70
C-5	74.40	76.50	74.20	72.30	73.50
C-6	63.40	70.10	63.50	70.10	179.10
Acetyl CH_3	24.70	24.80			
Acetyl CO	177.40	177.30			

[a] Measured at 200 MHz at ambient temperature relative to external TSP-d_4 at 0 ppm. [b] Measured at 500 MHz, 20°C, relative to internal TSP-d_4 at 0 ppm. [c] Values from Forster and Mulloy[25]. [d] Measured at 50 MHz at ambient temperature relative to external TSP-d_4 at 0 ppm.

The presence of a proportion of β anomers in the samples did not give rise to difficulties in assignment.

The $^3J_{H,H}$ values were measured from multiplet splittings in the 1D spectra. The $^3J_{H5,H6}$ values could only be measured (from the H-5 multiplet) in **3** and **4**, and $^2J_{H6proR,H6proS}$ values could not be determined due to signal overlap. For **3**, the stereospecific assignment of H-6 pro-*S* and H-6 pro-*R* is based on the observation of Nishida et al.[13] that the H-6 pro-*R* signal occurs upfield of the H-6 pro-*S* signal in glucopyranosides, and has the higher value for $^3J_{H5,H6}$.

NH doublets of the two *N*-acetylated compounds (**1** and **3**) were observed in the spectra recorded in 80% H^2O and $^3J_{H2,NH}$ of 8.6 Hz for both these compounds was observed.

The 500 MHz ^1H spectrum of α-D-IdoA-OMe (**5**) was also assigned by means of a COSY spectrum, and chemical shifts and $^3J_{H,H}$ values are listed in Table I; the values are consistent with those reported by Perlin et al.[14] given the differences in conditions between the two sets of measurements. It is expected that the spectrum of α-D-IdoA-OMe is identical with that of α-L-IdoA-OMe, the monosaccharide corresponding to the iduronate residues in heparin and the modified heparins.

The ^{13}C NMR spectra of all the monosaccharides were recorded at 50 MHz, and assigned by means of ^1H–^{13}C correlated spectra (not shown). Chemical shifts are listed in Table I.

^1H and ^{13}C NMR spectroscopy of the modified heparins.—^1H NMR spectra of the modified heparins (**8–10**) were assigned by means of COSY spectra and chemical shifts are summarized in Table II, together with corresponding data[2] for heparin (**6**) and de-*N*-sulphated re-*N*-acetylated heparin (**7**). The $^3J_{H,H}$ values, where these could be measured, are also given in Table II, but their accuracy is variable due to the width of all the lines in these spectra. Assignment of the H-6 resonances was based largely on chemical shift grounds as $^3J_{H5,H6}$ values could not be measured for most of the compounds[13], and where they could be measured, in **6** and **8**, $^3J_{H5,H6proS}$ and $^3J_{H5,H6proR}$ magnitudes were not markedly different. The $^3J_{H2,NH}$ values for **7** and **8** were measured from spectra of the polysaccharides in 4:1 H_2O–D_2O.

^{13}C NMR spectra of **1–10** were assigned using ^{13}C–^1H correlated spectra and the chemical shifts are listed in Table III together with values for **6** and **7** taken from ref 2.

The variation of ^{13}C chemical shift with temperature was investigated for all the compounds in the study (**1–10**). For **1–5**, ^{13}C spectra were recorded at 27, 37, 57, and 77°C; for **6–10**, ^{13}C spectra were recorded at 10°C intervals from 27 to 77°C. An external TSP-d_4 reference was used, which gave an invariant chemical shift over the temperature range used for the *N*-acetyl methyl signal in the spectra of **1**, **2**, **7**, and **8**. ^{13}C Chemical shifts for each compound were found to vary approximately linearly with temperature; the total changes in chemical shift over the range 27C–77°C for **1–10** are listed in Table IV.

^1H–^1H NOEs—For each of the polysaccharides truncated driven NOEs were

TABLE II

Proton chemical shifts and $^3J_{H,H}$ values for heparin and the modified heparins (6–10)

Atom(s)	^{1}H shifts (ppm) [a]				
	6	**7**	**8**	**9**	**10**
Iduronate residue					
H-1	5.22	5.14	4.89	4.92	5.20
H-2	4.35	4.33	3.65	3.69	4.32
H-3	4.20	4.22	3.83 [b]	4.09	4.22
H-4	4.10	4.06	4.04	4.04	4.05
H-5	4.81	4.87	4.68	4.72	4.78
Glucosamine residue					
H-1	5.39	5.11	5.14	5.35	5.28
H-2	3.29	4.0 [b]	3.93	3.25	3.25
H-3	3.67	3.73 [b]	3.73	3.67	3.66 [b]
H-4	3.77	3.73 [b]	3.69	3.63	3.66 [b]
H-5	4.03	4.0 [b]	3.85 [b]	3.83	3.8–3.9
H-6 pro*S*	4.39	4.3 [b]	3.85[b]	3.84	3.8–3.9
H-6 pro*R*	4.27	4.3 [b]	3.79	3.79	3.77
NH		7.82	8.02		
Acetyl		2.04	2.00		
Atom(s)	$^3J_{H,H}$ (Hz) [d]				
	6	**7**	**8**	**9**	**10**
Iduronate residue					
H-1,H-2	2.90	< 2	3.80	4.10	< 3
H-2,H-3	5.90	3.90	5.0 [c]	4.70	4.0 [c]
H-3,H-4	3.70	3.30	4.0 [c]	3.30	4.0 [c]
H-4,H-5	3.00	2.90	3.00	3.10	< 3
Glucosamine residue					
H-1,H-2	3.60	4.00	3.70	3.80	3.90
H-2,H-3	10.3 [c]	10.00	9.5 [c]	9.0 [c]	
H-3,H-4	9.60	– [d]	9.60	–	
H-4,H-5	9.60	–	10.0 [d]	–	–
H-5,H-6 pro*S*	2.30	–	3.60	–	–
H-5,H-6 pro*R*	< 3	–	4.60	4.70	4.50
H-6 pro*S*, H6pro*R* [f]	− 11.60	–	− 12.30	− 13.40	− 12.40
H-2,NH	9.40	9.40			

[a] Measured at 70°C relative to internal TSP-d_4. [b] Overlapping resonances; estimate. [c] ±0.5 Hz. [d] Unmeasureable due to overlapping resonances. [e] $^2J_{H,H}$; negative sign assumed, not determined.

measured with 200-ms irradiation of H-1 of iduronate (I) and H-1 of glucosamine (A) residues. The intraresidue NOE of H-1 to H-2 of the two residues was observed as well as interresidue NOEs; all these are listed in Table V. Interresidue NOEs directly across the linkage were observeable between H-1A–H-4I and H-1A–H-3I, and H-1I–H-4A and H-1I–H-6A, for all the polysaccharides.

^{13}C Spin-lattice relaxation rates.—^{13}C Spin-lattice relaxation rates (R_1s) at 50 MHz were calculated from inversion recovery data for ring carbons of polysaccharides **8–10**, and for C-6 of the glucosamine residues, and are listed in Table VI.

TABLE III

^{13}C Chemical shifts of heparin (**6**) and four modified heparins (**7–10**)

Atom	Chemical shifts (ppm) [a]				
	6	**7**	**8**	**9**	**10**
Iduronate residue					
C-1	102.2	100.7	104.5	104.2	102.3
C-2	79.0	75.3	72.9	72.4	78.3
C-3	72.2	66.0	72.6	71.7	71.2
C-4	79.1	72.8	77.8	78.0	78.9
C-5	72.0	69.2	72.9	72.5	72.7
C-6	177.1	176.5 [b]	177.1 [b]	177.4	177.7
Glycosamine residue					
C-1	99.6	95.1	97.5	98.4	100.1
C-2	60.8	54.6	56.6	60.6	60.9
C-3	72.9	71.3	72.6	72.5	72.7
C-4	79.1	77.8	80.2	80.2	80.7
C-5	72.0	70.6	74.0	73.6	74.0
C-6	69.3	67.7	62.9	62.7	63.0
C=O		176.7 [b]	177.1 [b]		
CH_3		23.2	24.8		

[a] Measured in D_2O solution at 70°C relative to internal TSP-d_4. [b] Assignments may be interchanged.

The mean values for **8** (5.5 s^{-1}), **9** (5.6 s^{-1}), and **10** (5.7 s^{-1}) are a little lower than those for **6** (6.3 s^{-1}) and **7** (6.4 s^{-1}) in ref 2.

CD Spectra of heparin and the modified heparins.—The CD spectra of **6** and **8–10** (Figs. 1a, b, c, and e, respectively) are similar in so much as they present a negative CD centred around 210 nm with a positive CD around 190 nm. The CD reported here of heparin itself (**6**) compares well with previously published data[15,16] within the wavelength range 260–190 nm. The CD spectrum of **7** (Fig. 1d) is clearly different. The short wavelength positive CD is retained but the pronounced negative CD around 210 nm is replaced by a bisignate curve centred at 221 nm with a weak negative maximum at 229 nm and an equally weak positive maximum at 210 nm. α-D-IdoA-OMe (**5**) was the only monosaccharide in the study for which a CD spectrum was recorded, as the others were available only as a mixture of anomers. The CD of **5** is presented in Fig. 2 showing a strong positive band at 209 nm ($\Delta\epsilon = +5.2$) with a weak negative band at ~194 nm and a strong positive band below 190 nm. The spectrum is in overall qualitative agreement with that of the sodium salt of α-D-IdoA-OMe reported by Morris et al.[17] except that their minimum at 194 nm has positive dichroism rather than crossing the zero. The more relevant model, α-L-IdoA-OMe, would have a CD spectrum that is the inverse of the D isomer. Accordingly the signs in Fig. 2 ought to be reversed for comparison purposes. The precise spectroscopic assignments of the CD spectrum in Fig. 2 are open to debate. The 209 nm positive peak can only be assigned to an $n \rightarrow \pi^*$ transition localised on the carboxylate group[18]. The half-height width of this band

TABLE IV

Variations in ^{13}C chemical shift with temperature

Atom	Difference in chemical shift (ppm) over the range 17–77°C [a]				
	1	**2**	**3**	**4**	**5**
(i) Monosaccharides					
C-1	0.08	0.05	0.11	0.08	0.00
C-2	0.06	0.05	0.06	0.07	0.46
C-3	0.17	0.11	0.19	0.23	0.46
C-4	0.26	0.30	0.26	0.35	0.43
C-5	0.11	0.15	0.08	0.18	0.24
C-6	0.26	0.15	0.24	0.15	n.d.
	6	**7**	**8**	**9**	**10**
(ii) Polysaccharides					
Iduronate residue					
C-1	0.04	0.11	0.00	−0.09	0.23
C-2	0.40 [b]	0.57	0.43 [c]	0.49 [b]	0.35
C-3	0.36 [b]	1.26	0.12 [b]	0.52	0.63
C-4	0.40 [b]	1.08	0.43	0.45	0.24
C-5	0.36 [b]	0.47	0.43 [c]	0.49 [b]	0.18 [b]
C-6	−0.58	−0.76	−0.40 [d]	−0.67	−0.45
Glucosamine residue					
C-1	0.26	0.58	0.22	0.23	0.00
C-2	0.04	−0.03	−0.07	−0.05	−0.09
C-3	0.31	−0.07	0.12 [b]	0.09	0.18 [b]
C-4	0.40 [b]	0.51	0.75	0.50	0.54
C-5	0.04	0.00	−0.07	−0.09	0.01
C-6	0.13	0.11	0.33	0.27	0.27

[a] A positive figure indicates a downfield shift. [b] and [c] Overlapping signals. [d] 17–67°C.

TABLE V

Experimental and calculated ^1H–^1H NOEs [a] (%) at 500 MHz for three modified heparins

	8			**9**			**10**		
	Exptl	Calcd		Exptl	Calcd		Exptl	Calcd	
		$^1C_4$	$^2S_0$		$^1C_4$	$^2S_0$		$^1C_4$	$^2S_0$
H-1A to:									
H−3I	−19.0	−31.0	−2.0	−4.0	−9.0	−2.0	−4.0	−8.0	−1.0
H−4I	−5.0	−2.0	−6.0	−10.0	−13.0	−6.0	−7.0	−13.0	−14.0
H−2A	−20.0	−26.0	−21.0	−12.0	−19.0	−20.0	−13.0	−15.0	−22.0
H−1I to:									
H−6 pro*S* A	−7.0	−9 [b]	−8 [b]	−4.0	−13 [b]	−5 [b]	−9 [c]	−6.0	−10 [b]
H−6 pro*R* A	−7.0	−4 [b]	−1 [b]	−4.0	−2 [b]	−1.0		−2.0	−1 [b]
H−4A	−4.0	−2.0	−2.0	−2.0	−6.0	−2.0	−3.0	−3.0	−2.0
H−2I	−5.0	−6.0	−2.0	−5.0	−3.0	−3.0	−2.0	−5.0	−3.0

[a] Mean of four measurements. [b] Calculated for the *g,g* rotamer of the C-5–C-6 bond only. [c] H-6 pro*S* and H-6 pro*R* signals overlap.

TABLE VI

Experimental and calculated ^{13}C spin-lattice relaxation rates (s^{-1}) for three modified heparins

	8			**9**			**10**		
	Exptl	Calcd		Exptl	Calcd		Exptl	Calcd	
		$^1C_4$	$^2S_0$		$^1C_4$	$^2S_0$		$^1C_4$	$^2S_0$
C-1A	6.10	7.00	5.80	6.20	7.00	5.70	4.90	6.80	5.80
C-2A	6.00	6.90	7.90	5.80	6.90	7.80	5.70	7.00	7.90
C-3A	6.10	6.40	7.50	5.40	6.50	7.60	5.40	6.60	7.60
C-4A	6.10	7.20	7.90	6.20	8.00	7.10	6.10	7.20	7.90
C-5A	5.90	7.00	7.90	5.40	7.00	7.90	5.50	7.10	7.90
C-6A	8.50	14.90	13.60	8.50	15.00	13.60	9.90	14.80	13.60
C-1I	4.80	4.90	5.00	4.80	4.90	4.90	6.60	4.90	5.10
C-2I	5.20	7.90	6.90	5.40	6.60	7.60	7.10	7.80	7.00
C-3I	5.20	5.70	6.90	4.80	6.10	6.50	5.60	5.90	7.00
C-4I	5.10	4.90	4.70	6.20	4.80	4.90	4.70	4.80	4.70
C-5I	5.20	7.90	7.90	5.40	7.80	8.00	5.40	7.80	7.90

is ~18 nm, although it ought typically to be ~30 nm[18]. The negative band peaking at 194 nm must therefore be the result of a band cancelling the short wavelength side of the 209 nm $n \rightarrow \pi^*$ transition. This negative spectral component must peak at a wavelength greater than 200 nm and therefore has to be assigned as also being of $n \rightarrow \pi^*$ origin. The carboxylate group in α-D-IdoA-OMe clearly generates at least two separate CD components, one at longer wavelengths with positive CD (negative CD in the L-model) and the other at shorter wavelengths with negative CD (positive in the L-model).

The CD spectra of **6**, **9**, and **10** (Figs. 1a, b, and c) can be assigned in a similar fashion. The negative band at 210 nm can be assigned to a single $n \rightarrow \pi^*$ carboxylate transition corresponding to the longer wavelength negative component in α-L-IdoA-OMe. The positive dichroism at 190 nm and below is based on oxygen[16] (glycosidic link, ring and hydroxyl) and/or the sulphoamino group[19]. The $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ amide transitions of the *N*-acetyl group of **7** and **8** give rise to CD features at 210 and 190 nm[20]; these will be superimposed on the spectrum of heparin (**6**). In agreement with NMR data (see below), the relatively large negative CD at 210 nm in **8** must be due to two reinforcing negative $n \rightarrow \pi^*$ bands (carboxylate and amide); the low CD at 210 nm in **7** results from cancellation as the shorter wavelength component changes sign due to an conformational changes in the iduronate residues. The CD bands observed between 230 and 240 nm in the case of **9** and **10** (Figs. 1b and c) are of unknown origin and must be due to an impurity introduced in the process of chemical modification.

Conformational energy calculations.—Disaccharide molecular models for both the glucosamine–iduronate (AI) and iduronate–glucosamine (IA) linkages were prepared for **8**–**10** as described previously[2]; as separate molecular models were used for the $^1C_4$ and $^2S_0$ ring forms of the iduronate residue, this gave a total of

four disaccharide models per polysaccharide. Using the ChemX software, maps of conformational energy vs. the glycosidic angles ϕ_H = H-1′–C-1′–O-4–C-4 ($\phi_H = 0$ when the H-1′–C-1′ and the O-4–C-4 bonds are eclipsed), and Ψ_H = C-1–O-4–C-4–H-4 ($\Psi_H = 0$ when the C-1–O-4′ and the C-4–H-4 bonds are eclipsed) were prepared; nonbonded energy alone was used, with twenty cycles of minimisation (allowing rotation of all exocyclic bonds other than those at the glycosidic linkages) at each step, varying the angles between 180° and −180° with a step size of 10°. The resulting maps are shown in Figs. 3 a, b, and c.

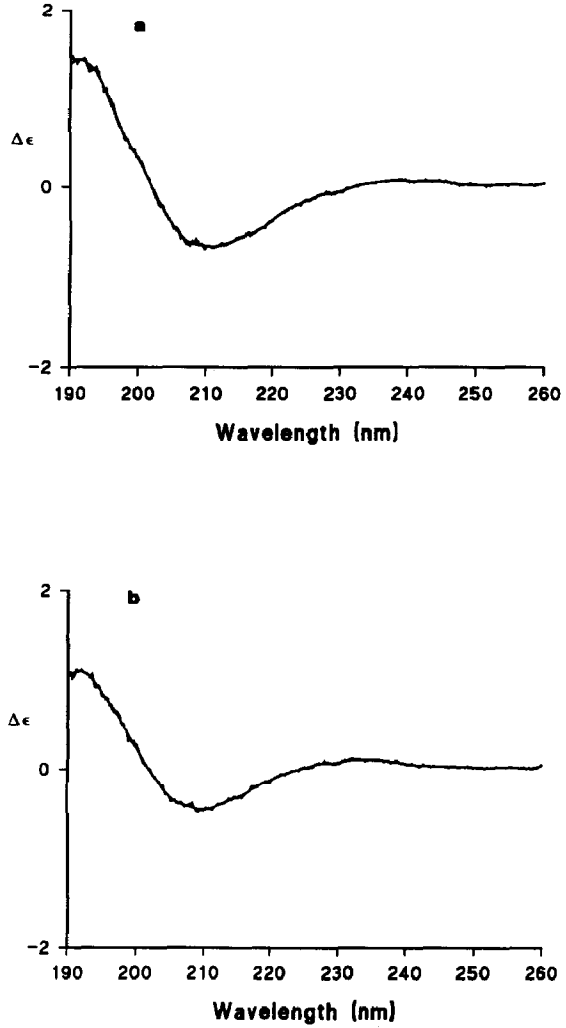
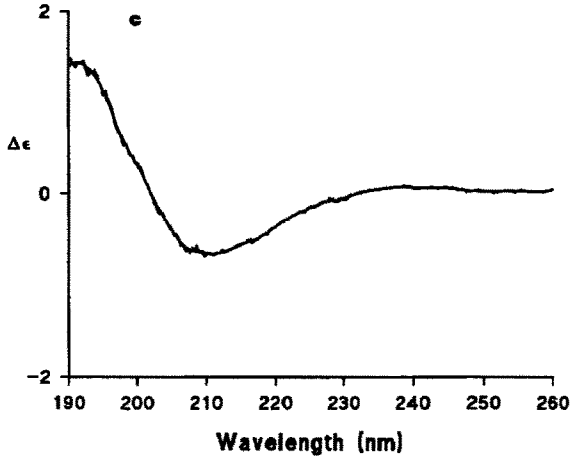
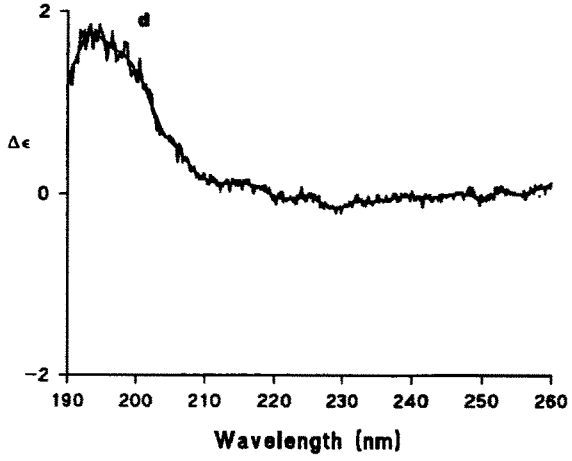


Fig. 1. CD spectra of (a) heparin (**6**); (b) 6-*O*-desulphated re-*N*-sulphated heparin (**10**); (c) *O*- and *N*-desulphated re-*N*-sulphated heparin (**9**); (d) *N*-desulphated re-*N*-acetylated heparin (**7**); and (e) *O*- and *N*-desulphated re-*N*-acetylated heparin (**8**). The spectra as recorded and after Fourier smoothing are overlaid.





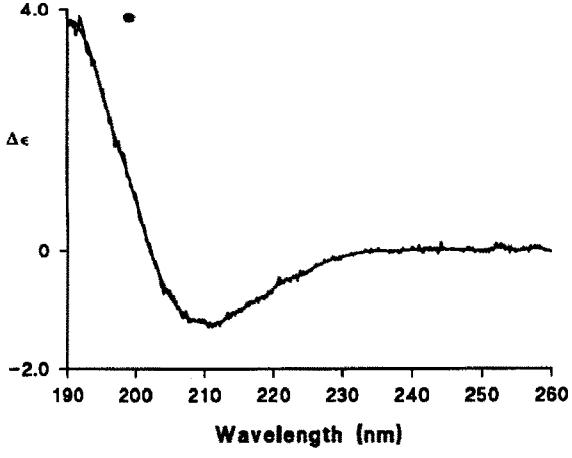


Fig. 1. (continued)

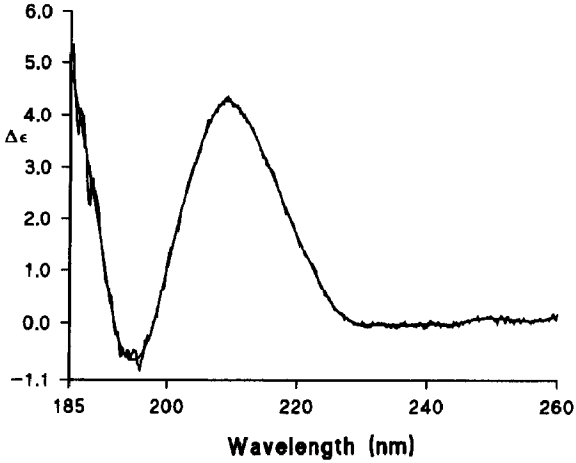


Fig. 2. CD spectrum of α-D-IdoA-OMe (**5**). The spectra as recorded and after Fourier smoothing are overlaid.

The maps for the two totally *O*-desulphated compounds (**8** and **9**) are similar. The AI linkage for both has a single energy minimum in the lower left quadrant of the map (with both ϕ_H and Ψ_H negative), surrounded by an energy well which is slightly more extensive with the iduronate residue in the $^2S_0$ form. The IA linkage has a broad area of low energy around the 0,0 point in the map, with minima both in the upper right quadrant (both ϕ_H and Ψ_H positive) and in or near the lower left quadrant (both ϕ_H and Ψ_H negative) of the map. The extra sulphate substituent at O-2 of the iduronate residue of **10** affects the energy maps in that the low energy areas become more restricted, particularly for the AI linkage with iduronate in the $^2S_0$ form. The maps are otherwise similar to those for **8** and **9**.

Structures corresponding to low energy conformations in the maps were further refined by minimization in the MM2 force field. A single minimum was refined for the AI linkages, and two minima for the IA linkage, as detailed in Table VII.

Conformation of the polysaccharide chain: interpretation of ^1H–^1H NOEs.—The conformation of the glycosidic linkages of the polysaccharides **8**–**10** was characterised by interpretation of the interresidue ^1H–^1H NOEs (Table V) in the light of the conformational energy calculations as previously described for polysaccharides **6** and **7** (ref 2). Briefly, dodecasaccharide models were built in which the glycosidic linkages were set at low energy conformations derived from the maps in Fig. 3 after MM2 minimization (Table VII). Several such models were produced for each polysaccharide with the iduronate residues set in the $^1C_4$ and $^2S_0$ ring forms (following the assumption[21] that these two conformations are available to iduronate residues linked at C-1 and C-4), and, for **8** and **9**, with the IA linkage set to both the identified low energy conformations. The program NOEMOL[6] was then used to perform a full multi-spin NOE simulation. NOEMOL performs a full multi-spin simulation of proton–proton NOEs for a molecular model, given as input the

TABLE VII

Glycosidic bond angles of initial and final molecular models of **8–10** used in ^1H–^1H NOE and ^{13}C R_1 simulation

Modified heparin	A-I linkage				I-A linkage			
	$^1C_4$		$^2S_0$		$^1C_4$		$^2S_0$	
	ϕ_H (°)	Ψ_H (°)	ϕ_H (°)	Ψ_H (°)	ϕ_H (°)	Ψ_H (°)	ϕ_H (°)	Ψ_H (°)
8								
Initial [a]	−43	−39	−27	−46	−19	−36	10	−42
					51	27	60	5
Final [b]	−43	−35	−28	−47	41	14	60	5
9								
Initial [a]	−25	−33	−30	−42	−16	−36		
					61	32	60	11
Final [b]	−25	−33	−30	−42	41	14	60	11
10								
Initial [a]	−30	−24	11	−7	58	29	77	32
Final [b]	−29	−24	−13	−45	41	14	59	12

[a] MM2 minimised structure from lowest energy conformations in the gridsearch. [b] Structure for which the ^1H–^1H NOEs and ^{13}C R_1s shown in Tables VII and VIII were calculated.

Cartesian coordinates of the model, the magnetic field strength of the spectrometer, a correlation time or times, and details of the experiment to be simulated (for example a NOESY spectrum, or, as here, a one-dimensional truncated driven NOE experiment). It is usual for such calculations to assume isotropic tumbling of the molecules in solution, but, as previously found for **6** and **7** (ref 2), it was not possible to simulate the intraresidue H-1–H-2 NOEs successfully in this way. The assumption that the molecule reorients as a cylindrical symmetric top (rotation parallel to the major axis being much faster than rotation perpendicular to it) resolved the difficulty. For the sake of consistency, the same correlation times were used as in ref 2, that is a correlation time for motion perpendicular to the main axis of the symmetric top, $\tau_\perp$, of 8 ns and for motion parallel to the main top axis, $\tau_\parallel$, of 0.16 ns. These correlation times were chosen to be consistent with experimentally determined ^{13}C spin-lattice relaxation rates, and the differences between these values for **6** and **7**, and for **8–10** were too small to justify a change. All the compounds have similar molecular mass distributions. Dodecasaccharide molecular models provided a sufficiently long oligosaccharide chain for the program to compute the position of the main axis accurately, and do not reflect any assumptions about the length of the polysaccharide chains in the samples under investigation or the length of chain which would give rise to the correlation times used in the simulation. Small adjustments of the linkage conformations in the dodecasaccharide models were made by hand to improve the fit between experimental and calculated data.

For **8** and **9**, the IA linkage conformations with both ϕ_H and Ψ_H positive gave predicted H-1I–H-6A NOEs near the experimental values; the alternative confor-

mation considered did not, and indeed predicted a H-1I–H-3A NOE which was not observed. This need not be taken to mean that the polysaccharides are rigid at the glycosidic linkages; the IA energy wells shown in Fig. 3 are broad and can accomodate a degree of flexibility, giving $^1H-^1H$ NOEs averaged over the conformational equilibrium. The AI linkage conformations identified by the energy calculations needed very little adjustment for **8** and **9**, but for **10**, with the iduronate ring in the $^2S_0$ form, the apparent global energy minimum gave a poor prediction of the observed NOEs, and manual adjustments to fit the NMR data took the glycosidic torsional angles closer to the values found for the other models. The conformational energy calculations, though quite unsophisticated, served very well as a means of generating starting structures for fitting to experimental data.

The calculated $^1H-^1H$ NOEs are shown with experimental values in Table V, and the ^{13}C R_1s in Table VI. No attempt has been made to calculate the

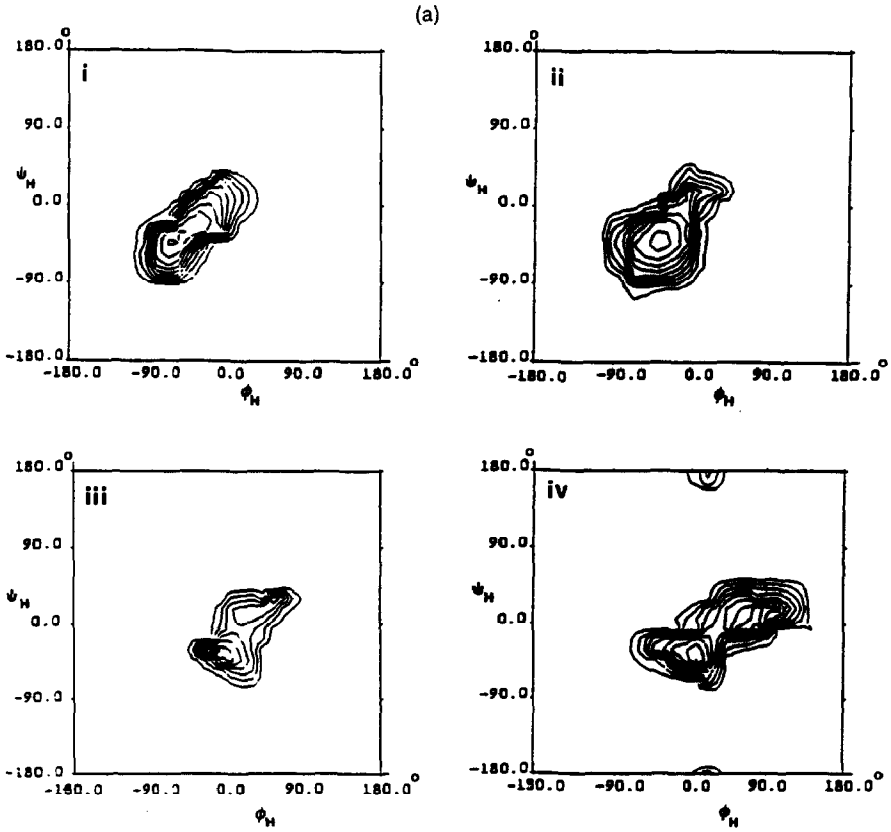


Fig. 3. Contour plots of nonbonded energy (2 kcal/mol between contours) against ϕ_H and Ψ_H for (a) **8**, (b) **9**, and (c) **10**. With (i) the A-I disaccharide with the iduronate residue in the $^1C_4$ chair conformation; (ii) the A-I disaccharide with the iduronate residue in the $^2S_0$ twist-boat conformation; (iii) the I-A disaccharide with the iduronate residue in the $^1C_4$ chair conformation; and (iv) the I-A disaccharide with the iduronate residue in the $^2S_0$ twist-boat conformation.

proportions of $^1C_4$ and $^2S_0$ forms of the iduronate ring present in the polysaccharides. The level of agreement between experimental and predicted data, for the two previously described polysaccharides as well as the three which are the main subject of this study, is satisfactory. Quantitative criteria of the quality of fit of calculated to experimental data are complex to define, as alteration of glycosidic bond angles affects both interresidue proton-proton distances and the orientation of H–H and C–H vectors in the symmetric top model for the whole of the molecule; so the linkages cannot be varied independently. The dependence of proton–proton NOEs and ^{13}C R_1s on orientation in the symmetric top model for these compounds has previously been discussed. These considerations represent extra constraints which must be satisfied in addition to the necessity for short proton–proton distances for observed NOEs.

DISCUSSION

Two representative dodecasaccharide molecular models, of **6** (heparin, with three sulphate groups per disaccharide) and **8** (*O*- and *N*-desulphated, re-*N*-

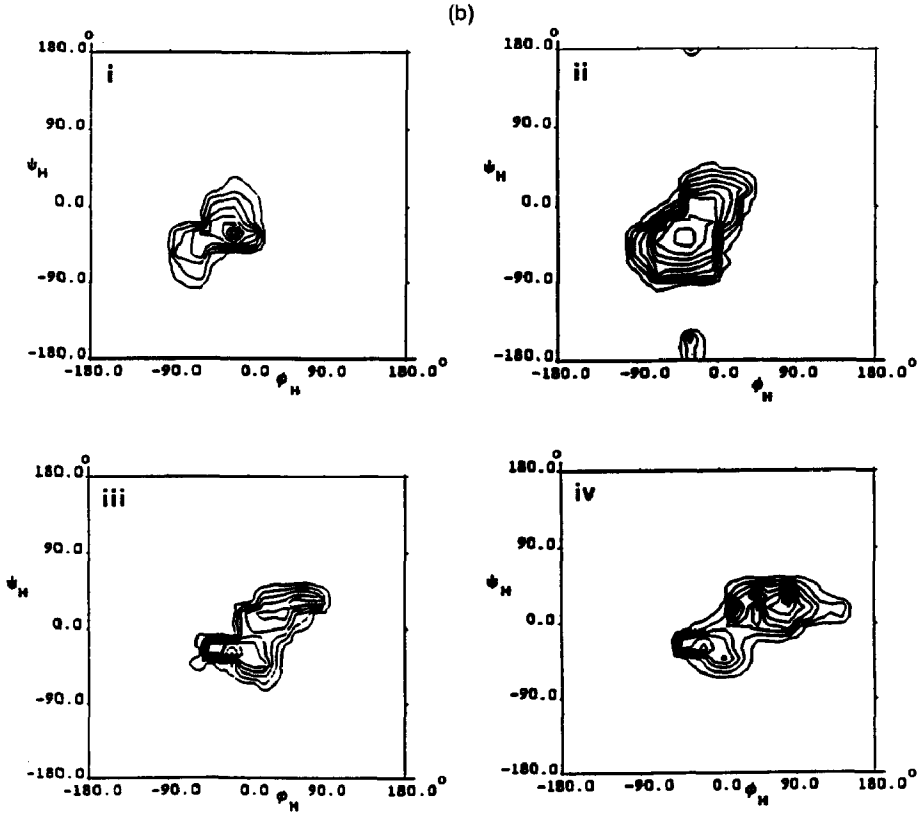


Fig. 3. (continued).

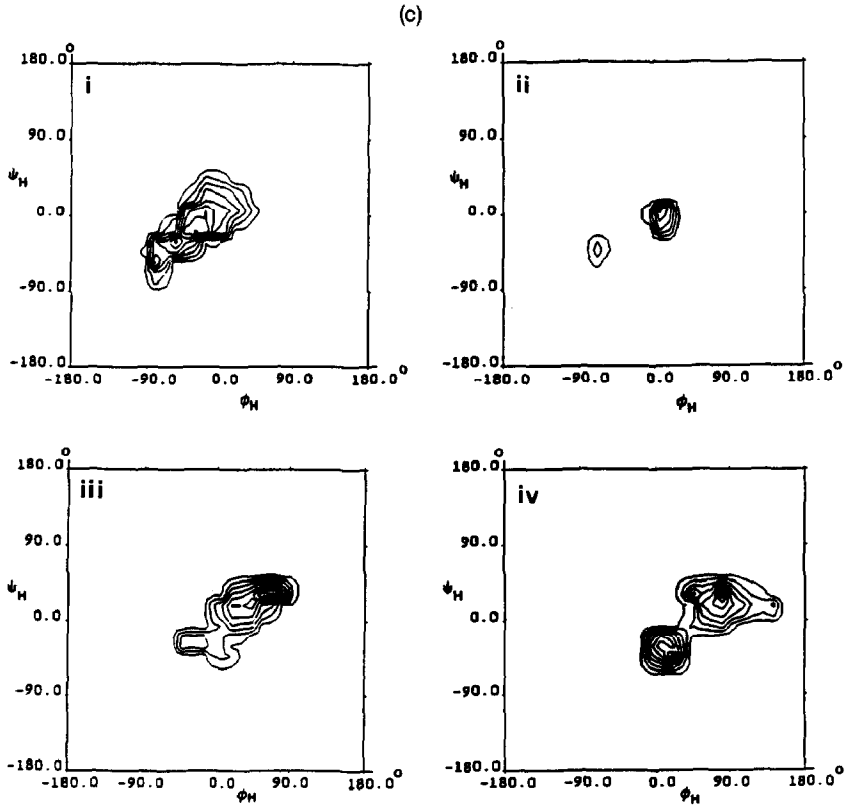


Fig. 3. (continued).

acetylated heparin, with no sulphate substitution at all) are illustrated in Fig. 4a with their iduronate residues in the $^1C_4$ form and in Fig. 4b (with the exception of **7**) with the iduronate residues in the $^2S_0$ form. All the models are linear helices like those illustrated. These models are not intended to represent real molecular species; the chain length of the samples in this study is mainly in the range 10–100 residues, and the IdoA ring forms will presumably be distributed at random along the chain. The consequences of the polydispersity of the samples for analysis of relaxation data has previously been discussed[2].

The conformations of the polysaccharides are similar to each other, as the similarities in their glycosidic torsional angles would imply (Table VII). As has already been discussed[2] for **6** and **7**, the overall length of each dodecasaccharide is near 50 Å, implying a rise per disaccharide of ~ 8.3 Å, close to the value determined by Nieduszynski et al.[1] for heparin in the fibrous state. The pitch of the helices is ~ 17 Å, corresponding to ~ 4 residues per turn.

Though the uncertainties surrounding this interpretation are as valid for **8–10** as discussed for **6** and **7** in ref 2, the data are consistent with the existence of these compounds in solution in a well-defined conformation close to those shown in Fig.

4. There is little effect of varying substitution on the overall molecular shape; the presence of bulky, charged sulphate substituents has little effect on the glycosidic linkage conformations, as demonstrated by the similar conformation of the totally desulphated **8** to the conformation of the trisulphated disaccharide of heparin (**6**). The disposition of the sulphate groups on the polysaccharides tends to form a linear array on opposite sides of the helix, as can be seen for **6** in Figs. 4a and b. This is true of all the sulphated polysaccharides; where the number of disaccharides per turn is not an exact integer the lines of sulphate groups slowly curl round the helix.

In heparin and all the modified heparins other than **7**, our data is consistent with a mixture of $^1C_4$ and $^2S_0$ ring forms for the IdoA pyranose ring. The precise way in which the $^1C_4$ ring form for IdoA in **7** is stabilized cannot be deduced from

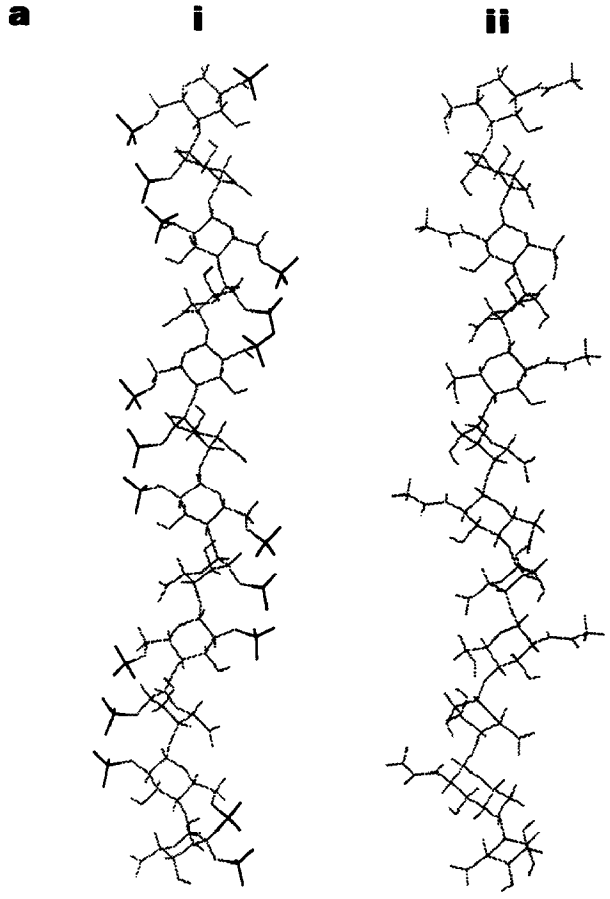


Fig. 4. Molecular models with the iduronate residue in (a) the $^1C_4$ chair and (b) the $^2S_0$ twist-boat, of the main repeating unit of (i) heparin (**6**) and of (ii) *O*- and *N*- desulphated, re-*N*-acetylated heparin (**8**). Positions of the sulphate groups are emphasised by bold lines. The conformations which gave best agreement with observed NMR data are similar for the heavily sulphated **6** and the totally unsulphated **8**.

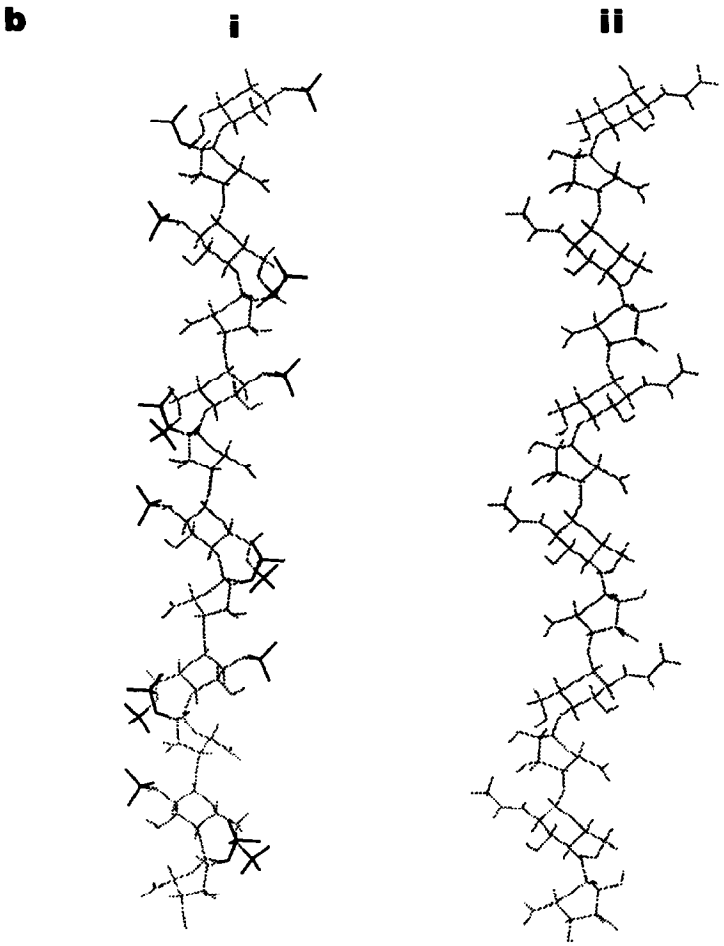


Fig. 4. (continued).

our data. van Boeckel et al.[24] have shown that both the 2-*O*-sulphate group and acetylation of the GlcN residue on the nonreducing side of an IdoA residue are needed to maintain the IdoA ring form almost entirely in the $^1C_4$ form. In our molecular model (Fig. 4a) O-2 of IdoA is less than 4 Å from N-2 of the residue attached at C-4, so some form of direct interaction between the *N*-acetyl and *O*-sulphate groups is possible.

Sulphation and glycosidation shifts in the ^1H and ^{13}C spectra. Comparison of mono- and poly-saccharides.—In the spectra of the glucosamine monosaccharides, sulphation at the 6-position causes a downfield shift of ~0.45 ppm to H-6 and 0.2 ppm for H-5 in both the *N*-acetylated (**1** and **2**) and *N*-sulphated (**3** and **4**) cases. The $^3J_{H5,H6proR}$ and $^3J_{H5,H6proS}$ values for the 6-*O*-sulphated monosaccharides are small and approximately equal, indicating that rotation around the C-5–C-6 bond is hindered with the *g*,*g* rotamer heavily favoured; the same phenomenon has been described for heparin[22]. It is not possible to make the same comparison with

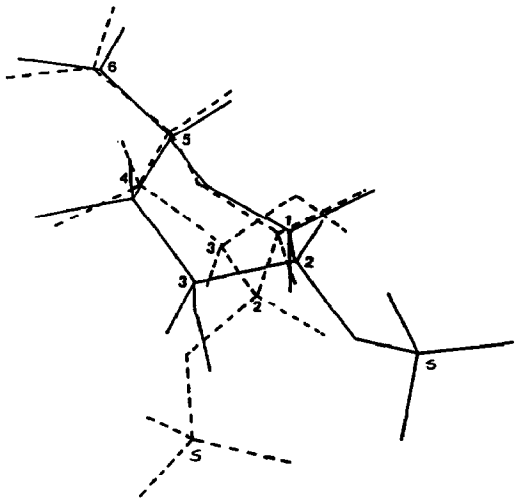


Fig. 5. The $^1C_4$ chair and $^2S_0$ twist-boat forms of the iduronate residue in heparin, overlaid with the C-1–O-1 and C-4–O-4 bonds matched as closely as possible, to show the changes in the positions of ring carbons and substituents: (———) $^2S_0$ and (- - - - -) $^1C_4$.

the *N*-acetylated compounds **1** and **2** as H-6 signals overlap in their spectra. In the *N*-acetylated polysaccharide **8**, the H-6 signals are separated due to interactions with neighbouring residues, and for this compound the difference between $^3J_{H5,H6proR}$ and $^3J_{H5,H6proS}$ is a little greater, thought not as much so as for the typical glucopyranose, in which an equilibrium between *g*,*g* and *g*,*t* rotamers gives $^3J_{H5,H6proS}$ 2 Hz and $^3J_{H5,H6proR} > 5$ Hz[13]. Sulphation shifts for H-6 for the polysaccharides are similar to those for the monosaccharides, though smaller (< 0.1 ppm) for H5.

The differences in chemical shift of H-2 between the *N*-acetylated and *N*-sulphated compounds is substantial, as expected; the deshielding effect of the acetamido group takes the H-2 signal 0.70–0.75 ppm downfield of its position in the *N*-sulphated compounds.

Changes in chemical shift due to incorporation in the polymer occur at the linkage positions (H-1 and H-4), showing downfield glycosylation shifts; and at H-6 of the glucosamine residues of the *N*-acetylated polymers **8**–**10**.

Iduronate ring conformation: ^{13}C NMR.—The ring conformation of the iduronate residues can change between the $^1C_4$ chair and $^2S_0$ twist-boat forms without any substantial alteration of the glycosidic linkage conformations (Fig. 5)[23]. The existence of an equilibrium between these ring forms in internal residues in oligosaccharides has been deduced from $^3J_{H,H}$ values[22–24]. A molecular dynamics study of the iduronate ring has introduced the possibility that the apparent $^2S_0$ twist-boat conformation may be a rapidly interconverting ensemble of boat and twist-boat forms[25]; for the sake of simplicity we have used only the $^2S_0$ form in this study. Linewidths in the ^1H spectra of the polysaccharide compounds **6**–**10** are too great to determine the small coupling constants typical of the iduronate ring with

sufficient accuracy to derive conformational information. On the other hand, ^{13}C chemical shifts can be measured and can be correlated approximately with the proportions of chair and twist boat; an upfield shift has been found to indicate a high proportion of $^1C_4$ chair in a series of heparin-like oligosaccharides[24]. Comparing ^{13}C chemical shifts of the iduronate ring carbons of **6–10** (Table III), it is clear that C-3 and C-4 of **7** resonate at higher field than C-3 and C-4 of the other polysaccharides, by ~ 6 ppm. This indicates that the contribution of the twist-boat form to the conformational equilibrium of the iduronate ring in this compound is small, as predicted for a 2-*O*-sulphated IdoA glycosylated at C4 with GlcNAc[24]. For the other modified heparins (**8–10**), ^{13}C chemical shifts of iduronate C-3 and C-4 differ only by 1–2 ppm from the equivalent values for heparin (**6**), indicating $^2S_0$ proportions of "up to 40%"[21].

Variation of ^{13}C chemical shift with temperature.—If variations in ^{13}C chemical shift for the iduronate residues of the different polysaccharides (and in the monomer) reflect different conformational equilibria, the chemical shifts of the resonances affected should alter with changes in temperature; with an increase in temperature the population of a conformation of higher energy should increase according to a Boltzmann distribution. The data shown in Table IV bear out this supposition, and show that temperature dependence of ^{13}C chemical shift can be a sensitive indicator of the existence of a dynamic equilibrium between two or more different conformations.

Comparison of the temperature coefficients for the glucosamine residues as monomers (**1–4**) and in the polysaccharides shows that temperature coefficients for C-1 and C-4 are greater in the polysaccharides than in the monosaccharides, indicating that the environment of a carbon atom at a linkage position changes more with temperature than that of the same atom in the monosaccharide. For the other glucosamine ring carbons, C-2, C-3, and C-5, the temperature coefficients are on the whole smaller in the polysaccharide, even showing some very slight upfield shifts.

Temperature coefficients for the ring carbons of α-D-IdoA-OMe are, with the exception of C-1, higher than for the glucosamine residues, reflecting the equilibrium between chair and twist-boat ring forms for this compound[23]. For the iduronate residue in the polysaccharides, the temperature coefficients are similar to those for the monosaccharide, with one exception; for C-3 and C-4 of 2-*O*-sulphated iduronate in **7**, temperature coefficients are more than twice as large as in the monosaccharide **5**. According to the arguments outlined above, the conformational equilibrium of the iduronate ring of **7** should contain only a small proportion of twist-boat (even at the high end of the temperature range); as that proportion rises with temperature the ^{13}C chemical shift changes markedly.

The rate of this interchange between ring forms is difficult to determine. ^{13}C R_1 values show no systematic differences between iduronate and glucosamine residues such as might be expected if the rate of conformational interconversion was on the order of, or more rapid than, overall molecular rotation ($\tau_\perp$ 8 ns). Molecular

dynamics calculations also predict that the conversion between chair and twist-boat ring forms is not rapid compared to overall molecular tumbling (during a number of 200 ps MD runs conversion between chair and twist-boat forms occurred infrequently), but does predict some rapid interconversion between boat and twist-boat forms[25] for which the present study offers no experimental support. At the other end of the timescale, the interchange is rapid enough to average ^{13}C and ^{1}H chemical shifts, so must be faster than 10^4 Hz. Rates of interchange between these two extremes are difficult to probe using NMR techniques. However, it seems clear that ^{13}C chemical shift temperature coefficients can distinguish between molecules, or even parts of the same macromolecule, with different degrees of flexibility.

Circular dichroism spectroscopy.—The CD spectra of polysaccharides are determined by the nature and conformation of the constituent monosaccharide residues and may be influenced by interresidue interactions[26]. Analysis can be undertaken in two ways. A spectrum can be treated as a linear algebraic sum of CD components associated with repeating units or conformational states (cf. protein secondary structure analysis[27]). Alternatively, a more analytical approach can be used to relate CD features to specific spectroscopic transitions. If the CD spectrum of a polysaccharide is the simple sum of the CD spectra of its constituent monosaccharides, it is reasonable to assume that there is no interaction between chromophores on neighbouring residues, and no changes in the conformation of individual residues. If this summation does not hold, either the chromophores are interacting or the conformation of the monomers in the polymer is different from that in free solution. In the 200–230 nm region, the $n \rightarrow \pi^*$ transitions are weak (low electric dipole moment) and do not couple strongly; CD changes associated with these bands are the direct result of local conformational changes. CD below 200 nm originates from transitions in the polysaccharide framework, including[16] $n \rightarrow \pi^{**}$ contributions sensitive to the glycosidic torsional angles ϕ_H and Ψ_H, that are not present in the monosaccharides. In the main, these short wavelength effects are outside the range of measurements made in this study. The CD reported here cannot be interpreted as the simple sum of the monosaccharide spectra. Summation of the published CD spectra of α-D-GlcNAc-OMe[28] with α-L-IdoA-OMe (the inverse of Fig. 2) gives a result which differs from the CD spectra of **7** or **8**, implying that local conformations (changes in ring pucker) are sensed by the 200–230 nm $n \rightarrow \pi^*$ transitions. NMR evidence indicates that the α-D-IdoA-OMe ring exists as a mixture of $^4C_1$ and $^1C_4$ chairs, with a smaller amount of twist-boat conformation[25], and the two carboxylate environments identified in the CD spectrum may reflect this conformational heterogeneity. The iduronate ring conformation in **6**, **9**, and **10** is shifted in favour of a mixture of $^1C_4$ and twist-boat forms. This loss of $^4C_1$ contribution corresponds to the loss of the shorter wavelength, $n \rightarrow \pi^*$, positive CD signal in the spectrum of α-L-IdoA-OMe. The $^3J_{H,H}$ data indicate that the *N*-acetylated glucosamine adopts the $^4C_1$ chair conformation in both the monosaccharides **1** and **2** and in the polysaccharides **7**

and **8**. The amide contributions to the CD spectra of **7** and **8** should therefore be similar. The increased proportion of the $^1C_4$ chair form of the iduronate residues in **7** can now be related to positive CD at 210 nm, which cancels out a negative CD associated with the unchanging amide $n \rightarrow \pi^*$ circular dichroism present in **7** and **8**. The predominant negative carboxylate CD in **8** must therefore correlate with the twist-boat form.

Combining the NMR and CD results leads to the conclusion that a $^1C_4$ ring conformation confers a positive CD on the carboxylate $n \rightarrow \pi^*$ transition; a $^4C_1$ ring conformation confers a negative sign, as does the twist boat form of the iduronate ring.

CONCLUSIONS

1. The conformation of those parts of the heparin polysaccharide chain consisting of alternating glucosamine and iduronate residues is well defined. The degree and position of *O*-sulphation (or replacement of *N*-sulphation by *N*-acetylation to give sequences not found in native heparin), affect the conformation round the glycosidic linkages only to a small extent.

2. The iduronate residues in such sequences may adopt either the $^1C_4$ chair or the twist-boat conformations without causing major changes to the conformations of the glycosidic linkages. ^{13}C Chemical shifts are consistent with a contribution from both forms for all but one of the modified heparins studied. For *N*-desulphated re-*N*-acetylated heparin (**7**), ^{13}C chemical shifts are consistent with a predominantly $^1C_4$ conformation.

3. Changes of ^{13}C chemical shift with temperature are a sensitive monitor of conformational flexibility. The chemical shifts of signals from ring carbons of glucosamine residues with stable ring conformations have low temperature coefficients, unless they are at linkage positions where the environment of the carbon atom is affected by changes in the conformational equilibrium at the glycosidic linkage. For iduronate residues the chemical shifts of signals from ring carbons have high temperature coefficients. An increase in the proportion of the twist-boat form at higher temperatures is indicated by a downfield change of the chemical shift; this is most marked for *N*-desulphated re-*N*-acetylated heparin (**7**), for which the twist-boat contribution is the smallest.

4. The rate of interchange of conformations of the iduronate ring is not sufficently rapid to affect ^{13}C R_1s, but is rapid enough to average ^{13}C chemical shifts, and ^1H shifts and coupling constants; perhaps in the range 10^4–10^7 Hz.

5. The circular dichroism spectra of the iduronate-containing polysaccharides are strongly affected by the conformation of the iduronate ring. The CD spectrum of **7** indicates that the $^1C_4$ form has a positive band at $\sim$ 210 nm strong enough to cancel out the negative contribution at that wavelength from GlcNAc. The $^4C_1$ and twist-boat forms induce negative CD at this wavelength.

6. The results of this study can be used to predict the conformation of most of the iduronate-containing sequences found in native heterogeneous heparin and heparan sulphate, though not the specific antithrombin binding pentasaccharide which has been the subject of study elsewhere[29]. Descriptions of heparin interactions with proteins at the molecular level are slow to come forward, but as the structures of more heparin-binding proteins are determined it may become possible to describe such phenomena in detail using molecular models of the type described here.

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