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α-L-Iduronate Ring Conformations in Heparin and Heparin Derivatives

^{13}C NUCLEAR-MAGNETIC-RESONANCE ANALYSES AND TITRATION DATA FOR VARIOUSLY DESULPHATED AND PERIODATE-OXIDIZED HEPARINS

By LARS-ÅKE FRANSSON,*† THOMAS N. HUCKERBY‡ and IAN A. NIEDUSZYNSKI*
**Department of Biological Sciences, University of Lancaster, Bailrigg, Lancaster LA1 4YQ, U.K., and ‡Department of Chemistry, University of Lancaster, Bailrigg, Lancaster LA1 4YA, U.K.*

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A heparin derivative that had been *O*/*N*-desulphated and re-*N*-acetylated was investigated by ^{13}C n.m.r. spectroscopy and potentiometric titration. Three forms of uronic acid were observed, tentatively identified as β-D-glucuronate, and two different forms of α-L-iduronate. A comparison of the n.m.r. spectra of heparin, an oligosaccharide (β-D-glucuronate-2-acetamido-2-deoxy-α-D-glucose)$_n$, and heparin that had been subjected to selective oxidation of β-D-glucuronate, enabled the position of the anomeric carbon of the latter residue to be assigned [δ 102.9 (p.p.m.)]. Periodate oxidation of *O*/*N*-desulphated heparin destroyed in addition, approx. 40% of the α-L-iduronate content. The remainder of the α-L-iduronate residues displayed only one anomeric resonance, at δ 99.7 (p.p.m.). In another preparation, after sequential desulphation of heparin (*N*-desulphation, re-*N*-acetylation and *O*-desulphation) the anomeric resonance of the α-L-iduronate residue shifted downfield [from δ 99.7 (p.p.m.) to δ 102.3] indicating a change in ring conformation. These data support the interpretation that the unsulphated α-L-iduronate residues may adopt two conformations. It was shown that the proportions of α-L-iduronate conformers are determined by the sequence of desulphation operations. Also minor components of heparin were assigned.

The various glycosaminoglycuronans that are largely found in connective tissues are composed of alternating hexuronic acid and *N*-acetylhexosamine residues. The unusual sugar residue L-iduronic acid (IdUA) is found in heparin, heparan sulphate and dermatan sulphate. Heparin is stored in mast-cell granules (for a review see Lindahl, 1976), heparan sulphate seems to be a ubiquitous component of cell surfaces and, in addition, a matrix constituent of many tissues (see Kraemer, 1977), whereas dermatan sulphate is chiefly an extracellular component of fibrous connective tissues (see Fransson, 1976). Heparin is endowed with unique biological properties. It inhibits blood coagulation principally by accelerating the reaction between antithrombin and thrombin (Damus *et al.*, 1973). Moreover, heparin interacts with the extracellular receptors of lipoprotein lipase resulting in release of this enzyme into the circulating blood (Olivecrona *et al.*, 1977). Affinity for lipoprotein lipase is also displayed by heparan sulphate and dermatan sulphate (Olivecrona *et al.*, 1977). Similarly, all three IdUA-containing glycans interact strongly with serum lipoproteins (Iverius, 1972).

The shapes, molecular interactions and functions of the IdUA-containing glycans depend on the conformation of this unique sugar residue. The two most commonly considered conformations for α-L-iduronate are the $^4C_1$ [Reeves C1, see also Carbohydrate Nomenclature Committee (1973)], which disposes the carboxy group axially, and the two linkage bonds C-1–O and C-4–O equatorially, and the $^1C_4$ (Reeves 1C), which conversely sets the carboxy group equatorially and the two linkage bonds axially. These two conformations dictate very different macromolecular shapes, the former ($^4C_1$) giving rise to an 'extended' conformation and the latter ($^1C_4$) to a 'crimped' one. With such a conformationally versatile residue the fascinating possibility exists that an environmental or biosynthetic mechanism might control macromolecular conformation.

In heparin the α-L-iduronate residue is generally sulphated in the 2-position (Lindahl, 1976) and the interpretation of both the n.m.r. data of Perlin *et al.*

Abbreviations used: IdUA, α-L-iduronic acid; GlcUA, β-D-glucuronic acid; $GlcNH_3^+$, 2-amino-2-deoxy-α-D-glucose; GlcNAc, 2-acetamido-2-deoxy-α-D-glucose; $GlcNSO_3$, 2-sulphamido-2-deoxy-α-D-glucose; GlcN, α-D-glucosamine with unspecified 2-substituent; $-OSO_3$, ester sulphate group; aMan, 2,5-anhydro-D-mannose.

†Permanent address: Department of Physiological Chemistry, University of Lund, P.O. Box 750, S-220 07 Lund 7, Sweden.

(1970, 1972) and the X-ray fibre diffraction data of Nieduszynski & Atkins (1973) has favoured the $^1C_4$ over the $^4C_1$ chair conformer. However, other skew-boat conformations have been considered by Nieduszynski *et al.* (1977) and cannot readily be rejected.

In dermatan sulphate, where most α-L-iduronate residues are non-sulphated, the X-ray studies of Atkins & Isaac (1973) and Arnott *et al.* (1973) strongly favour the $^4C_1$ chair conformation. This is also supported by the marked sensitivity of this residue to periodate oxidation (Fransson, 1974). However, the n.m.r. data of Perlin *et al.* (1970) and Hamer & Perlin (1977) have been interpreted in terms of the $^1C_4$ chair conformation for α-L-iduronate.

Heparan sulphate embraces a wide family of molecules with differing IdUA/GlcUA ratios and sulphate contents (Lindahl, 1976). Although X-ray and n.m.r. studies have been performed (Perlin, 1974; Atkins & Nieduszynski, 1976, 1977; Elloway & Atkins, 1977) on this polymer no information on the α-L-iduronate conformation is available.

In the present study the conformations of the α-L-iduronate residues in desulphated heparins have been examined by ^{13}C n.m.r. spectroscopy, potentiometric titration and periodate oxidation methods.

Experimental

Materials

Heparin, batch number 5H 488 and of anticoagulant activity 157units/mg, was supplied by Dr. H. G. Hind of Evans Biologicals, Runcorn, Cheshire, U.K.

Another heparin preparation (Inolex, Glenwood, IL, U.S.A.; sulphate/hexosamine molar ratio 2.56; anticoagulant activity 125units/mg) and an *O*/*N*-desulphated and re-*N*-acetylated derivative thereof [sulphate/hexosamine molar ratio, 0.31; desulphation carried out by the method of Kantor & Schubert (1957)] were generously given by Dr. L. Rodén, University of Alabama, Birmingham, AL, U.S.A. Heparan sulphate from pig mucosa was obtained from Dr. J. A. Cifonelli, University of Chicago, Chicago, IL, U.S.A.

Analytical methods

Free hexuronic acids were released from heparin and derivatives thereof by the procedure of Höök *et al.* (1974), which involves acid hydrolysis, deaminative cleavage and repeated acid hydrolysis. Uronic acids (IdUA and GlcUA) were then separated by ion-exchange chromatography (Fransson *et al.*, 1968; Fransson, 1978). Sulphate was determined by the method of Terho & Hartiala (1971).

Degradation methods

Deaminative cleavage of heparan sulphate and heparin (500mg of each) was performed with the low pH HNO_2 method of Shively & Conrad (1976). After evaporation of excess HNO_2 with methanol, degradation products were fractionated by gel chromatography on a column of Sephadex G-25 superfine (size, 30mm×1350mm; eluent, 0.2M-pyridine/sodium acetate, pH5.0; elution rate, 15ml/h). The effluent was analysed for uronic acid by the orcinol method (Brown, 1946) and appropriate fractions were pooled and freeze-dried. (The void volume fraction, which contained octasaccharides or higher was eluted between 450 and 500ml, whereas disaccharides emerged between 750 and 950ml.) Disaccharides were subsequently resolved into non-sulphated, mono-sulphated and di-sulphated species by ion-exchange chromatography on a column (15mm×300mm) of DE-52 DEAE-cellulose (equilibrated with 0.1M-sodium acetate, pH5.0), which was eluted with a linear gradient (0.1–2.0M-sodium acetate, pH5.0; total elution volume, 500ml) at a rate of 20ml/h. The three disaccharide species (V_e = 15–17ml and 22–30ml respectively) were finally desalted by chromatography on a Sephadex G-15 column (30mm×400mm; eluent, aq. 10% ethanol; elution rate, 20ml/h; analysis, orcinol) and freeze-dried. The ion-exchange chromatography technique was also used to isolate desulphated disaccharides of heparin (see below).

Periodate oxidation of heparin and heparin derivatives was carried out as follows. Solutions of polymers (2mg/ml) in 20mM-sodium metaperiodate in 50mM-sodium citrate buffers (pH3.0) or 50mM-sodium phosphate buffer (pH7.0) were incubated at 4 or 37°C in the dark for 0–24h. In the case of heparin, oxidations were performed in 0.2M-sodium perchlorate to minimize periodate–polyanion repulsion (Scott, 1968). Samples were treated with a molar excess of D-mannitol and analysed for uronic acid by the Dische (1949) carbazole method. For preparative-scale oxidations the terminated reaction mixtures were dialysed and freeze-dried or desalted on Sephadex G-15 (see above). Oxyglycans were degraded in alkaline solution (adjusted to pH12 with 2M-NaOH; 5mg/ml) at room temperature for 30min. The degradation products were re-isolated by gel chromatography on Sephadex G-15 (see above) or Sephadex G-25 superfine (column size 12mm×1800 mm) with 0.2M-pyridine acetate, pH5.0, as eluent at an elution rate of 10ml/h. The effluent was analysed by the orcinol method. Further details of periodate oxidation are given by Fransson (1978).

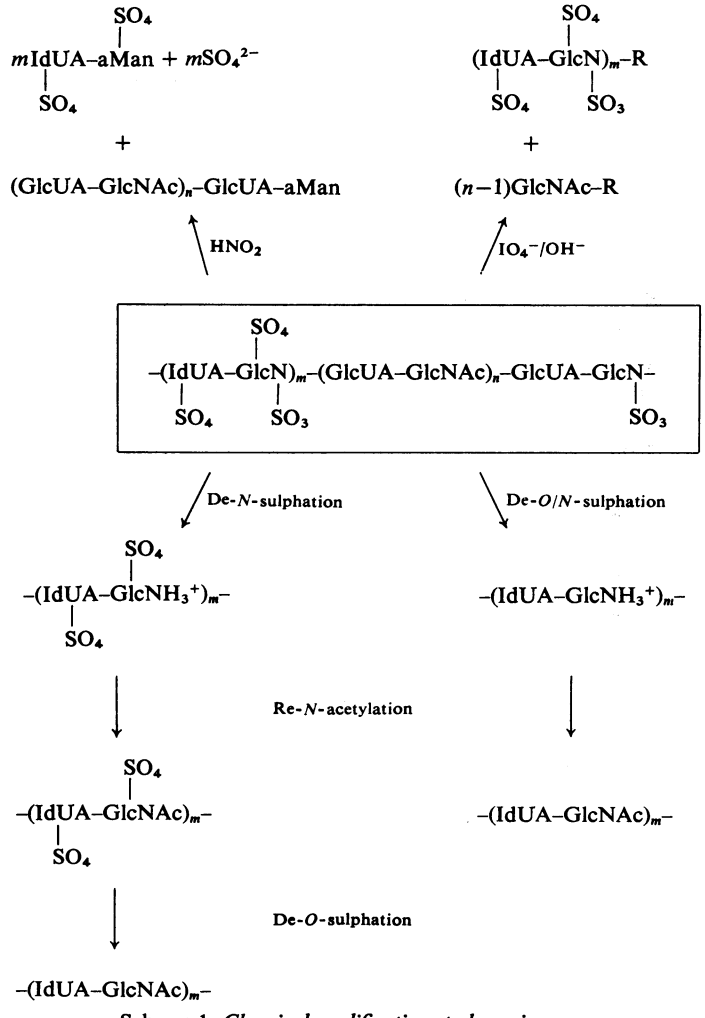
Modification methods

Solvolytic *N*-desulphation of heparin was carried out by treatment of its pyridinium salt with dimethyl sulphoxide containing 5% methanol for 1.5h at 50°C as described by Inoue & Nagasawa (1976). *O*/*N*-Desulphation was obtained by treating heparin

or *N*-desulphated, re-*N*-acetylated heparin (pyridinium salts) with dimethyl sulphoxide containing 10% methanol for 2h at 100°C (Nagasawa *et al.*, 1977; Kamata, 1977). The latter method was also used to desulphate disaccharides of heparin (produced by deaminative cleavage and isolated as described above). Re-*N*-acetylations were carried out with acetic anhydride (ice-cold; buffered with 10% $NaHCO_3$) as described by Levvy & McAllan (1959). Re-*N*-sulphation was carried out by treatment with trimethylamine/sulphur trioxide (Lloyd *et al.*, 1971). The various procedures are outlined in Scheme 1.

Potentiometric titration

Samples (approx. 60mg) were dissolved in 5ml of 0.15M-NaCl and dialysed against the solvent, where applicable, to achieve Donnan equilibrium. Samples (4.5ml) of the solution were titrated in a cell thermostatted to 25.0°C. The pH-meter Beckman model 4500 (Beckman, Irvine, CA, U.S.A.) and electrode (Beckman combination 539504) were calibrated at pH1.10 with 0.10M-HCl (BDH Chemicals, Poole, Dorset, U.K.), pH4.00 and 7.00 with Soloid buffer tablets (Burroughs Wellcome and Co., London, U.K.) and pH drift over the course of the experiment was <0.01 pH unit. Additions of the 2M-HCl and 2M-NaOH (BDH) for back titration were made with Beckman titrators (Beckman) capable of accurately dispensing 0.01 μl of solution. For each experiment the sample was titrated from pH7.0 to pH1.70 and then back to pH7.0 and a solvent blank was performed.



Scheme 1. *Chemical modifications to heparin*

The data are presented as the derivatives of the volume of acid added with respect to pH against pH. Data analysis was carried out with a du-Pont 310 (E. I. du Pont de Nemours and Co. Inc., Wilmington, DE, U.S.A.) curve analyser by using curves of Gaussian profile.

Carbon nuclear-magnetic-resonance spectroscopy

Carbon n.m.r. spectra were determined at 20 MHz with a Varian CFT-20 Fourier-transform spectrometer. The spectra, which were normally of 4000 Hz (200 p.p.m.) spectral width, were accumulated in 4k (4096) data points with acquisition times of 0.512 s. Carbon-relaxation times for CH residues were about 50 ms, and thus 90° pulses could be routinely used. For proton-coupled spectra a gated decoupling technique was used, so that the nuclear Overhauser enhancement could be retained.

In a typical experiment, polysaccharide (about 100 mg) was dissolved in ^2H_2O (0.8–1.0 ml), with adjustment of solution pH where necessary, and placed in a 10 mm outside diameter sample tube together with a vortex suppressor. After acquisition of the free induction-decay patterns from between 1×10^5 and 5×10^5 pulses, the data were exponentially weighted to decrease noise, and Fourier-transformed. Chemical shifts were determined relative to tetramethylsilane by using a secondary referencing procedure as follows: the pulse position relative to dilute dioxan in ^2H_2O was accurately known and invariant over a period of 2 years, thus the pulse position relative to tetramethylsilane was calculated by using the known chemical shift for dioxan.

Chemical shifts (quoted throughout with units of p.p.m.) have probable errors of ±0.1 p.p.m., and coupling constants are considered accurate to not more than ±2 Hz. Signal positions, particularly for the hexosamine anomeric carbons, are sensitive to solution pH; spectra were run at pH values of about 7–8 where the changes are minimal.

Results

The Innolex heparin, which had an IdUA/GlcUA molar ratio of 3:1 (Table 1), yielded the n.m.r. spectrum shown in Fig. 1(*a*). The Evans heparin, which gave a closely similar n.m.r. spectrum (Table 2), on titration (Fig. 2*a*) demonstrated the presence of two components in a ratio of 3:1 with apparent carboxy pK_a values of 3.95 and 2.85 respectively. These components must correspond in mol proportion to sulphated IdUA and GlcUA. Spectra of heparin at higher field strengths (75 MHz) resolve each of the individual carbons in the heparin disaccharide repeat unit both in terms of chemical shift and relative proportions. Our peak assignments, which are in agreement with those of Perlin *et al.* (1972), are given in Table 2.

In this study especial emphasis has been placed on the separate anomeric resonances observed for uronic acids and hexosamines. Other significant resonances include those of hexosamine C-6 with or without sulphate and hexosamine C-2 bearing either *N*-sulphate or *N*-acetyl groups. All data were analysed by the hypothesis that anomeric resonance positions would be sensitive both to the constituents of their own residue and to the structure of the residue across the adjacent glycosidic linkage.

Totally desulphated (*O*/*N*) and re-*N*-acetylated heparin should, in principle, contain one type of amino sugar (GlcNAc) and two types of uronic acids (GlcUA and IdUA). It would be expected that the major influence on the chemical shift of the hexosamine anomeric carbon would arise from the configuration at C-5 of the uronic acid to which it is attached. Therefore, one would predict two anomeric resonances for the hexosamine residues of relative intensities 1:3. Since the uronic acids are both attached via C-1 to identical amino sugar residues they would also be expected to give two signals reflecting the uronic acid composition. In contrast with the prediction of four anomeric components the

Table 1. *Uronic acid composition and susceptibility to periodate oxidation of heparin and heparin derivatives*
Heparin (H) was totally desulphated by methanolysis, H(−*N*/*O*-sulphate), and subsequently either re-*N*-acetylated, H(−*N*/*O*-sulphate) (+*N*-Ac), or re-*N*-sulphated, H(−*N*/*O*-sulphate) (+*N*-sulphate). The uronic acid composition was determined by ion-exchange chromatography of free uronic acids released from the polymers by acid hydrolysis, deaminative cleavage and rehydrolysis. The extent of oxidation of uronic acids by periodate was monitored by carbazole measurements.

Preparation	IdUA/(IdUA+GlcUA) (%)	Oxidation of uronic acids (%)	
		At pH 3 and 4°C	At pH 7 and 37°C
H	75	20	30
H(−*N*/*O*-sulphate)	73	50*	90
H(−*N*/*O*-sulphate) (+*N*-Ac)	75	30	90
H(−*N*/*O*-sulphate) (+*N*-sulphate)	77	20	90

* Estimation of the uronic acid composition after periodate oxidation indicated approx. 40% destruction of IdUA.

Table 2. *^{13}C n.m.r. spectral parameters of heparin derivatives*

Part (*A*) shows the anomeric resonances for the heparin derivatives used in this investigation. Thus, in the example GlcUA → (GlcN) the chemical shift of C-1 in a glucuronic acid residue adjacent to that glucosamine that occurs across the glycosidic linkage involving the glucuronate C-1 is tabulated. Part (*B*) lists some of the non-anomeric carbon resonances and their assignments. Chemical shifts are expressed in p.p.m. with reference to tetramethylsilane. Abbreviations: H, heparin; HS, heparan sulphate; D and ND, dialysable and non-dialysable respectively; (−*N*-sulphate) and (−*N*/*O*-sulphate), de-*N*-sulphated and de-*N*/*O*-sulphated respectively; (+*N*-Ac) and (+*N*-sulphated), re-*N*-acetylated and re-*N*-sulphated respectively; P, periodate-oxidized (pH 3 and 4°C); A, alkali-treated; R, borohydride-reduced; HNO_2, subjected to deaminative cleavage; oligo, oligosaccharide; Di-diS and Di-0S, disulphated and non-sulphated disaccharide respectively; IdUA-I and IdUA-II, the original and new conformations of (non-sulphated) iduronate respectively. Notes: (*a*) This small component possibly represents the anomeric carbon of galactose, present in the linkage region of heparin. In the smaller, dialysable heparin it is proportionally larger. (*b*) This minor signal may represent a small proportion of glucuronate linked through C-1 to C-6 in a glucosamine residue (T. N. Huckerby & I. A. Nieduszynski, unpublished observations). (*c*) These two columns represent residues attached to IdUA-I and IdUA-II, but have not been individually assigned. (*d*) The signal ascribed to glucuronate in heparin is a broad envelope of resonances, separable by curve-resolver methods, more clearly defined in the smaller dialysable material and in 75 MHz spectra. The position δ 102.6 represents the top of the envelope; a shoulder (see Fig. 1) coincides with the more usual position for glucuronate. Possibly this signal contains a component from IdUA-II; non-sulphated IdUA is known to be present to a small extent in heparin. Small shoulders corresponding to $GlcNSO_3^-$ attached to non-sulphated IdUA may also be seen (approx. δ96–97). (*e*) This resonance is hidden under the larger $GlcNSO_3^- \rightarrow$ (IdUA-2-SO_4) component; curve resolver studies show that the latter is an envelope of several resonances. (*f*) These parameters are for material prepared by the method of Kantor & Schubert (1957). The Inoue & Nagasawa (1976) method produces a desulphated heparin of closely similar spectral appearance. (*g*) Unresolved shoulders; positions assigned by using a curve resolver. (*h*) Numbers in brackets are $^1J_{CH}$ coupling constants. (*i*) Broad and unresolved. (*j*) Unresolved doublet; the other component might possibly be $GlcNSO_3^- \rightarrow$ (GlcUA). (*k*) Next to a sulphated IdUA. (*l*) Small residual components. (*m*) This peak is diminished in magnitude compared with the same component in H (−*N*/*O*-sulphated) (+*N*-Ac). (*n*) This small component possibly represents the C-6 -CH_2OH resonance in galactose (see *a*). (*o*) Broad resonance. (*p*) Very small component. (*q*) Shoulder.

		GlcUA → (GlcN)	IdUA-II → (GlcN)		IdUA-I → (GlcN)	IdUA-2-SO_4 → (GlcN)	GlcN-SO_3^- → (IdUA-2-SO_4)	GlcNAc → (GlcUA)	$GlcNH_3^+$ → (GlcUA)	GlcNAc → (IdUA-II)	GlcNAc → (IdUA-I)	GlcN-SO_3^- → (IdUA)	GlcN-SO_3^- → (IdUA)	$GlcNH_3^+$ → (IdUA-II)	$GlcNH_3^+$ → (IdUA-I)
(*A*)	(*a*)			(*b*)								(*c*)	(*c*)		
H, ND (*h*)	104.3	102.6 (*d*)				99.6 (173)	97.7 (174)	(*e*)							
H, D	104.2_5	102.6 (*d*)				99.8	97.4	(*e*)							
H, (−*N*/*O*-sulphate) (*f*)	104.3	103.0 (*g*)	102.6	102.2_5 (*g*)	99.5				96.5 (*g*)					93.6	92.3_5
H, (−*N*/*O*-sulphate) (+*N*-Ac) (*h*)	104.2	102.9 (163) (*g*)	102.4_5 (165)	101.9 (165) (*g*)	99.9 (171)			97.6 (174)		95.1 (174)	94.5 (172)				
H, (−*N*/*O*-sulphate) (+*N*-sulphate)	104.5_5	102.9	102.5	101.9	99.9					94.9$_5$ (*i*)	94.9$_5$ (*i*)	97.7$_5$ or 97.5$_5$ (*j*)	96.2		
HS, HNO_2, oligo (*h*)		102.9 (163)		102.0				97.4 (175)							
H						99.9	98.1								
H, (−*N*-sulphate) (+*N*-Ac)		103.0				99.9		97.8			94.9 (*k*)				
H, P, R, (−*N*/*O*-sulphate), P, A, (+*N*-Ac)					99.7$_5$						94.4				
H, (−*N*/*O*-sulphate), P, A, (+*N*-Ac)		102.8 (*l*)	102.5 (*l*)		99.8			97.5 (*l*)			94.3				
H, (−*N*-sulphate) (+*N*-Ac), P, A, (−*O*-sulphate) (*h*)		102.9 (*l*)	102.3 (170)					97.6$_5$ (*l*)		95.2 (173)					
H, (−*N*-sulphate) (+*N*-Ac) (−*O*-sulphate)	104.4$_5$	102.7$_5$	102.2					97.5		95.1					
H, (−*N*-sulphate) (−*O*-sulphate) (+*N*-Ac)		102.8$_5$	102.3$_5$ (*m*)	102.0	99.9			97.5		95.0 (*m*)	94.3				
H, HNO_2, Di-diS						100.1									
H, HNO_2, Di-0S			102.2												

Table 2 *continued*

	Carbonyls	GlcN C-6-SO_4	(*n*)	GlcN C-6-OH	Hexosamine C-2 NSO_3^-	Hexosamine C-2 NAc	Hexosamine C-2 NH_3^+	Acetyl CH_3
(*B*)								
H, ND	175.5	67.2	61.7	60.5(*o*)	58.5	54.1		22.6
H, D	175.4	67.1	61.7	60.3	58.5	54.3		22.8
				60.8			55.0	
H, (−*N*/*O*-sulphate)	175.8 175.5	66.6(*p*)	61.5	60.0				
H, (−*N*/*O*-sulphate) (+*N*-Ac)	175.3		61.6(*q*)	60.3		54.3		22.6
H, (−*N*/*O*-sulphate) (+*N*-sulphate)	175.8 175.1		61.6	60.4	58.6_5	54.7(*o*)		22.7
HS, HNO_2, oligo	175.6 175.1			60.0		53.8_5		22.7
H, P	174.3	66.9		60.1	58.5	54.1		23.1(*p*)
H, (−*N*-sulphate) (+*N*-Ac)	175.6	67.3				54.3		23.0
H, P, R, (−*N*/*O*-sulphate), P, A, (+*N*-Ac)	175.5			60.4		54.4		22.9
H, (−*N*/*O*-sulphate), P, A, (+*N*-Ac)	175.4			60.5		54.3		22.9
H, (−*N*-sulphate) (+*N*-Ac), P, A, (−*O*-sulphate)	175.6 174.9		61.5	60.4		54.3		22.6
H, (−*N*-sulphate) (+*N*-Ac) (−*O*-sulphate)	175.6 175.0		61.6	60.4		54.5		22.7
H, (−*N*-sulphate) (−*O*-sulphate) (+*N*-Ac)	176.2 175.5 175.1		61.5	60.4		54.4		22.8
H, HNO_2, Di-diS								
H, HNO_2, Di-0S								

carbon spectrum of *O*/*N*-desulphated and re-*N*-acetylated heparin (Fig. 1*b*) showed six major (and two minor) anomeric components. Only the major anomeric resonances will be discussed here. Both desulphation methods (methanolysis and solvolytic desulphation) yielded the same result. Assignments of hexosamine and uronic acid components are facilitated by comparison of the spectra for heparin, *O*/*N*-desulphated heparin and *O*/*N*-desulphated and re-*N*-acetylated heparin (Table 2), since removal of *N*-sulphate causes upfield shifts of the hexosamine C-1 resonances. This chemical-shift change is decreased on subsequent re-*N*-acetylation, and after re-*N*-sulphation the signals move towards their original positions as seen in heparin.

The uronic acid anomeric carbons fall into two groups at δ 99.9 (one resonance) and δ 102–103 (two

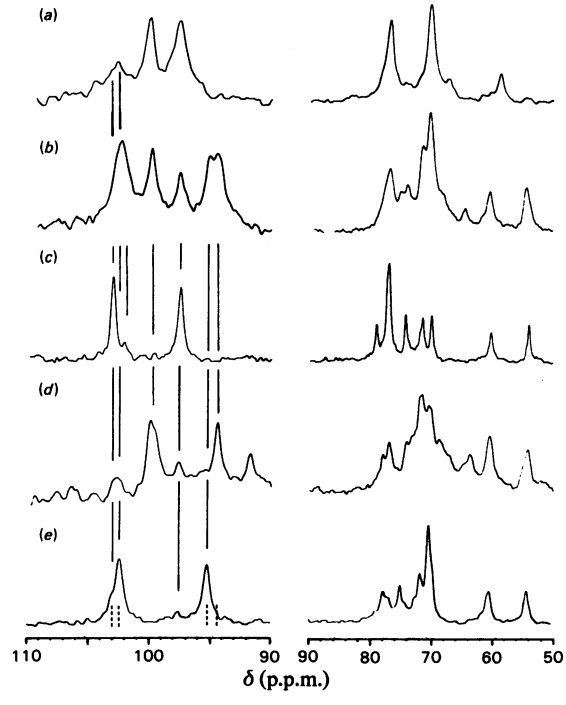


Fig. 1. *Carbon spectra for heparin and some modified heparins*

The left column illustrates the anomeric region (δ 90–110) and the right column shows resonances from the other ring carbons that fall in the range δ 50–90. The materials are as follows: (*a*) Innolex heparin; (*b*) *N*/*O*-desulphated re-*N*-acetylated heparin; (*c*) $(GlcUA–GlcNAc)_n$ oligosaccharide; (*d*) heparin, periodate cleaved at pH3, reduced, *N*/*O*-desulphated, again periodate cleaved at pH3, alkali-treated and re-*N*-acetylated; (*e*) heparin, de-*N*-sulphated, re-*N*-acetylated, and de-*O*-sulphated.

resonances). Examination of the spectrum (Fig. 1*c*) from a heparan sulphate oligosaccharide of principal sequence $(GlcUA–GlcNAc)_n$, sequences that are present in heparan, identifies the GlcUA anomeric carbon (δ 102.9) and its GlcNAc counterpart (δ 97.4). This was confirmed by selective periodate oxidation of GlcUA residues in heparin (see Scheme 1), which causes the anomeric resonances at δ 102–103 to disappear (Table 2).

Inspection of the mole proportions of IdUA/GlcUA in both the parent heparin and the desulphated heparins together with the assignment in the latter of the anomeric resonance at δ 102.9 to GlcUA makes it clear that the remaining uronic acid anomeric resonances both at δ 99.9 and δ 102.5 are ascribable to IdUA (IdUA-I and IdUA-II respectively). The possibility that either of these peaks is attributable to residual sulphate can be eliminated by examination of the degree of sulphation. In the *O*/*N*-desulphated heparins the desulphation of IdUA was virtually complete since only 10% of the uronic acid resisted periodate oxidation at pH7.0 and 37°C (Table 1) and this 10% of IdUA-OSO_3 is much less than the 30% of uronic acid material represented in either of the IdUA peaks, whether in n.m.r. or potentiometric titration. Similarly, there is n.m.r. evidence that the residual sulphate content of 0.31 sulphate groups per hexosamine should be divided between C-6 of the amino sugar residue (Fig. 1*b*, right) and C-2 of the IdUA moiety. Therefore, the proportion of residual sulphated IdUA *per se* is insufficient to account for any of the three uronic acid peaks observed in the n.m.r. or titration studies. Since the IdUA/GlcUA ratio was not appreciably altered (Table 1) one must conclude the desulphation of IdUA produces two conformational forms of this sugar residue. This was further supported by titration data (Fig. 2*b*). *O*/*N*-Desulphated and re-*N*-acetylated heparin had three distinct carboxy apparent pK_a values at 2.45, 3.3 and 4.2 respectively. The latter two values are ascribed to two forms of IdUA, designated IdUA-II and IdUA-I respectively.

It proved possible to isolate materials in which the proportion of either IdUA form could be enriched. Periodate oxidation at pH3.0 and 4°C of *O*/*N*-desulphated and re-*N*-acetylated heparin destroyed 30% of the uronic acid residues (Table 1). It has been shown elsewhere (Fransson, 1978) that GlcUA residues are oxidized under these conditions, whereas IdUA residues are resistant. However, the uronic acid content of *O*/*N*-desulphated heparin (containing a large proportion of amino sugars with unsubstituted amino groups) was markedly decreased (to about 50%) after periodate oxidation (Table 1). Accordingly, about 40% of the IdUA residues were destroyed under these conditions. The carbon n.m.r. spectrum of an *O*/*N*-desulphated heparin, which had been oxidized by periodate, treated with alkali and

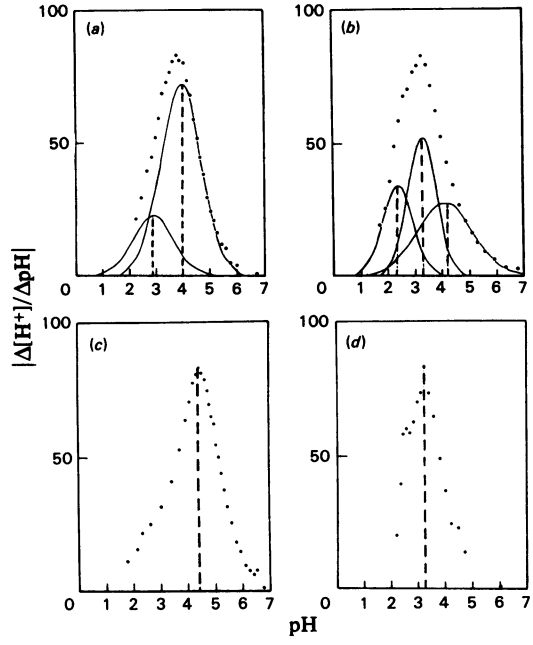
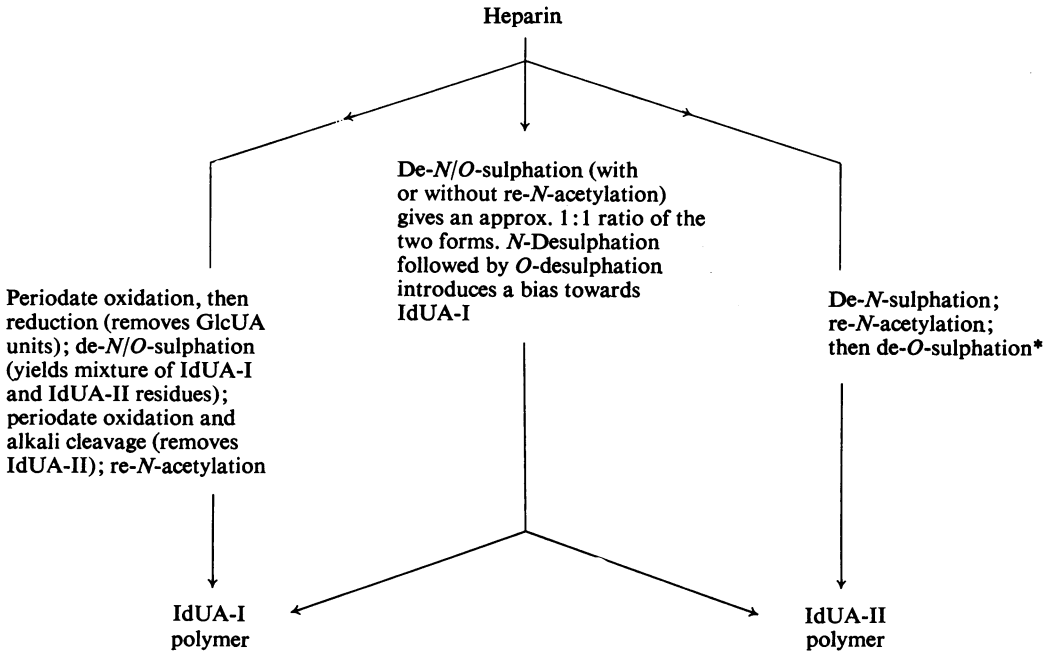


Fig. 2. *Titration data for heparin and some modified heparins*

Plots of change in volumes of acid added divided by change in pH (ordinate) against pH (abscissa). Filled circles are data points, solid lines enclose the areas representing proportions of individual classes of sites and broken lines indicate the mean apparent pK_a of each class of site. (*a*) Evans heparin; (*b*) *O*/*N*-desulphated, re-*N*-acetylated heparin; (*c*) *O*/*N*-desulphated heparin, oxidized by periodate, treated with alkali and subsequently re-*N*-acetylated; (*d*) *N*-desulphated heparin, re-*N*-acetylated and *O*-desulphated.

subsequently re-*N*-acetylated (Fig. 1*d*), contained only two major anomeric resonances (at δ 99.8 and δ 94.3 respectively) corresponding to IdUA-I and its associated GlcNAc (Table 2). Clearly, periodate oxidation of *O*/*N*-desulphated heparin selectively destroyed one (IdUA-II) of the two IdUA conformers (presumably a different form from that present in heparin as judged by the chemical shift) when the amino groups of adjacent glucosamine residues were unsubstituted. The fragments obtained after *O*/*N*-desulphation, periodate oxidation and alkaline cleavage were of tri- to hexa-saccharide size (gel chromatography on Sephadex G-25) showing that the periodate-susceptible IdUA conformer had been randomly distributed throughout the heparin chain. The sulphate/hexosamine molar ratio of this material after re-*N*-acetylation was 0.32. The apparent pK_a value of IdUA-I was 4.5 (Fig. 2*c*).



* By appropriate periodate oxidation the $(GlcUA–GlcNAc)_n$ component may be eliminated, leaving the isolated $(IdUA\text{-}II–GlcNAc)_n$ unit.

Scheme 2. *Sequential desulphation of heparin*

Complete desulphation of heparin may be achieved in a sequential mode, whereby *N*-desulphation is followed by *O*-desulphation. When heparin was *N*-desulphated, re-*N*-acetylated and then *O*-desulphated the spectrum (Fig. 1*e*) again contained only two major anomeric resonances, but now at positions δ 102.3 and δ 95.2 respectively. Clearly sequential desulphation has produced the other form of IdUA, IdUA-II. Accordingly, sequentially desulphated heparin had an apparent IdUA pK_a value of 3.25 (Fig. 2*d*), which was distinctly different from that of the other IdUA (4.5). This preparation of desulphated heparin had a sulphate/hexosamine molar ratio of 0.04. In another procedure, the IdUA-II form also appeared in *N*-desulphated and re-*N*-acetylated heparin that had been subjected to periodate oxidation of its GlcUA residues before the final *O*-desulphation (Table 2). Sequential *N*-desulphation and *O*-desulphation before re-*N*-acetylation favoured a retention of the original conformation (IdUA-I; Table 2). A summary of these results is given in Scheme 2.

Attempts to change the relative proportions of the two IdUA conformers in *O*/*N*-desulphated and re-*N*-acetylated heparin by lowering the pH to 2, raising it to 11.5, or by dissolving the material in 4M-guanidinium chloride were unsuccessful.

In addition a disulphated disaccharide obtained from heparin by deaminative cleavage was shown to have only one anomeric component at δ 101.1 (Table 2). Upon desulphation this component shifted downfield to δ 102.2, a position similar to that of IdUA-II.

Discussion

The two forms of IdUA observed in *O*/*N*-desulphated heparin showed the following features (see Table 3). The anomeric carbon of IdUA-I had a chemical shift (δ 99.7) and a ($^1J_{CH}$) coupling constant (171 Hz) similar to those of sulphated IdUA (δ 99.6; 173 Hz). The apparent pK_a value of IdUA-I was 4.5 (that of sulphated IdUA was 3.95), and it was resistant to periodate oxidation at pH 3.0 and 4°C when the adjacent GlcN moieties were unsubstituted at the C-2 amino group. Collectively, these observations reinforce the suggestion that IdUA-I has retained the $^1C_4$ conformation (see Fig. 3). The other IdUA form (II) had a chemical shift (δ 102.3) similar to that of GlcUA (δ 102.9). However, both the $^1J_{CH}$ coupling

Table 3. *Summary of iduronate potentiometric titration and n.m.r. data*

	IdUA-OSO_3	IdUA-I	IdUA-II
Apparent carboxy pK_a	3.95	4.2–4.5	3.25
Chemical shift [δ (p.p.m.) C-1]	99.6–99.9	99.5–99.9	102.3–102.6
$^1J_{CH}$ (Hz)	173	171	170

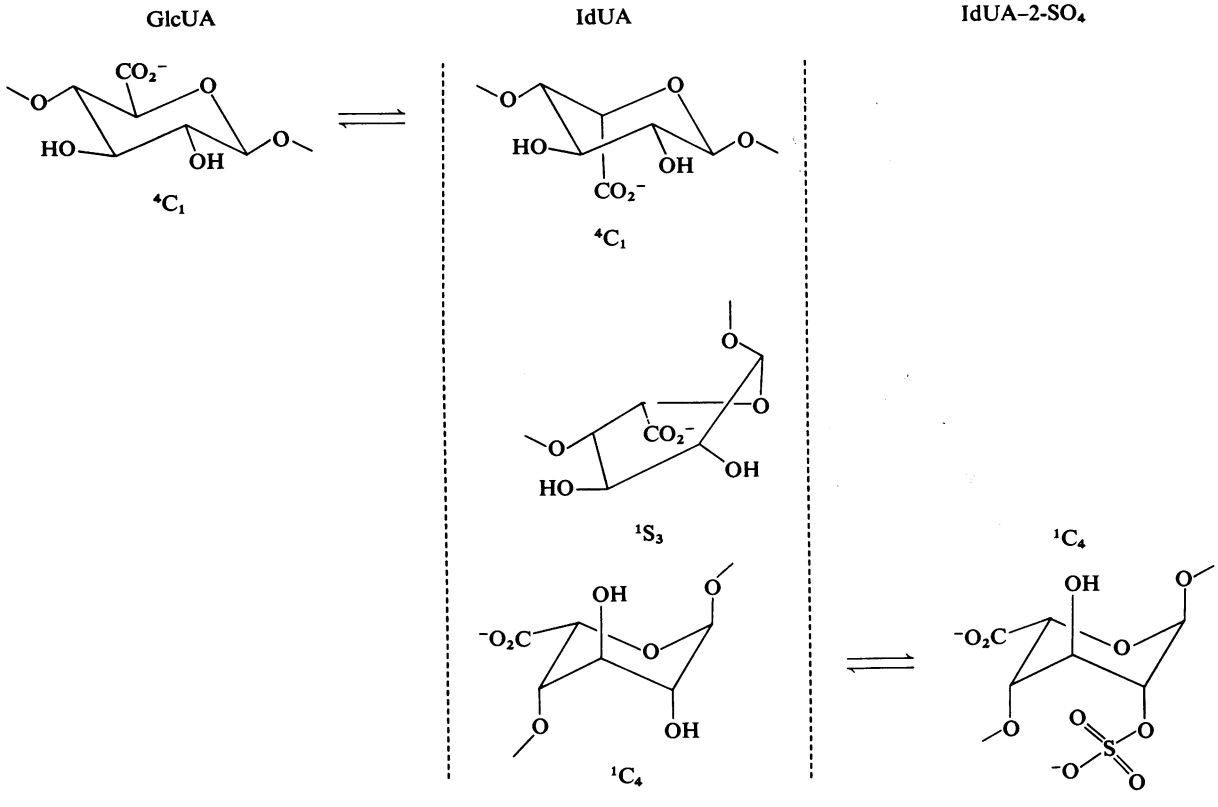


Fig. 3. *Ring conformations of uronic acid residues in heparin*
During biosynthesis of heparin GlcUA is converted into IdUA by C-5 inversion on the polymer level and subsequently into sulphated IdUA by 2-*O*-sulphation (Lindahl, 1976). The ring conformation of GlcUA is almost certainly $^4C_1$, whereas sulphated IdUA occupies the opposite chair conformation ($^1C_4$) (see also the introduction). Non-sulphated IdUA may adopt several ring conformations at closely similar energy levels. The $^4C_1$, $^1S_3$ and $^1C_4$ conformers depicted in the centre may constitute intermediates in the transformation of GlcUA into sulphated IdUA.

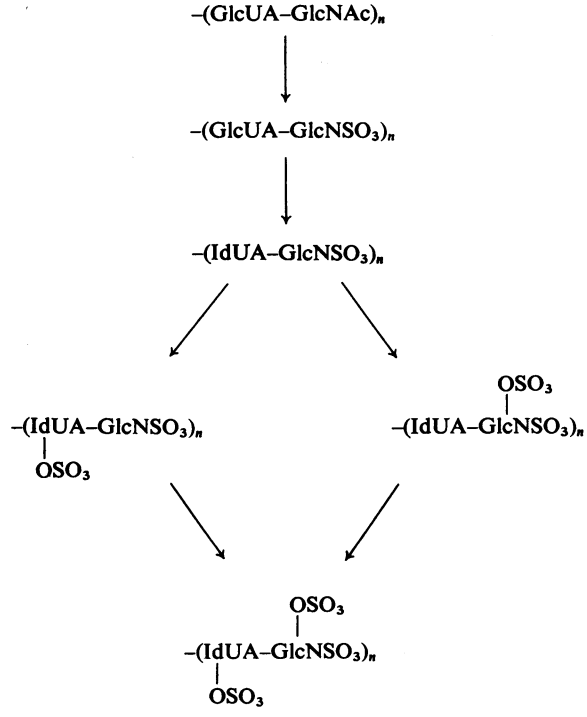
constant (165–170Hz; the value for IdUA-II in *O*/*N*-desulphated re-*N*-acetylated heparin will be subject to a large error because of severe multiplet overlap) and the apparent pK_a value (3.25) were significantly higher than those of GlcUA (163 Hz and about 2.5 respectively). Since IdUA-II was sensitive to periodate oxidation, the 2- and 3-hydroxy groups should be equatorially disposed as in, say, the $^4C_1$ and $^1S_3$ conformers shown in Fig. 3. The lower apparent pK_a value of IdUA-II does not favour the $^4C_1$ conformer since it suggests an equatorial carboxy group (Stolow, 1959), and the $^1J_{CH}$ coupling constant for IdUA-II suggests an axially disposed C-1–O glycosidic bond as in the $^1S_3$ conformer (Fig. 3).

The conformational state of IdUA is apparently dependent on the route of desulphation. Desulphation of sulphated IdUA residues when the neighbouring GlcN moieties are *N*-sulphated or carry unsubstituted amino groups results in the retention of the $^1C_4$ conformation in approximately half of the IdUA residues. However, desulphation of IdUA-SO_3 residues surrounded by GlcNAc moieties alters the

ring conformation of all the IdUA residues. Since direct *O*/*N*-desulphation leaves a small proportion of sulphated IdUA in the polymer, whereas sequential *N*- and *O*-desulphation appears to be much more complete, it is also conceivable that the presence of a few sulphated IdUA residues in the $^1C_4$ conformation causes the retention of ring shape in some of the non-sulphated residues by a co-operative mechanism.

The existence of two conformations of IdUA in desulphated heparins invites enquiry into the shape of the iduronate moiety whenever it is not conformationally anchored by a sulphate ester group. In heparin the majority of the IdUA residues are 2-*O*-sulphated, but clearly a few non-sulphated residues have potential versatility and a single such residue in, say, the opposite $^4C_1$ conformation could confer a distinctive 'recognition site' for other molecules, e.g. antithrombin. In heparan sulphates the wide variation in degree of sulphation might give rise to an ever greater conformational variability among non-sulphated IdUA residues. The ability of dermatan sulphate samples to yield X-ray fibre diffraction patterns of high quality (Atkins & Isaac, 1973; Arnott *et al.*, 1973) almost certainly indicates that the entire IdUA–GalNAc–OSO_3 block regions occur with the IdUA residue in the $^4C_1$ conformer, but conformational variability may well be introduced into this glycan by the presence of sulphated IdUA residues (Cöster *et al.*, 1975).

In the present study it was also possible to identify the anomeric resonances of minor disaccharide repeats in heparin, i.e. GlcUA–GlcNAc, GlcUA–$GlcNSO_3$ and IdUA–$GlcNSO_3$. These minor repeating units arise during biosynthesis (Lindahl, 1976), which proceeds through the steps shown in Scheme 3. Since the various steps are incomplete, the presence of a small number of intermediate disaccharide repeats is to be expected. Such disaccharide repeats are common in heparan sulphate. Therefore the results of the present work should be particularly useful in the elucidation of the complex structure of this polysaccharide. The inversion of configuration at C-5 of GlcUA to form IdUA is a vital step in the biosynthesis of both heparin and heparan sulphate (see Fig. 3). The equilibrium of this reaction is assumed to be in favour of D-GlcUA. Subsequent *O*-sulphation markedly increases the yield of IdUA. Presumably the immediate product of the inversion is an IdUA in the $^4C_1$ conformer, i.e. the same as in GlcUA. However, the sulphated IdUA occurs in the opposite chair form ($^1C_4$). It is conceivable that non-sulphated IdUA residues adopt other ring shapes (e.g. $^1S_3$) when converted from the $^4C_1$ into the $^1C_4$ chair conformation.



Scheme 3. *Steps in the biosynthesis of heparin*

This study illustrates the power of the n.m.r. approach to conformational problems and an extension of this work, now in progress, is intended to define the IdUA conformers present in heparan sulphate.

It is likely that the conformation of non-sulphated α-L-iduronate in native polymers is dependent on the sequence of biosynthetic modifications in the same way as it has been shown to be dependent on the sequence of desulphation operations.

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