Carbohydrate Research, 223 (1992) 81–98
Elsevier Science Publishers B.V., Amsterdam

One- and two-dimensional ^{13}C-n.m.r. characterization of two series of oligosaccharides derived from porcine intestinal mucosal heparin by degradation with heparinase

Peter Gettins* and Angela P. Horne
Department of Biochemistry and Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, TN 37232–0146 (U.S.A.)

(Received June 3rd, 1991; accepted July 25th, 1991)

ABSTRACT

Two tetrasaccharides, two hexasaccharides, and a disaccharide have been purified from heparinase digests of porcine intestinal mucosal heparin in sufficient quantities to permit ^{13}C-n.m.r. characterization of the species. The two tetrasaccharides are the sulfated iduronic acid-containing 4en-Hex*p*A2SO$_3$-(1→4)-α-D-Glc*p*NSO$_3$;6SO$_3$-(1→4)-α-L-Ido*p*A2SO$_3$-(1→4)-D-Glc*p*NSO$_3$;6SO$_3$ and the non-sulfated glucuronic acid-containing 4en-Hex*p*A2SO$_3$-(1→4)-α-D-Glc*p*NSO$_3$;6SO$_3$-(1→4)-β-D-Glc*p*A-(1→4)-D-Glc*p*NSO$_3$;6SO$_3$. The two hexasaccharides are related to the two tetrasaccharides by the insertion of α-linked L-Ido*p*A2SO$_3$-(1→4)-D-Glc*p*NSO$_3$;6SO$_3$ after the non-reducing end sulfated glucosamine residue. The disaccharide is 4en-Hex*p*A2SO$_3$-(1→4)-α-D-Glc*p*NSO$_3$;6SO$_3$. The disaccharide, together with each of the iduronate-containing oligosaccharides, form one series of related di-, tetra-, and hexa-saccharides, while the disaccharide together with the glucuronate-containing oligosaccharides form a second series. Using inverse detection as a means of increasing sensitivity, two-dimensional n.m.r. ^{13}C–^{1}H heterocorrelation spectra have been obtained for all five oligosaccharides. The use of two-dimensional heterocorrelation n.m.r. spectroscopy offers a much less ambiguous means of making ^{13}C resonance assignments than do traditional one-dimensional methods, while the use of inverse detection gives both greater sensitivity than direct detection, as well as values for the one-bond ^{13}C–^{1}H coupling constants. From a knowledge of the assignments of resonances in the ^{1}H spectra of these species, it has been possible to assign almost all of the ^{13}C resonances of these five oligosaccharides. Some corrections to previously published assignments for the tetrasaccharides have been made. In addition, one-bond ^{13}C–^{1}H coupling constant data have been obtained for all of the anomeric protons.

INTRODUCTION

Heparin is a heterogeneous, highly sulfated, linear polymer of alternating glucosamine and uronic acid residues in 1→4 linkages. The heterogeneity arises from the various post-synthetic modifications that occur to the initially biosynthesized copolymer of *N*-acetyl-D-glucosamine and D-glucuronic acid. The modifications include epimerization of D-glucuronate to L-iduronate, deacetylation, and subsequent *N*-sulfation of the glucosamine, as well as *O*-sulfation of the 2-position of L-iduronate, and of the 6-, and, to a limited extent, the 3-positions of the D-glucosamine[1]. Heterogeneity

*To whom all correspondence should be addressed.

0008-6215/92/$ 05.00

arises from the fact that not all residues are completely modified, which leads to many possible sequences within a given heparin chain.

The physiological and pharmacological importance of heparin lies in its ability to bind to many different proteins. Thus it has long been used as an anticoagulant, which is attributable to its ability to greatly enhance the rate at which the serine protease inhibitors antithrombin III and heparin cofactor II inhibit proteases of the blood coagulation cascade, in particular thrombin. It is known to increase levels of lipoprotein lipase, through stabilization of the enzyme to degradation[2], to bind to platelet activating factor[3], and to have a possible role in atherosclerosis through its interaction with apolipoproteins[4,5] B and E. In addition, heparin is known to bind to other components of the circulatory system, including fibronectin[6] and laminin[7].

While some of these interactions appear to be relatively non-specific, in that they depend mainly on the high negative charge of the heparin rather than on the existence of a particular sequence of sugars, other interactions appear to be highly specific. A particular pentasaccharide sequence[8] is required for activation of antithrombin III towards factor X_a, though longer heparin species containing this pentasaccharide are required to activate antithrombin III against thrombin[9]. Heparin-derived oligosaccharides with almost no anticoagulant activity have also been identified that are potent inhibitors of C-3 convertase (classical-complement-pathway C-3–C-5 convertase, EC 3.4.21.43) and are thus of complement activity[10].

Given this mediation of divers physiological processes by different sequences within heparin, it is important to have a sensitive means of determining the structures of these species. ^{13}C-N.m.r. is one such method that is sensitive to residue type, anomeric linkage, and substitution pattern. Although extensive systematic compilations of ^{13}C-n.m.r. chemical shift data have been published for many oligosaccharides[11], relatively few such data are available for heparin-derived oligosaccharides[8,12–15]. This is in part due to the large quantities of material necessary to obtain sufficiently good quality ^{13}C-n.m.r. spectra to permit more traditional means of assignment, such as selective proton decoupling, to be employed. Even the use of ^{1}H–^{13}C two-dimensional heterocorrelation spectroscopy, to afford better resonance separation as well as simultaneous assignment of the ^{13}C resonances through correlation with the chemical shift of the directly bonded proton, suffers from the intrinsic insensitivity of natural abundance ^{13}C. It is estimated that an improvement in sensitivity of 8-fold is expected for indirect detection of carbon compared to direct detection[16,17], with a further factor of 2 increase resulting from ^{13}C decoupling during acquisition. We report herein the use of the more sensitive two-dimensional inverse ^{1}H–^{13}C heterocorrelation n.m.r. method to obtain ^{13}C resonance assignments for nearly all the H-bonded carbons in five related heparin-derived oligosaccharides, ranging in size from disaccharide to hexasaccharide. These data permit the effects of incremental changes in structure to be analyzed.

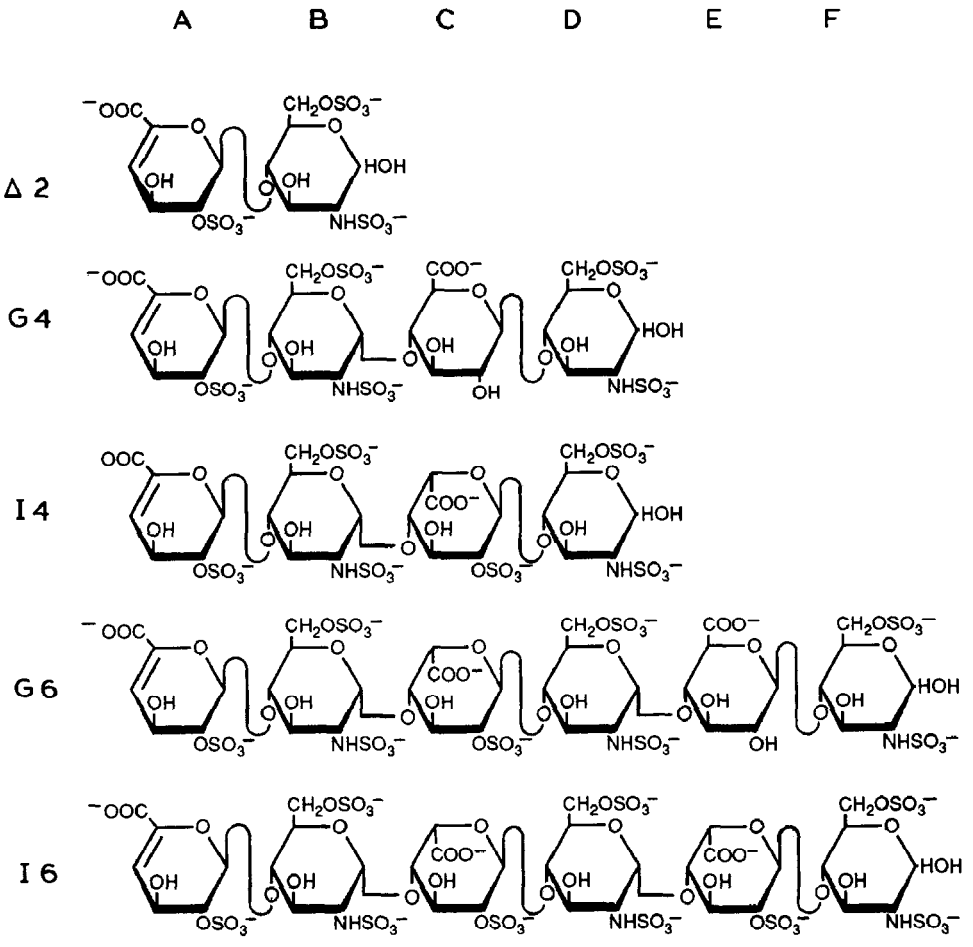

Fig. 1. Structures of the five oligosaccharides examined in the present study, together with the abbreviations used to designate each and the lettering system used to specify particular sugar residues in each oligosaccharide.

RESULTS

Inverse-detected ^{13}C-^{1}H two-dimensional heterocorrelation spectra of heparin oligosaccharides. — The structures of the five heparin oligosaccharides examined in the present study, together with the abbreviations used to designate the different sugar residues, are given in Fig. 1. This system of lettering results in residue A always being the 4,5-unsaturated sugar and residue B being the sulfated D-glucosamine residue adjacent to it. For these five oligosaccharides, inverse-detected ^{13}C–^{1}H heterocorrelation spectra were obtained overnight on solutions with concentrations in the range 10 to 20mM, corresponding to 4 mg of disaccharide, 8 mg of tetrasaccharide, and 12 mg of hexasaccharide at the 20mM concentration. This illustrates the sensitivity of the inverse-detection method over more conventional direct ^{13}C detection.

The inverse heterocorrelation spectrum of the disaccharide *Δ2*, together with

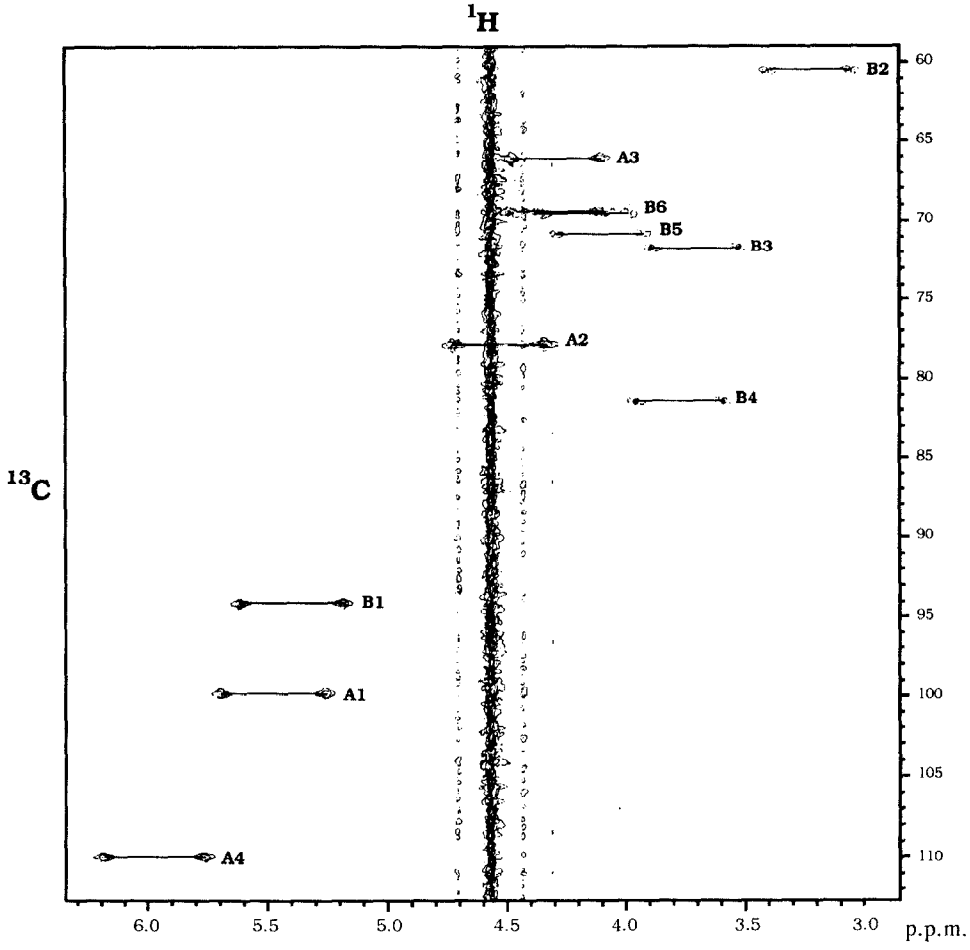

Fig. 2. Inverse-detected ^{13}C–^1H heterocorrelation contour plot of disaccharide *Δ*2 obtained at 400 MHz. For each of 256 t_1 values, 32 scans were collected at a temperature of 310 K. The sample pH was pH 7.0. A sweep width of 1400 Hz in the F_2 dimension (^1H) and 5400 Hz in the F_1 dimension (^{13}C) were employed. Data were weighted with a shifted sine bell apodization function prior to Fourier transformation. All 10 protonated carbons give clearly resolved pairs of correlated peaks. Assignments are shown for each resonance, using the nomenclature given in the text, and were made from knowledge of the ^1H assignments[18].

assignment of the cross-peak pairs is given in Fig. 2. Assignments for this disaccharide have been previously published[12] and are in agreement with the present determinations, given in Table I. Fig. 3 shows the inverse heterocorrelation spectrum for tetrasaccharide **I4**. All of the protonated carbons give resolved pairs of cross peaks. The ^{13}C assignments are given in Tables I, II, and III. Although assignments have also been published for this oligosaccharide[12], based on a combination of more traditional approaches, there are a number of revisions necessary as a result of the present study. Thus the assignments within each of the three pairs of resonances B4 and D4, C5 and B5, and B2 and D2 need to be reversed. In each case the earlier error is understandable, since it was based upon the effects of proton decoupling in a very crowded region of the ^1H spectrum. The ability

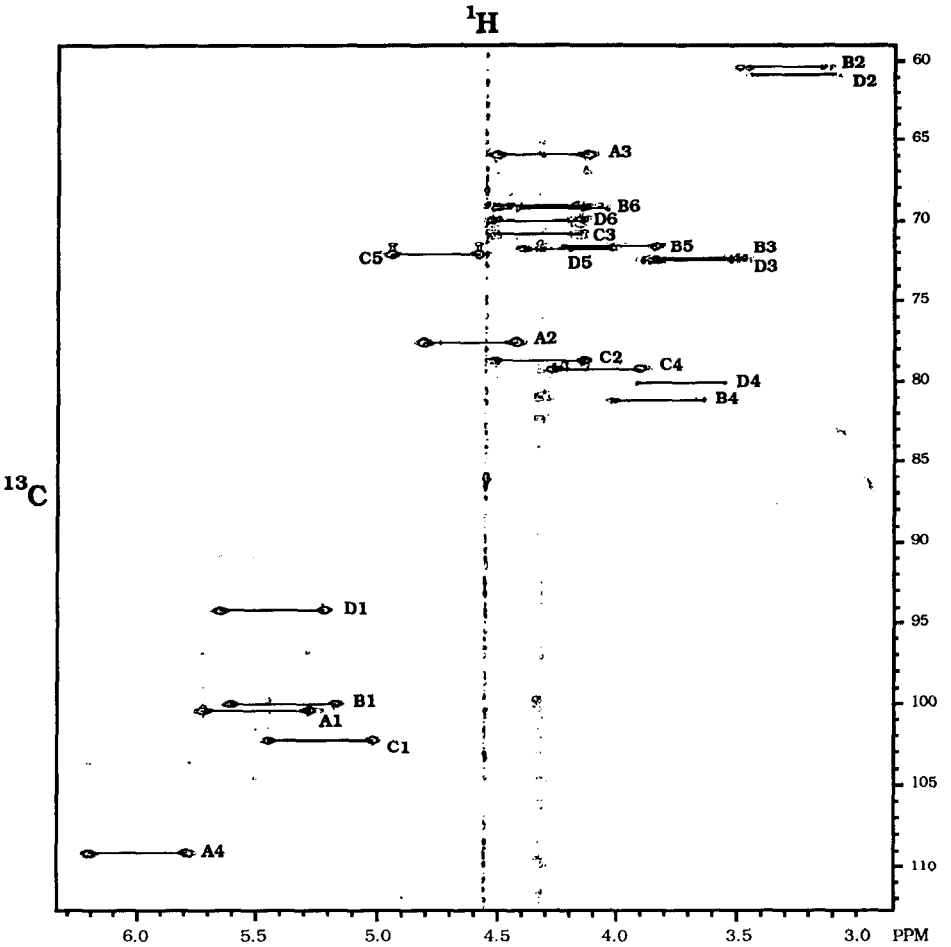

Fig. 3. Inverse-detected ^{13}C–^{1}H heterocorrelation contour plot of tetrasaccharide **I4** obtained at 400 MHz. For each of 256 t_1 values, 64 scans were collected at a temperature of 310 K. The sample pH was pH 7.0. A sweep width of 1400 Hz in the F_2 dimension (^{1}H) and 5400 Hz in the F_1 dimension (^{13}C) were employed. Data were weighted with a shifted sine bell apodization function in both dimensions. All 21 protonated carbons give clearly resolved pairs of correlation peaks. Lines connecting the related halves of the C–H doublets are shown for all carbons. Assignments are shown for all resonances using the nomenclature given in the text. Assignments were made from knowledge of the ^{1}H resonance assignments[18].

to resolve individual cross-peak pairs in the present two-dimensional inverse heterocorrelation study, however, illustrates one of the advantages of this method of resonance assignment. In both the previous[12] and present[18] studies, knowledge of the ^{1}H assignments was a prerequisite.

The inverse heterocorrelation spectrum of the tetrasaccharide **G4** is shown in Fig. 4. As with tetrasaccharide **I4**, all protonated carbons give resolved pairs of cross peaks, allowing unambiguous assignments to be made from knowledge of the proton chemical shift assignments[18]. These ^{13}C resonance assignments are given in Tables I, II, and III. For this tetrasaccharide there are several major differences from the assignments

TABLE I

^{13}C-N.m.r. resonance assignments for the 4,5-unsaturated residue and the reducing-end sulfated D-glucosamine in five heparin oligosaccharides[a]

	Chemical shift											
	4,5-Unsaturated residue (A)						*Reducing-end glucosamine*					
	C-1	C-2	C-3	C-4	C-5	C-6	C-1	C-2	C-3	C-4	C-5	C-6
Δ2	99.55	77.68	65.95	109.74	147.30	171.53	93.80	60.47	71.66	81.32	70.72	69.42
I4	100.12	77.34	65.71	108.83	147.56	171.89	93.84	60.67	72.37	79.94	71.62	69.74
G4	99.87	77.26	65.53	108.67	147.47	171.89	93.70	60.32	72.05	81.54	70.80	69.37
I6	99.92	77.21	65.49	108.51	147.56	171.87	93.74	60.61	72.22	79.50	71.11	69.64
G6	99.95	77.19	65.53	108.60	147.45	171.76	93.72	60.34	72.12	81.62	70.84	69.44

[a] Determined at 310 K.

TABLE II

^{13}C-N.m.r. resonance assignments for the internal sulfated D-glucosamine residues of four heparin oligosaccharides[a]

	Residue B chemical shift (p.p.m.)						*Residue D chemical shift (p.p.m.)*					
	C-1	C-2	C-3	C-4	C-5	C-6	C-1	C-2	C-3	C-4	C-5	C-6
I4	99.72	60.34	72.25	81.03	71.53	68.98			n.a.[b]			
G4	100.34	60.17	72.25	80.86	71.32	68.81			n.a.[b]			
I6	99.62	60.32	72.22	80.93	71.44	68.90	99.06	60.61	72.22	78.59	71.91	69.08
G6	99.79	60.19	72.45	80.86	71.49	68.91	100.19	60.69	72.12	78.62	71.94	69.02

[a] Determined at 310 K. [b] Residue D for the two tetrasaccharides is the reducing-end sulfated D-glucosamine and is therefore given in Table I.

TABLE III

^{13}C-N.m.r. resonance assignments for the uronic acid residues of four heparin oligosaccharides[a]

	L-*Iduronate chemical shift (p.p.m.)*						D-*Glucuronate chemical shift (p.p.m.)*					
	C-1	C-2	C-3	C-4	C-5	C-6[b]	C-1	C-2	C-3	C-4	C-5	C-6
I4	101.99	78.52	71.19	78.99	71.95	177.30			n.a.[c]			
G4			n.a.[c]				104.79	75.59	78.62	80.19	79.16	177.30
I6(i)[d]	101.93	79.50	72.39	79.16	72.47	177.01			n.a.[c]			
I6(ii)[d]	101.93	78.76	72.09	78.84	72.09	177.32			n.a.[c]			
G6[d]	101.96	78.50	71.80	79.13	71.80	176.83	104.84	75.69	78.62	79.92	79.13	177.18
Heparin[e]	100.12	76.64	70.05	77.10	70.15	175.51						

[a] Determined at 310 K. [b] The specific assignment of the carboxyl resonances has not been established for **I6** and **G6**. [c] n.a. = not applicable. [d] The two iduronate residues (C and E) could not be distinguished for **I6**. [e] Data from Gatti *et al.*[31]. Chemical shifts are referenced to external tetramethylsilane.

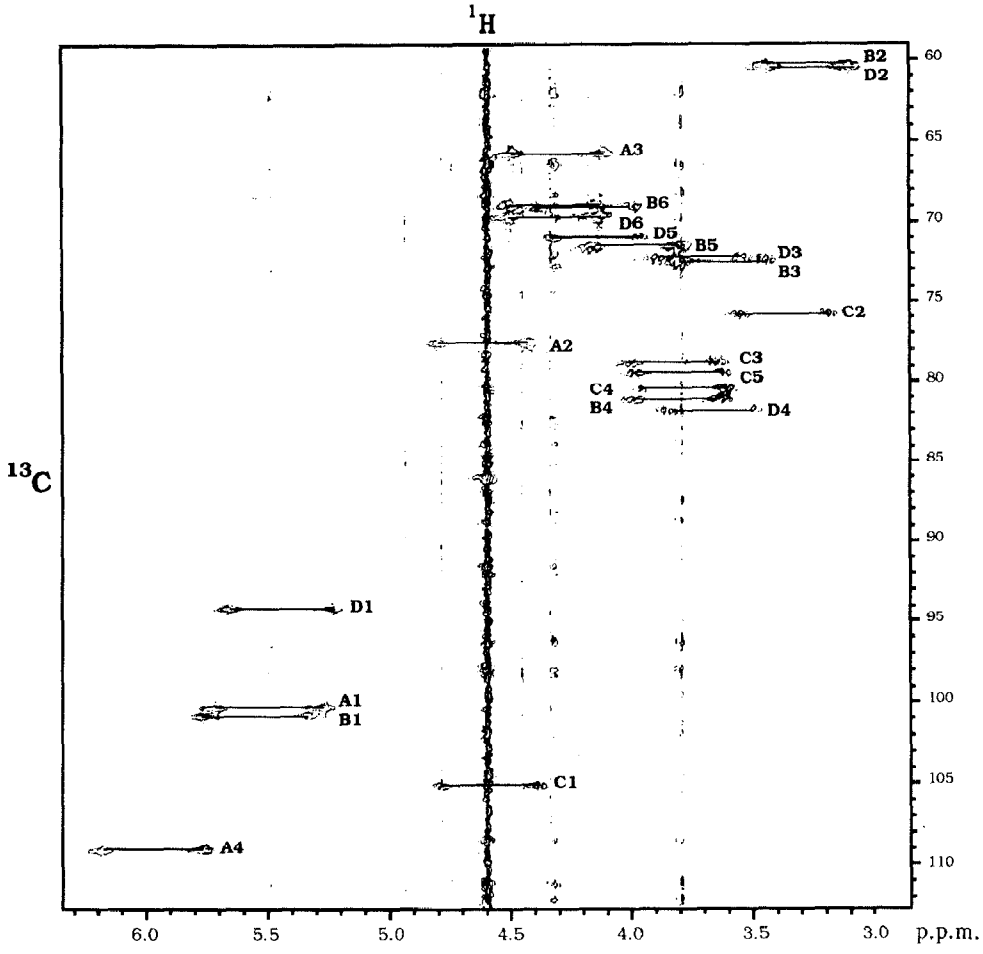

Fig. 4. Inverse-detected ^{13}C–^{1}H heterocorrelation contour plot of tetrasaccharide **G4** obtained at 400 MHz. For each of 512 t_1 values, 64 scans were collected at a temperature of 310 K. The sample pH was pH 7.0. A sweep width of 1400 Hz in the F_2 dimension (^{1}H) and 5400 Hz in the F_1 dimension (^{13}C). Data were apodized with a shifted sine bell weighting function. All 21 protonated carbons give resolved pairs of correlation peaks. Lines connecting the related halves of each C–H doublet have been drawn in for all resonances.

published by Merchant *et al.*[12]. In the present work the D-glucuronate C-5 is found to resonate at 79.16 p.p.m. and the C-3 at 78.62 p.p.m., whereas Merchant *et al.*[12] reported chemical shifts of 70.4 p.p.m. and 74.2 p.p.m., respectively, representing differences of 8.76 p.p.m. and 4.42 p.p.m.. These differences seem to result primarily from mis-assignment of H-5 of the D-glucuronate residue in the ^{1}H spectrum, as well as the very crowded nature of the ^{1}H-n.m.r. spectrum in the region from 3.6 to 3.9 p.p.m. This makes selective decoupling in this region difficult to accomplish, and thus makes interpretation of selectively ^{1}H-decoupled ^{13}C-n.m.r. spectra ambiguous. It appears that the assignment of the H-5 resonance given by Merchant *et al.*[12] was based upon that given by Linker and Hovingh[15], which in turn was based upon comparison with model compounds, *viz.*, hyaluronic acid. However, our assignment, based upon a 2-

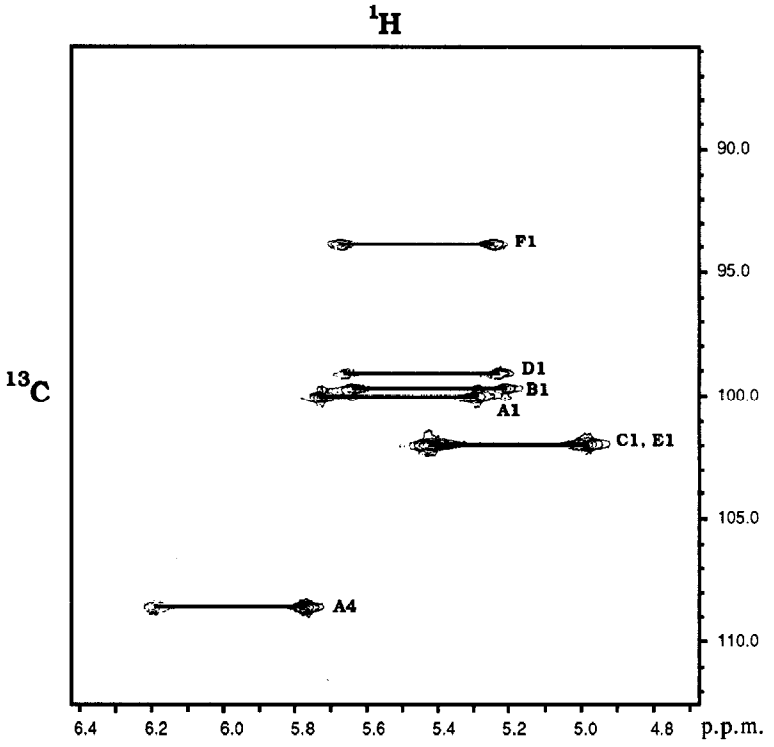

Fig. 5. Inverse-detected ^{13}C–^{1}H heterocorrelation contour plot of the anomeric region of hexasaccharide **I6** obtained at 400 MHz. The sample pH was pH 7.0. A sweep width of 1400 Hz in the F_2 dimension (^{1}H) and 5400 Hz in the F_1 dimension (^{13}C). For each of 246 t_1 values, 80 scans were collected at a probe temperature of 310 K. Six resolved correlation peak pairs can be identified, which correspond to the seven protonated carbons in this region. The two L-iduronate C-1 resonances are exactly superimposed. Assignments are based on knowledge of the ^{1}H chemical shift assignments.

dimensional COSY analysis, is in fact in much better agreement with the chemical shifts observed in hyaluronic acid and also for the D-glucuronate residue in the high-affinity synthetic heparin pentasaccharide of Choay and coworkers[19]. These workers also based their ^{1}H-n.m.r. assignment on a 2-dimensional n.m.r. analysis. This further emphasizes the need to use 2-dimensional assignment methods, rather than comparison with model compounds, in establishing definitive assignments for such oligosaccharide fragments.

Whereas all protonated carbons give resolved cross peaks for the disaccharide and the two tetrasaccharides, this is no longer true for the two hexasaccharides examined in this study. Even in the anomeric region, complete resolution of each carbon is achieved only for **G6**. For **I6** the two internal L-iduronate residues give superimposed cross peaks, reflecting the near equivalence of the flanking 2,6 sulfated D-glucosamine residues for each L-iduronate. The anomeric region of the inverse heterocorrelation spectrum of **I6** is shown in Fig. 5 and that of **G6** is shown in Fig. 6. Assignments are given in Tables I, II, and III. Since these are the first ^{13}C-n.m.r. data to be reported for the two hexasaccharides, no comparisons can be made with earlier assignments.

One-bond C–H coupling constants were determined from the inverse hetero-

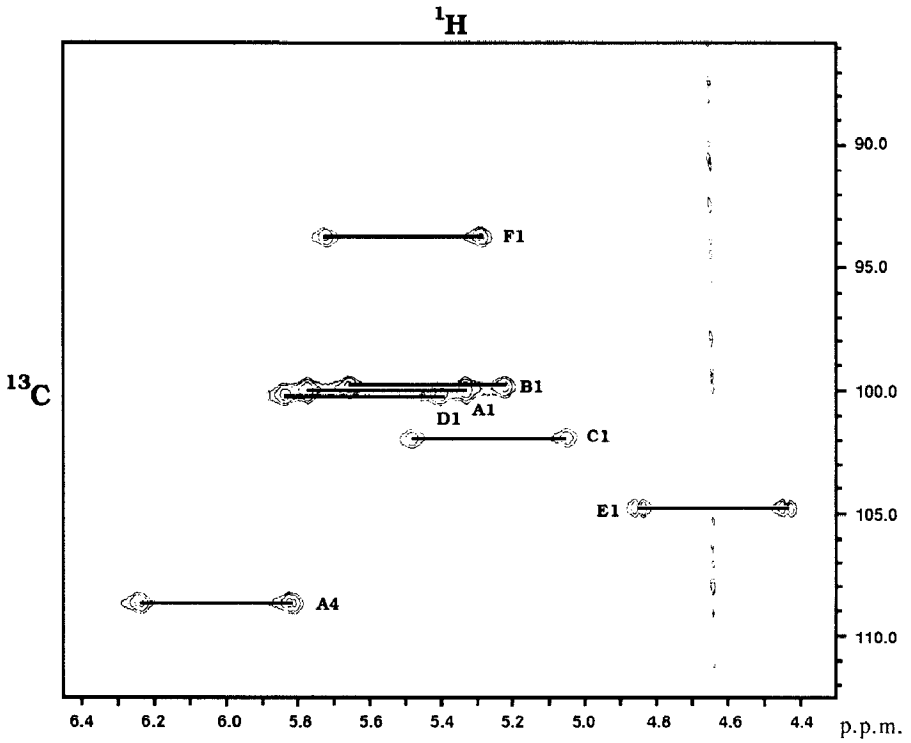

Fig. 6. Inverse-detected ^{13}C–^1H heterocorrelation contour plot of the anomeric region of hexasaccharide **G6** obtained at 400 MHz. The sample pH was pH 7.0. A sweep width of 1400 Hz in the F_2 dimension (^1H) and 5400 Hz in the F_1 dimension (^{13}C). For each of 128 t_1 values, 208 scans were collected at a probe temperature of 310 K. All seven protonated carbons in this region give resolved pairs of correlation peaks. Assignments for all resonances are indicated and are based on knowledge of the ^1H chemical shift assignments.

TABLE IV

One-bond C–H coupling constants for anomeric carbons (Hz)[a]

	Unit					
	A	*B*	*C*	*D*	*E*	*F*
Δ2	175	172				
I4	176	172	174	172		
G4	175	174	163	172		
I6	175	174	174	174	174	172
G6	176	173	175	172	164	171

[a] The estimated error in the coupling constants is ± 1 Hz.

correlation spectra for those of the resonances that gave clearly resolved pairs of cross peaks. The data for the anomeric carbons are summarized in Table IV, since these coupling constants are sensitive to the glycosidic linkage[20].

One-dimensional ^{13}C-n.m.r. spectra of heparin oligosaccharides. — Although as-

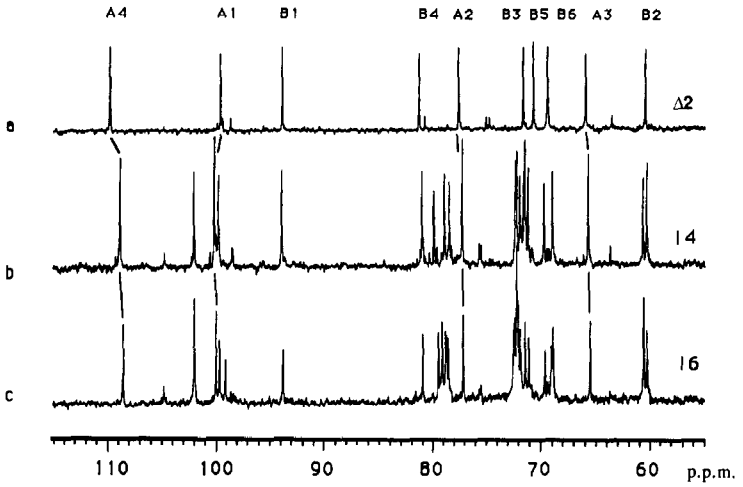

Fig. 7. Proton-decoupled 100 MHz ^{13}C-n.m.r. spectra of heparin oligosaccharides **Δ2**, **I4**, and **I6**, collected at 310 K and pH 7.0. Spectra were recorded using a 60° pulse angle, a repetition rate of 0.32 s^{-1}, a block size of 16 384 data points, and low-power composite pulse proton decoupling, applied continuously, so that nuclear Overhauser enhancements are present. The sweep width was 13 888 Hz in each case and covered the region from 55 to 193 p.p.m. It was known from other ^{13}C spectra collected with a wider sweep width, as well as from the ^{1}H-n.m.r. spectra, that no acetyl resonances were missed in this manner. A line broadening of 4 Hz was applied to each free-induction decay prior to Fourier transformation to improve the signal-to-noise ratio. The spectra and the number of scans collected for each are as follows: (a) disaccharide **Δ2**, 2600 scans; (b) tetrasaccharide **I4**, 6560 scans; (c) hexasaccharide **I6**, 17 400 scans. Complete assignments are indicated for the disaccharide. The solid lines connecting resonances in (a), (b), and (c) indicate the positions of the A residue resonances in each spectrum as a reference aid.

signments of ^{13}C chemical shifts, and determination of one-bond C–H coupling constants were achieved from the 2-dimensional data sets described above, 1-dimensional, proton-decoupled ^{13}C-n.m.r. spectra of the five heparin fragments provide an easier visual means of comparing the changes that occur upon increasing fragment size, or upon substituting D-glucuronate for L-iduronate. Proton-decoupled ^{13}C-n.m.r. spectra of the protonated carbons of the oligosaccharides **Δ2**, **I4**, and **I6** are shown in Fig. 7. For the disaccharide, all 12 carbon resonances are resolved for the α anomer, of which 10 occur in the region shown (Fig. 7a). In addition, smaller resonances can be discerned that arise from the β anomer. Interestingly, it is estimated that the relative proportions of the α and β anomers are 85% and 15%, respectively. Such a preponderance of the α anomer has been remarked upon previously for the disaccharide[21], though without quantitation of the relative proportions. Such a distribution, strongly favoring the α anomer, has also been reported for 2-acetamido-2-deoxy-D-glucose[21] and is in marked contrast to the situation for D-glucose, where the β anomer is predominant in solution. The resonance at the unusual chemical shift of 109.74 p.p.m. is from the C-4 position of the 4,5-unsaturated sugar, while the resonances at 99.55 p.p.m. and 93.80 p.p.m. arise from the anomeric carbons of the 4,5-unsaturated and reducing-end D-glucosamine residues, respectively. The unsaturation in residue A also results in distinctive chemical shifts for the C-2 and C-3 carbons.

Upon increasing the size of the oligosaccharide by adding first one (Fig. 7b) and then a second (Fig. 7c) L-iduronate-disulfated D-glucosamine disaccharide unit to the disaccharide **Δ2**, to give oligosaccharides **I4** and **I6**, respectively, the unusual C-2, C-3, and C-4 resonances from the 4,5-unsaturated A residue can still be clearly discerned in each case, and are indicated for each spectrum. For the tetrasaccharide **I4**, all carbon resonances can again be resolved. In the anomeric region two additional resonances are thus seen (Fig. 7b), arising from the non-reducing D-glucosamine and the L-iduronate. In the hexasaccharide **I6** individual resonances from all of the carbons are no longer resolved, resulting in only one additional resonance in the anomeric region, but with an increase in the relative intensity of the resonance at 101.93 p.p.m., which arises from the two L-iduronate residues (see above).

In the region from 77 to 81 p.p.m., the effect of increasing size is to first introduce three resolved resonances, in the tetrasaccharide, between the extrema delimited by the resonances of carbons A2 and B4 (Fig. 7b) and then, in the hexasaccharide, to superimpose an additional three resonances. These sets of three resonances are from the L-iduronate C-2 and C-4 and from glucosamine C-4 from D-glucosamine residues other than residue B. Since the two internal, disulfated D-glucosamine–L-iduronate units in the hexasaccharide have almost identical ^{13}C chemical shifts, the expectation is that, for octasaccharide or larger species in this series, this region of the spectrum would consist of outrigger resonances from A2 and B4, at high- and low-field extremes, respectively, and an increasingly poorly resolved central region containing three additional resonances for each disaccharide unit increase in size. A similar superpositioning is seen in the next group of resonances between 70 and 74 p.p.m. In going from disaccharide to tetrasaccharide, new resonances occur upfield from B3. However, upon increasing the size further to the hexasaccharide, the effect is to add intensity to resonances already present in the tetrasaccharide.

In **I4** the two glucosamine C-6 resonances are well resolved, being separated by 0.76 p.p.m.. Further increase in size to give the hexasaccharide maintains the difference in chemical shift between the C-6 resonances from the reducing-end D-glucosamine and the internal D-glucosamines, though the difference in chemical shift between the latter two is very small (0.18 p.p.m.).

Finally, near 62 p.p.m., where the D-glucosamine C-2 carbons resonate, the addition of a non-reducing D-glucosamine in going from disaccharide to tetrasaccharide results in a second, well-resolved resonance. Upon addition of another non-reducing D-glucosamine, to give the hexasaccharide, the extra C-2 resonance superimposes on that of the other non-reducing D-glucosamine residue.

Fig. 8 shows the ^{1}H-decoupled ^{13}C-n.m.r. spectra of the protonated carbons of the series of oligosaccharides **Δ2**, **G4**, and **G6** (the spectrum of **Δ2** is identical to that of Fig. 7a and is repeated for ease of comparison). This series can be considered to be generated by first adding a D-glucuronate–disulfated D-glucosamine unit to the disaccharide to give the tetrasaccharide **G4**, and then by inserting an L-iduronate–disulfated D-glucosamine between the internal D-glucosamine and the D-glucuronate of **G4** to give the hexasaccharide **G6**. Thus, in contrast to the first series, where the hexasaccharide differs

from the tetrasaccharide by the same disaccharide unit as the tetrasaccharide differs from the disaccharide, there are two different internal disaccharide units in **G6**. Much greater differences are therefore expected, and seen, between the appearance of the ^{13}C spectra of **G4** and **G6** than between the spectra of **I4** and **I6**. In the anomeric region, the tetrasaccharide (Fig. 8b) differs from the disaccharide by the appearance of two new resonances, at 104.79 p.p.m. and 100.36 p.p.m. from the anomeric carbons of the D-glucuronate and internal D-glucosamine residues, respectively. In the anomeric region of the hexasaccharide **G6** (Fig. 8c) two additional, resolved resonances are seen compared to the tetrasaccharide spectrum. These occur at 101.96 p.p.m. and 99.79 p.p.m., and arise from the L-iduronate and internal sulfated D-glucosamine, respectively, of the extra disaccharide unit. The chemical shift of the L-iduronate is identical to within 0.03 p.p.m. of the C-1 chemical shifts of both L-iduronate residues in **I6**, reflecting the relative insensitivity of this carbon to differences more than one residue removed.

In the region between 75 and 82 p.p.m., the addition of the D-glucuronate–disulfated D-glucosamine disaccharide unit to Δ**2**, to give the tetrasaccharide **G4**, results in the appearance of five additional resonances, one from the reducing-end D-glucosamine C-4 and four from the D-glucuronate carbons 2, 3, 4, and 5. Upon addition of the sulfated D-glucosamine–L-iduronate to give **G6**, three new resonances are found in this region: those from C-4 of the glucosamine and from C-2 and C-4 of the L-iduronate. These are the same additions seen upon going from **I4** to **I6**.

In the next group of resonances, between 71 and 72 p.p.m., fewer additional resonances are seen in going from disaccharide to **G4** tetrasaccharide than are seen in going from disaccharide to **I4** tetrasaccharide. This is because the glucuronate C-3 and C-5 resonances occur much further downfield than those of the corresponding L-iduronate carbons. However, upon going from **G4** to **G6**, an additional four resonances, from the C-3 and C-5 carbons of both the D-glucosamine and L-iduronate are seen. The result is significant resonance overlap, so that all resonances are no longer resolved in this region of the hexasaccharide.

As was seen for **I4**, a significant difference in chemical shift for the C-6 resonances of the two D-glucosamines of **G4** is found, although in this case it is somewhat smaller (0.56 p.p.m. *vs.* 0.76 p.p.m.). Also similar to the difference between **I4** and **I6**, the third D-glucosamine C-6 resonance of **G6** is only barely resolved from that of the other internal glucosamine.

DISCUSSION

The aim of the present study was to obtain definitive ^{13}C resonance assignments for five related heparin oligosaccharides and to do this by means of inverse-detected 2-dimensional heterocorrelation spectroscopy. Use of 2-dimensional n.m.r. techniques helps remove the degeneracy that is so frequently a problem in spectra of carbohydrates composed of similar repeating units, while the use of inverse detection provides greatly enhanced sensitivity over conventional heterocorrelation spectroscopy, and it can also simultaneously give one-bond C–H coupling constants. The data given in the inverse

heterocorrelation plots shown in Figs. 2–6 demonstrate the success of this approach in providing complete assignments for the oligosaccharides **Δ2**, **I4**, and **G4**, as well as nearly complete assignments for the hexasaccharides **I6** and **G6**. The close structural relationships between the various fragments results in degeneracy or near degeneracy for several resonances, which is not completely resolved even in two dimensions. However, the use of higher field (14.1 T *vs.* the 9.4 T used here), as well as carbon decoupling during data acquisition, would help alleviate this problem. These definitive assignments for the two tetrasaccharides necessitate changes in the published assignments described above. Although some of these changes are relatively small, there are two major differences involving C-5 and C-3 of the D-glucuronate of **G4**, where the differences in chemical shift between the present study and that of Merchant *et al.*[12] are 8.76 and 4.42 p.p.m., respectively.

Since the present study examines five closely related oligosaccharides, some useful comparisons of chemical shifts can be made using the data compiled in Tables I–III. Residue A is not a naturally occurring heparin residue, but results from the enzymatic cleavage of the glycosidic linkage between a sulfated D-glucosamine and a sulfated L-iduronate. The unsaturation in the ring results in useful u.v. absorption properties for such heparinase-derived species[22], as well as ^{1}H and ^{13}C chemical shifts that are distinctly different from the parent L-iduronate. Table I gives the chemical shifts for the four protonated carbons of residue A in the five oligosaccharides. For **I4**, **G4**, **I6**, and **G6**, the chemical shifts of each carbon in residue A vary by no more than 0.25 p.p.m. amongst the oligosaccharides. In contrast, there is substantial difference between the chemical shifts in **Δ2** and the remaining four species. This presumably reflects the basic difference between these two classes, *viz.* that the disaccharide has residue A linked to a reducing disulfated D-glucosamine, whereas the other species have residue A linked to a non-reducing disulfated D-glucosamine. The reducing-end D-glucosamine also reflects this distinction (Table I). Thus C-4 has a chemical shift of 81.32 p.p.m. in the disaccharide, but chemical shifts of 79.94 and 79.50 in **I4** and **I6**, respectively. There is a further distinction shown by the chemical shift of the reducing-end D-glucosamine C-4. This is between the D-glucuronate-containing species **G4** and **G6** and the L-iduronate-containing species **I4** and **I6**. A difference of 1.60 p.p.m. in chemical shift of the D-glucosamine C-4 between **G4** and **I4** and of 2.12 p.p.m. between **G6** and **I6** is seen. Such large differences are perhaps not surprising, given the change in both the glycosidic linkage and the sulfation of the uronic acid residues between the D-glucuronate series and the L-iduronate series. The resulting large downfield shift on the C-4 D-glucosamine residue upon substituting β-D-glucuronate for α-L-iduronate makes this resonance the most downfield in this group of resonances, occurring at lower field even than the B residue C-4 resonance (Fig. 8). It should also be noted, however, that there is a difference between the chemical shift differences that is itself substantial. This difference of 0.52 p.p.m. must arise from distinct tertiary structures for the hexasaccharides and tetrasaccharides. Other examples of this are discussed below. The resonance position of the glucosamine C-5 also differs for attachment of the D-glucosamine to D-glucuronate or L-iduronate, although the magnitude of the difference is less.

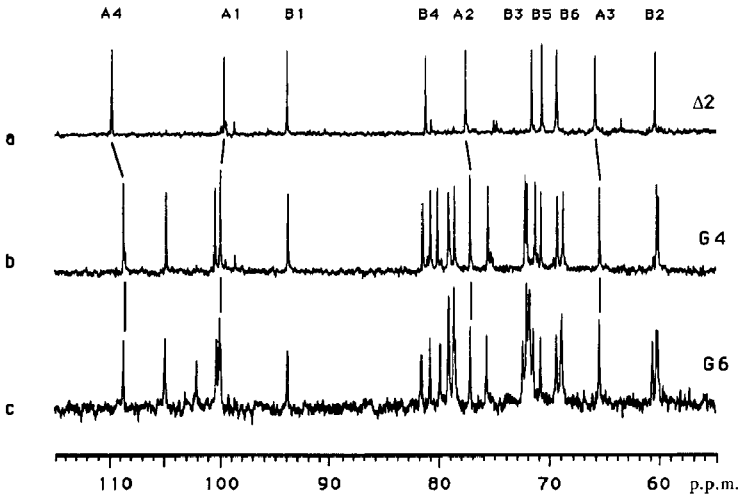

Fig. 8. Proton-decoupled 100 MHz ^{13}C-n.m.r. spectra of heparin oligosaccharides **Δ2**, **G4**, and **G6**, collected at 310 K and pH 7.0. Spectra were recorded using a 60° pulse angle, a repetition rate of 0.32 s^{-1}, a block size of 16 384 data points, and low-power composite pulse proton decoupling, applied continuously. The sweep width was 13 888 Hz in each case. A line broadening of 4 Hz was applied to each free-induction decay prior to Fourier transformation. The spectra, and the number of scans collected for each sample are as follows: (a) disaccharide **Δ2**, 2600 scans (this is the same spectrum as shown in Fig. 7a); (b) tetrasaccharide **G4**, 18 050 scans; (c) hexasaccharide **G6**, 16 600 scans. Complete assignments are indicated for the disaccharide. The solid lines connecting resonances in (a), (b), and (c) indicate the positions of the A residue resonances in each spectrum as a reference aid.

The remaining six disulfated D-glucosamine residues are all non-reducing and give ^{13}C chemical shifts (Table II) that can be first distinguished by whether the residue is of type B (*i.e.*, attached to the 4,5-unsaturated A residue) or type D. Within the group of four type B residues, there is very little variation in chemical shift, with the exception of C-1. The value of 100.34 p.p.m. for the C-1 resonance of **G4** is between 0.55 and 0.72 p.p.m. downfield of the other C-1 resonances and reflects the attachment of the former to a D-glucuronate and all three of the latter to L-iduronate residues. Similarly for the D type residues, the chemical shift of C-1 for **G6** is 1.13 p.p.m. downfield of the C-1 resonance for **I6**. This again reflects the attachment of D-glucuronate to the former and L-iduronate to the latter. In comparing the chemical shifts of the other carbons between the two classes B and D, it can be seen that C-4 shows the greatest differences (1.31–1.44 p.p.m.), with lesser, though significant, sensitivity shown by C-5 and C-6 resonances. That C-4 should exhibit such a large difference again reflects the unusual properties of the 4,5-unsaturated uronic acid residue to which it is attached.

The variations in chemical shift seen within the six uronic acid residues (Table III) are most prominently due to configurational and sulfation changes between sulfated α-L-iduronate and unsulfated β-D-glucuronate. Thus C-1 for the latter is approximately 2.8 p.p.m. downfield from C-1 of the former. C-2, which bears a sulfate group in the L-iduronate but not the D-glucuronate, resonates between 2.81 and 3.91 p.p.m. further downfield in the L-iduronate. Conversely, C-3 of L-iduronate lies between 6.23 and 7.43

p.p.m. upfield of C-3 in D-glucuronate and C-5 lies between 6.66 and 7.36 p.p.m. further upfield in the L-iduronate. Beyond these gross differences, the D-glucuronate residues in **G4** and **G6** vary little between each other. However, there are some large differences present in the four L-iduronate residues that are not readily explicable on the basis of primary structure of the oligosaccharide. Thus, while the C-1 resonances show almost no variation in chemical shift, and the C-4 resonances show variations of only 0.32 p.p.m., the C-2 resonances differ by as much as 1.00 p.p.m., the C-3 resonances by 1.20 p.p.m., and the C-5 resonances by 0.67 p.p.m.. Since the L-iduronate residue in **I4** is essentially equivalent to one of the L-iduronates in **I6**, with respect of primary structure, there should be similar chemical shifts for these two residues, unless other factors are also important. That there are significant differences, whether the residue designated **I6(i)** or **I6(ii)** is used for comparison, again suggests that the overall conformations of the tetra- and hexa-saccharides are not identical. Comparison of these chemical shifts with those previously reported for the L-iduronate residues in intact heparin (Table III) is complicated by the use of a different referencing method from the present study, such that the former values are consistently lower by approximately 1.8 p.p.m. from those reported here. If an adjustment of 1.8 p.p.m. is made to the values for the L-iduronate in heparin, the best agreement is found with the chemical shifts found here for the **I6** iduronate designated (**ii**), suggesting that the conformation of this L-iduronate and its neighbors most closely approximates the situation in intact heparin. Even here there are still differences in chemical shift between the hexasaccharide and intact heparin. The discrepancies are 0.01 p.p.m. for C-1, 0.32 p.p.m. for C-2, 0.24 p.p.m. for C-3, 0.06 p.p.m. for C-4, and 0.14 p.p.m. for C-5. As was found for the comparisons within the oligosaccharides, C-2, C-3, and C-5 show the greatest differences in chemical shift, indicating a particular sensitivity to conformation.

In addition to ^{13}C chemical shift assignments, the present study has provided one-bond C–H coupling constants, which can be compared within the various series of species. One-bond coupling constants have only been reported previously for the Δ**2** disaccharide[12]. These values are in agreement with our data obtained from the inverse heterocorrelation plot (Fig. 2) and given (for the anomeric carbons only) in Table IV. The values of 175 Hz and 172 Hz for residue A and B, respectively, are consistent with the α configuration for each residue. Very similar values are found for each disulfated D-glucosamine and for each L-iduronic acid, again confirming the α-D- and α-L-configurations, respectively, for these sugars. The only substantial difference is seen for the D-glucuronate residues of **G4** and **G6**, which exhibit ^1J values of 163 and 164 Hz, respectively. Since differences of approximately 10 Hz have been reported between the α and β anomeric pairs of a number of sugars[23–26], the difference of approximately 10 Hz seen here, in comparison with the ^1J values for the α-L-iduronate residues, is consistent with the β configuration of the D-glucuronate residues determined to be present in heparin[27,28].

EXPERIMENTAL

N.m.r. spectra were recorded at 310 K on a Bruker AM 400 narrow-bore spectrometer, operating at 400 MHz for ^1H and 100 MHz for ^{13}C. Sample concentrations were in the range of 10–20mM. Heparin oligosaccharide concentrations were determined spectrophotometrically using the absorbance at 232 nm and an extinction coefficient of $\varepsilon = 5.2 \times 10^3 M^{-1} cm^{-1}$ for all species[22]. For one-dimensional proton-decoupled ^{13}C-n.m.r. spectra, low-power composite pulse decoupling[29] was employed. Inverse-detected two-dimensional ^{13}C–^1H heterocorrelation spectra were recorded using the pulse scheme of Bax *et al.*[30] without decoupling of carbon during aquisition; a sweep width of 1400 Hz in F_2 and 5400 in F_1 were employed. Typically, between 32 and 128 scans were collected for each of 256 t_1 values. The data in the F_1 dimension were zero filled to 2048 data points prior to Fourier transformation. A shifted sine bell weighting function, with a shift of 90° was used in both dimensions. Spectra were recorded without sample spinning. Chemical shifts are given relative to an external standard of mM sodium 2,2-dimethyl-2-silapentane-5-sulfonate in D_2O at 0 p.p.m. for both ^{13}C and ^1H chemical shifts.

Heparin fragments were generated by heparinase (EC 3.2.1.19) treatment of porcine intestinal mucosal heparin. Typically 250 units (1 unit will form 0.1 μmole of unsulfated uronic acid per h at pH 7.5 and 298 K) of heparinase I (Sigma) were added to 1.5 g of heparin in a pH 7.0 solution of 0.1M sodium acetate, mM $CaCl_2$, pH 7.0, containing 0.02% sodium azide. The reaction was carried out at room temperature and was followed by monitoring the change in absorbance at 232 nm, corresponding to generation of 4,5-unsaturated residues at sites of cleavage. After 96 h of reaction the change in optical density at 232 nm corresponded typically to the generation of approximately 180 μmoles of fragments. The reaction was stopped at this stage by freeze-drying the solution. The freeze-dried heparin fragments and salts were redissolved in a minimum volume of water and applied in three separate batches to a 2.5 × 125-cm P6 sizing column run in 0.5M ammonium hydrogencarbonate buffer, pH 7.0. This was run at a flow rate of 15 mL h^{-1} with a fraction size of 2.3 mL. A typical digestion yielded 8% octasaccharide, 11% hexasaccharide, 12% tetrasaccharide, and 23% disaccharide, expressed as mole percentages of the total fragments generated. Fractions that were homogeneous by size were pooled, freeze-dried to remove the ammonium hydrogencarbonate and then applied to a Q25 quaternary ammonium ion-exchange column (Pharmacia). Heparin fragments were eluted with a linear NaCl gradient from 0 to 1.2M NaCl. Fractions containing a given heparin fragment were pooled and desalted in an Amicon ultrafiltration cell fitted with a YC05 membrane, which has a nominal molecular weight cut-off of 500 daltons. Where necessary, heparin fragments were rechromatographed on Q25 and/or P6 resins to remove minor contaminants. The disaccharide pool yielded >95% **Δ2**, the tetrasaccharide pool yielded approximately 54% **I4** and 30% **G4**, and the hexasaccharide pool yielded approximately 42% **I6** and 21% **G6**. Products from three separate heparin digests were used to generate the tetra- and hexa-saccharide n.m.r. samples. Prior to examination by n.m.r.

spectroscopy, the samples were freeze-dried three times from D_2O to remove exchangeable protons. The pH of all samples, uncorrected for deuterium isotope effects, was pH 7.0. All carboxyl groups are therefore expected to be fully deprotonated[31]. All samples characterized by ^{13}C- or ^{1}H-n.m.r. spectroscopy were >90% pure, such that assignments were obtained unambiguously for the major species in each case.

ACKNOWLEDGMENTS

Brenda Crews is thanked for help in preparation and purification of the heparin oligosaccharides. Financial support from the National Institutes of Health through Grant HL32595 is gratefully acknowledged. The n.m.r. facility is supported in part by the Center in Molecular Toxicology through Grant ES 00267 from the National Institutes of Health.

REFERENCES

1 B. Casu, *Adv. Carbohydr. Chem. Biochem.*, 43 (1985) 51–134.
2 M. Cupp, A. Bensadoun, and K. Melford, *J. Biol. Chem.*, 262 (1987) 6383–6388.
3 R. E. Jordan, L. V. Favreau, E. H. Braswell, and R. D. Rosenberg, *J. Biol. Chem.*, 257 (1982) 400–406.
4 W. Hollander, *Exp. Mol. Pathol.*, 25 (1976) 106–120.
5 S. R. Srinivasan, C. Yost, B. Radhakrishnamurthy, E. R. Dalferes, Jr., and G. S. Berenson, *Atherosclerosis*, 38 (1981) 137–147.
6 K. M. Yamada, *Ann. Rev. Biochem.*, 52 (1983) 761 799.
7 S. Sakashita, E. Engwall, and E. Ruoslahti, *FEBS Lett.*, 116 (1980) 243–246.
8 B. Casu, P. Oreste, G. Torri, G. Zoppetti, J. Choay, J.-C. Lormeau, M. Petitou, and P. Sinaÿ, *Biochem J.*, 197 (1981) 599–609.
9 A. L. Stone, D. Beeler, G. Oosta, and R. D. Rosenberg, *Proc. Natl. Acad. Sci. U.S.A.*, 79 (1982) 7190–7194.
10 R. J. Linhardt, K. G. Rice, Y. S. Kim, J. D. Engelken, and J. M. Weiler, *J. Biol. Chem.*, 263 (1988) 13090–13096.
11 K. Bock, C. Pedersen, and H. Pedersen, *Adv. Carbohydr. Chem. Biochem.*, 42 (1984) 193–225.
12 Z. M. Merchant, Y. S. Kim, K. G. Rice, and R. J. Linhardt, *Biochem. J.*, 229 (1985) 369–377.
13 R. J. Linhardt, K. G. Rice, Z. M. Merchant, Y. S. Kim, and D. L. Lohse, *J. Biol. Chem.*, 261 (1986) 14448–14454.
14 M. Petitou, J.-C. Lormeau, B. Perly, P. Berthault, V. Bossennec, P. Sié, and J. Choay, *J. Biol. Chem.*, 263 (1988) 8685–8690.
15 A. Linker and P. Hovingh, *Carbohydr. Res.*, 127 (1984) 75–94.
16 A. Minoretti, W. P. Aue, M. Reinhold, and R. R. Ernst, *J. Magn. Reson.*, 40 (1980) 175–190.
17 D. H. Live, D. G. Davis, W. C. Agosta, and D. Cowburn, *J. Am. Chem. Soc.*, 106 (1984) 6104–6105.
18 A. P. Horne and P. Gettins, *Carbohydr. Res.*, (1992) in press.
19 G. Torri, B. Casu, G. Gatti, M. Petitou, J. Choay, J.-C. Jacquinet, and P. Sinaÿ, *Biochem. Biophys. Res. Commun.*, 128 (1985) 134–140.
20 A. S. Perlin, N. M. K. Ng Ying King, S. S. Bhatta-Charjee, and L. F. Johnson, *Can. J. Chem.*, 50 (1972) 2437–2442.
21 A. S. Perlin, D. M. Mackie, and C. P. Dietrich, *Carbohydr. Res.*, 18 (1971) 185–194.
22 A. Linker and P. Hovingh, *Anal. Biochem.*, 11 (1972) 563–568.
23 A. S. Perlin and B. Casu, *Tetrahedron Lett.*, (1969) 2921–2924.
24 A. S. Perlin, B. Casu, and H. J. Koch, *Can. J. Chem.*, 48 (1970) 2596–2606.
25 J. A. Schwarcz and A. S. Perlin, *Can. J. Chem.*, 50 (1972) 3667–3676.
26 K. Bock and C. Pedersen, *J. Chem. Soc., Perkin Trans. 2*, (1974) 293–297.
27 T. Helting and U. Lindahl, *J. Biol. Chem.*, 246 (1971) 5442–5447.
28 M. Höök, U. Lindahl, and P.-H. Iverius, *Biochem. J.*, 137 (1974) 33–43.
29 M. H. Levitt and R. Freeman, *J. Magn. Reson.*, 43 (1981) 502–507.
30 A. Bax, R. H. Griffey, and B. L. Hawkins, *J. Magn. Reson.*, 55 (1983) 301–315.
31 G. Gatti, B. Casu, G. K. Hamer, and A. S. Perlin, *Macromolecules*, 12 (1979) 1001–1007.