Carbohydrate Research, 225 (1991) 43–57
Elsevier Science Publishers B.V., Amsterdam

^{1}H-N.m.r. spectral assignments for two series of heparin-derived oligosaccharides

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

(Received August 13th, 1991; accepted September 24th, 1991)

ABSTRACT

Six heparin-derived oligosaccharides, ranging in size from di- to octa-saccharide and forming two closely related series differing in structure by the substitution of an unsulfated D-glucuronate for a 2-sulfated L-iduronate residue, have been characterized by 2-dimensional ^{1}H-n.m.r. spectroscopy. In addition to providing new data on hexa- and octa-saccharides, several important changes to previously published data have been found for the two tetrasaccharides. The D-glucuronic acid H-5 proton is assigned to a resonance in the same region as resonances for the H-3 and H-4 D-glucuronate protons, rather than downfield from these resonances as earlier reported. The presence of D-glucuronic acid in the heparin sequence of the series-1 fragments affects the positions of neighboring D-glucosamine resonances, in particular shifting the anomeric proton signal in the preceding D-glucosamine 0.1–0.2 p.p.m. downfield. Resonances from the reducing-end D-glucosamines differ from internal D-glucosamine resonances both in relative position and in the degree of chemical shift difference between the H-6 and H-6′ protons. This work illustrates the usefulness of two-dimensional techniques in determining heparin structure and emphasizes the need for direct analysis, rather than assignment by comparison to model compounds.

INTRODUCTION

Heparin is a heterogeneous glycosaminoglycan which plays a role in several important physiological processes including platelet activation[1,2], fibronectin binding[3], lipoprotein lipase release[4,5] and inhibition of coagulation[6–8]. This polysaccharide is composed of alternating hexuronic acid and D-glucosamine residues, though variation in the hexuronic acid residue (L-iduronic *vs.* D-glucuronic) and in the *N*- and *O*-sulfation patterns generated during biosynthesis[9–11] gives rise to considerable heterogeneity. L-Iduronate-containing glycosaminoglycans such as heparin and dermatan sulfate appear to be more physiologically potent than their counterparts containing a larger percentage of D-glucuronic acid (heparan sulfate and chondroitin 4-sulfate). One reason postulated for this fact is that flexibility in the conformation of the L-iduronate ring is important in orienting the carboxyl groups[12]. The extent of sulfation plays an important role in protein interactions, as demonstrated by heparin's greater potential as an anticoagulant compared to the undersulfated heparan sulfate. Some interactions, such as those between heparin and fibronectin or thrombin, appear to be nonspecific

* To whom correspondence should be addressed.

0008-6215/92/$05.00

charge–charge interactions independent of the particular heparin composition[13]. Other interactions are much more dependent on heparin composition and specific sulfation patterns, the most studied example being the interaction between the plasma serine proteinase inhibitor antithrombin and specific antithrombotic heparin fragments[14–16]. Certain sequences within the heparin polysaccharide have high affinity for the antithrombin molecule and act by catalytically enhancing the basal rate of proteinase inhibition 1000-fold[17]. Further understanding of this and other physiological roles for heparin will depend on structural analysis of the interaction of biologically active heparin oligosaccharides with their target proteins.

N.m.r. spectroscopy is a useful tool in characterizing carbohydrate structure[18]. Structural parameters including sugar content, ring conformation, glycosidic linkage, and sulfation patterns can be determined from analysis of chemical shifts and coupling constants[12,19–24]. In addition, n.m.r. spectroscopy has the advantage of being a rapid as well as a nondegradative procedure. The present work describes the characterization by 2-dimensional n.m.r. (2D n.m.r.) spectroscopy of several low molecular weight heparin oligosaccharides (isolated using size-exclusion and anion-exchange chromatography) which form two series of heparin oligosaccharides related in structure. One series (designated "series-2", reflecting the position of elution from the anion-exchange column) is composed of the fully sulfated tetra-, hexa- and octa-saccharide, formed from multiple repeats of the disaccharide β-L-idopyranuronic acid 2-sulfate (1→4) 2-amino-2-deoxy-D-glucos 6,*N*-disulfate. The tetra- and hexa-saccharide in the second group (designated "series-1") differ in the substitution of α-D-glucopyranuronic acid for one of the β-L-idopyranuronic acid residues. The octasaccharide from series-1 was not obtained in sufficiently pure form to perform unambiguous n.m.r. analysis. A disaccharide containing the 4,5-unsaturated, sulfated uronic acid linked to 2-amino-2-deoxy-D-gluco 6,*N*-disulfate serves as the smallest member of each series, and is included for comparative purposes.

RESULTS AND DISCUSSION

^{1}H-n.m.r. spectral assignments. — The structures of the six heparin oligosaccharides examined in this study are given in Fig. 1. The abbreviations used to designate different sugar residues are arranged such that the 4,5-unsaturated sugar is always residue A, with the adjacent D-glucosamine labeled as residue B. The series-2 heparin fragments are fully-sulfated multiple repeats of the disaccharide β-L-idopyranosyluronic acid 2-sulfate-(1→4) 2-amino-2-deoxy-D-glucosamine 6,*N*-disulfate. The series-1 oligosaccharides, which elute from an anion-exchange column at a lower salt concentration, differ from the series-2 fragments in the substitution of α-D-glucopyranuronic acid for the β-L-idopyranuronic acid residue at the reducing end of the oligomer. The ^{1}H-n.m.r. spectra for the series-2 and series-1 fragments are given in Figs. 2 and 3, respectively.

Assignments of the ^{1}H resonances for the six oligosaccharides were made from analyses of two-dimensional correlation (COSY) spectra. These assignments are more

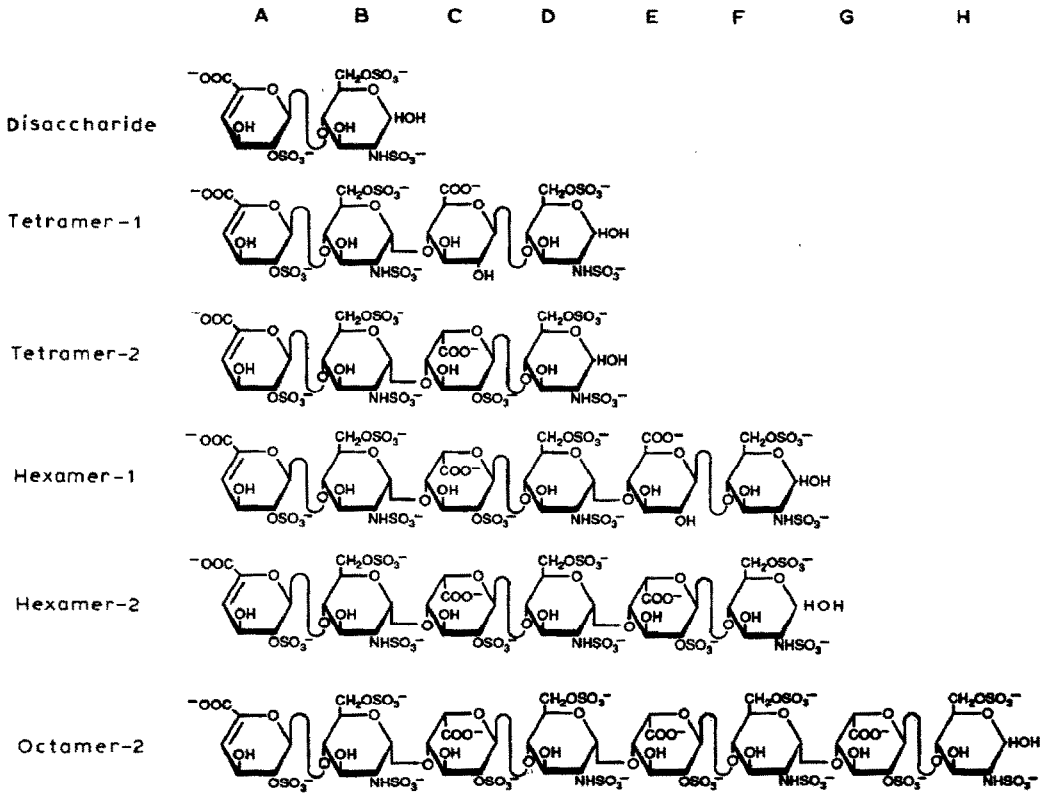

Fig. 1. Structures of the six oligosaccharides examined in the present study, and the lettering system used to specify particular sugar residues in each oligosaccharide.

definitive than those made using one-dimensional techniques, such as selective decoupling and comparison to model compound spectra, because unambiguous through-bond couplings are displayed. 2D-N.m.r. techniques also have the advantage of spreading the information in a densely populated region of the spectrum across a larger area, thus separating at least some of the overlapping resonances in the second dimension. Since all of the connectivities are examined in one experiment, there is no need for selective excitation of specific resonances, again made difficult by overlapping resonances. ^{1}H Assignments for the different component sugars are given for the 4,5-unsaturated residues (Table I), the reducing end D-glucosamines (Table II), the internal D-glucosamines (Table III), and internal hexuronic acid residues (Table IV). Coupling constants are given for the hexuronate and D-glucosamine residues in Tables V and VI, respectively).

Comparison of hexuronate assignments. — Hexasaccharide-1 contains all three types of hexuronic acid found in heparinase-cleaved heparin fragments, *viz.* L-iduronate, D-glucuronate, and the terminal 4,5-unsaturated residue. This oligosaccharide thus provides a good example for comparing the major differences observed in the ^{1}H resonances of the uronic acid residues. The COSY spectrum of this hexasaccharide is

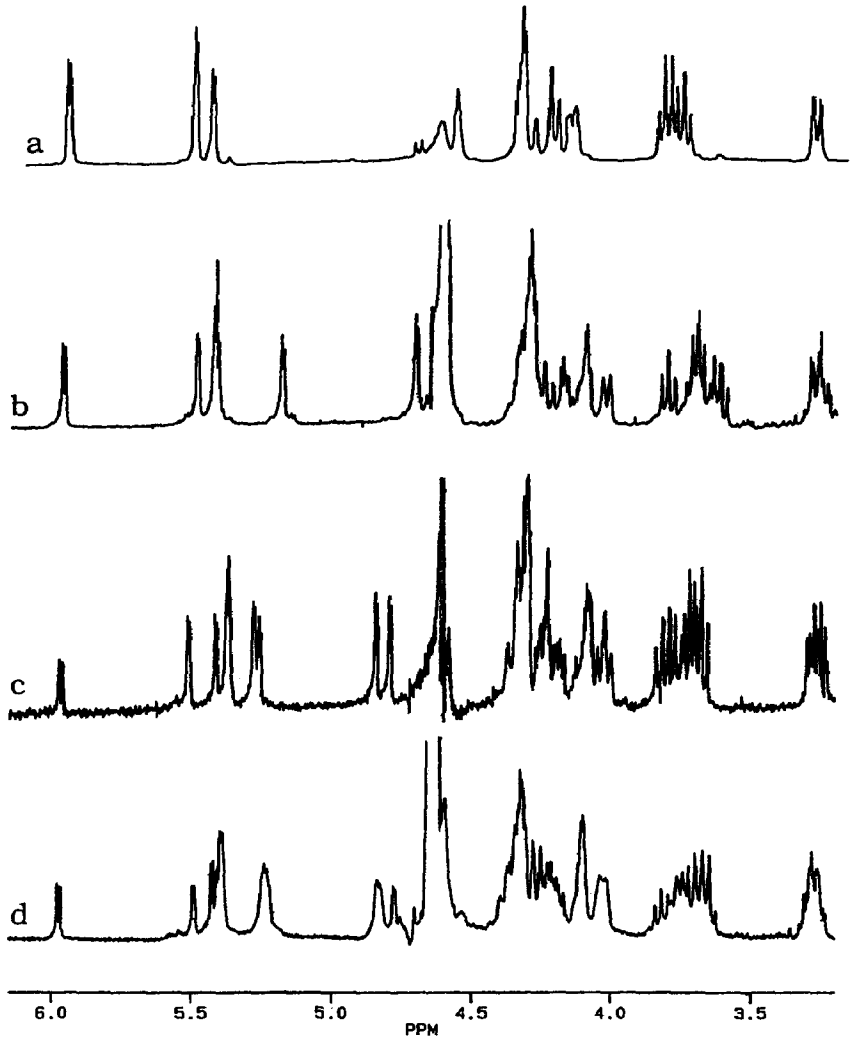

Fig. 2. 400 MHz ^{1}H-n.m.r. spectra of the disaccharide and the series-2 heparin oligosaccharides, collected at 310 K and neutral pH: (a) disaccharide; (b) tetrasaccharide-2; (c) hexasaccharide-2; (d) octasaccharide-2.

TABLE I

^{1}H-N.m.r. assignments for the protons of the 4,5-unsaturated residue in six heparin oligosaccharides at 310 K and pH 7.0[a]

Residue	*H-1*	*H-2*	*H-3*	*H-4*
Disaccharide	5.488	4.546	4.320	5.944
Tetrasaccharide-2	5.485	4.599	4.289	5.964
Tetrasaccharide-1	5.496	4.598	4.298	5.972
Hexasaccharide-2	5.494	4.594	4.302	5.970
Hexasaccharide-1	5.499	4.612	4.306	5.982
Octasaccharide	5.485	4.597	4.318	5.972

[a] The estimated error in the resonance assignments is ± 0.001 p.p.m.

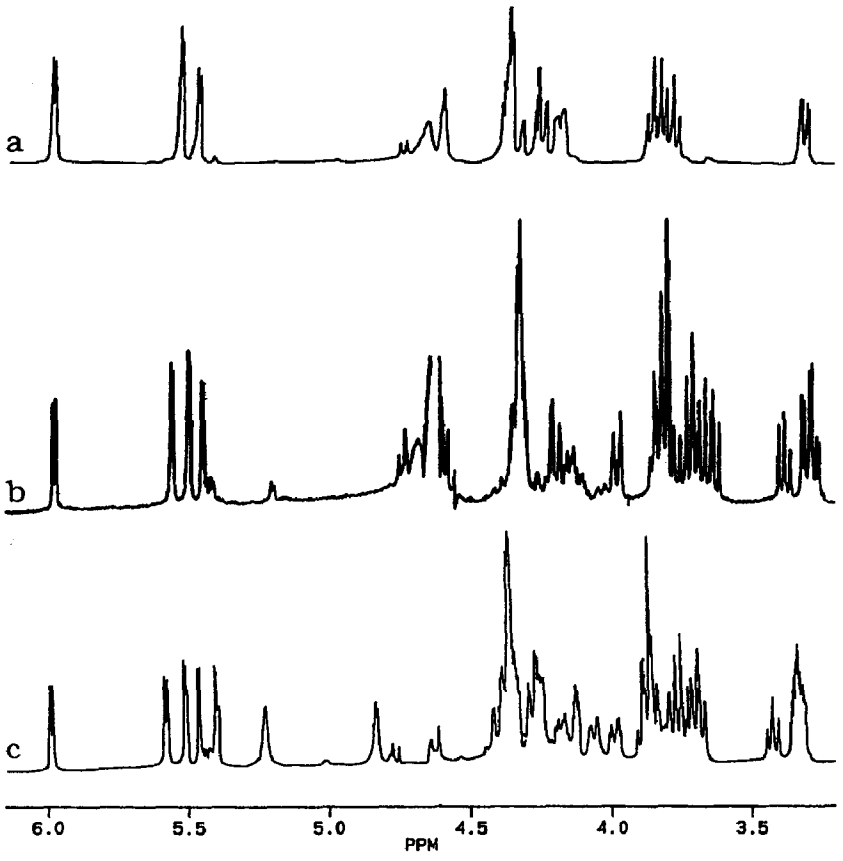

Fig. 3. 400 MHz ^{1}H-n.m.r. spectra of the disaccharide and the series-1 heparin oligosaccharides, collected at 310 K and neutral pH: (a) disaccharide; (b) tetrasaccharide-1; (c) hexasaccharide-1.

TABLE II

^{1}H-N.m.r. assignments for the protons of the reducing end D-glucosamine residue in six heparin oligosaccharides at 310 K and pH 7.0[a]

Residue	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6',6*
Disaccharide	5.436	3.252	3.732	3.799	4.133	4.195, 4.320
Tetrasaccharide-2	5.423	3.243	3.667	3.728	4.111	4.280, 4.327
Tetrasaccharide-1	5.446	3.265	3.727	3.695	4.146	4.315, 4.374
Hexasaccharide-2	5.421	3.252	3.707	3.744	4.115	4.272, 4.314
Hexasaccharide-1	5.443	3.263	3.721	3.698	4.154	4.301, 4.363
Octasaccharide-2	5.425	3.254	3.696	3.739	4.120	4.294, 4.347

[a] The estimated error in the resonance assignments is ± 0.001 p.p.m.

TABLE III

^{1}H-N.m.r. assignments for the protons of the internal hexuronic acid in six heparin oligosaccharides at 310 K and pH 7.0[a]

L-*Iduronate 2-sulfate residue*	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*
Tetrasaccharide-2[b]	5.181	4.293	4.176	4.089	4.707
Hexasaccharide-2[b]	5.191	4.31	4.190	4.097	4.802
[c]	5.191	4.325	4.237	4.081	4.852
Hexasaccharide-1[b]	5.221	4.340	4.222	4.087	4.796
Octasaccharide-2[d]	5.23	4.34	4.218	4.105	4.835
	5.23	4.34	4.236	4.095	4.826
	5.23	4.32	4.184	4.111	4.775
D-*Glucuronate residue*	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*
Tetrasaccharide-1[b]	4.573	3.373	3.836	3.773	3.8
Hexasaccharide-1[c]	4.595	3.389	3.850	3.805	3.8

[a] The estimated error in the resonance assignments is ±0.001 p.p.m. [b] Assignments are for residue C within the given oligosaccharide. [c] Assignments are for residue E within the given oligosaccharide. [d] Position within the octasaccharide has not been determined.

TABLE IV

^{1}H-N.m.r. assignments for the protons of the internal D-glucosamine residue in six heparin oligosaccharides at 310 K and pH 7.0[a]

Residue	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6',6*
Tetrasaccharide-2[b]	5.414	3.278	3.618	3.805	4.025	4.233, 4.320
Tetrasaccharide-1[b,e]	5.555	3.291	3.638	3.818	3.975	4.190, 4.337
Hexasaccharide-2[b]	5.384	3.293	3.683	3.824	4.046	4.248, 4.361
[c]	5.391	3.285	3.638	3.781	4.021	4.221, 4.325
Hexasaccharide-1[b]	5.378	3.297	3.653	3.825	4.035	4.252, 4.359
[c,e]	5.572	3.280	3.660	3.757	3.966	4.234, 4.371
Octasaccharide-2[d]	5.401	3.269	3.671	3.741	4.04	4.267, 4.387
[f]	5.391	3.289	3.647	3.817	4.04	4.257, 4.387

[a] The estimated error in the resonance assignments is ±0.001 p.p.m. [b] Assignments are for residue B within the given oligosaccharide. [c] Assignments are for residue D within the given oligosaccharide. [d] Position within the octasaccharide has not been determined. [e] D-glucosamine precedes a D-glucuronate acid. [f] Assignments represent more than one resonance.

shown in Fig. 4. The nonreducing-end sugar protons resonate at unique positions, due to the double bond in the sugar ring. These resonances are readily assigned in the COSY spectrum and occur at similar positions in five of the six heparin fragments (Table I). The chemical shifts of the disaccharide residue A resonances differ from those of the larger fragments by as much as 0.07 p.p.m., reflecting the atypical composition of the disaccharide fragment (having the reducing-end and nonreducing-end residues directly linked). Resonances from the internal L-iduronic acid 2-sulfate differ from fragment to

TABLE V

^{1}H-N.m.r. coupling constants for hexuronate residues[a]

4,5-Unsaturated residue	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$ *(Hz)*	
Disaccharide	3.2	3	4.4	
Tetrasaccharide-2	2.4	4	4.8	
Tetrasaccharide-1	2.0	4	4.6	
Hexasaccharide-2	2.3	5	4.7	
Hexasaccharide-1	2.4	5	4.8	
Octasaccharide-2	2.8	3	4.8	
Internal L-*iduronic acid*	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$ *(Hz)*
Tetrasaccharide-2[b]	3.5	6	4	3.0
Hexasaccharide-2[b]	2.6	5	4	2.6
[c]	3.1	5	3	2.9
Hexasaccharide-1[b]	2.5	6	3	2.7
Octasaccharide-2[d]	3.5	6	3	3.5
	3.5	6	3	3.5
	3.5	5	4	2.8
Internal D-*glucuronic acid*	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$ *(Hz)*
Tetrasaccharide-1[b]	8	8.4	8	n.d.
Hexasaccharide-1[c]	8	8.6	9	n.d.

[a] The estimated error in the coupling constants is ± 0.1 Hz for data obtained from the 16K spectra. *J*-values are given to one significant figure. Data from the COSY plots for resonances in crowded regions of the spectrum are resolved to 0.8 Hz and are rounded to ± 1 Hz. [b] ^3J-values are for residue C within the given oligosaccharide. [c] ^3J-values are for residue E within the given oligosaccharide. [d] Position within the octasaccharide has not been determined. [e] ^3J-values represent more than one resonance.

fragment, as well as from those of the 4,5-unsaturated uronic acid or the unsulfated D-glucuronate residue in the series-1 fragments. Even in fragments as large as octasaccharide-2, which contains three internal L-iduronate 2-sulfate residues, the resonances from the different L-iduronate 2-sulfate residues are distinguishable, indicating that their conformations differ within the oligosaccharide[25].

The presence of a D-glucuronic acid in the series-1 heparin fragments is indicated by a characteristic triplet from the D-glucuronate H-2 proton at 3.38 p.p.m. THe D-glucuronate resonances from tetrasaccharide-1 and hexasaccharide-1 differ to a lesser extent than the L-iduronate 2-sulfate resonances, due to the lessened conformational flexibility of the D-glucuronate structure[26]. The position of the D-glucuronic acid residue within hexasaccharide-1 can be unambiguously determined from the ^{13}C-n.m.r. resonances for the same fragment[25]. As a result of linkage to the D-glucuronic acid residue, the ^{13}C-n.m.r. resonance from the reducing end D-glucosamine C-4 is perturbed in the series-1 fragments as compared to that of the series-2 fragments. From the analysis of the COSY spectra, it was found that the D-glucuronate H-5 proton resonates in the same region of the n.m.r. spectrum (3.8 p.p.m.) as the D-glucuronate H-3 and H-4 resonances

TABLE VI

^{1}H-N.m.r. coupling constants for D-glucosamine residues[a]

Reducing D-*glucosamine*	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$	$J_{5,6'}$	$J_{6,6'}$
Disaccharide	3.4	10.0	9.0	9.5	3.9	2.1	11.0
Tetrasaccharide-2	3.6	10.1	8.8	10	n.d.	n.d.	12
Tetrasaccharide-1	3.2	10.2	9.2	n.d.	n.d.	n.d.	11
Hexasaccharide-2	3.5	10.9	9	10	<5	n.d.	12
Hexasaccharide-1	3.6	9.4	n.d.	n.d.	3	<2	10
Octasaccharide-2	3.5	10	11	n.d.	4	<3	11
Internal D-*glucosamine*							
$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$	$J_{5,6'}$	$J_{6,6'}$	
Tetrasaccharide-2[b]	3.6	10.5	9	10	n.d.	n.d.	11
Tetrasaccharide-1[b,f]	3.6	10.9	11	10	n.d.	n.d.	11
Hexasaccharide-2[b]	3.5	10	10	10	<5	<3	11
[c]	3.6	n.d.	10	9	<5	<3	11
Hexasaccharide-1[b]	3.6	10.2	10	10	<3	<3	11
[c,f]	3.6	10.4	11	10	<3	<3	11
Octasaccharide-2[d]	4.2	10	9	9	5	3	12
[d,e]	2.8	10	9	9	5	3	12

[a] The estimated error in the coupling constants is ± 0.1 Hz for data obtained from the 16K spectra. *J*-values are given to one significant figure. Data from the COSY plots for resonances in crowded regions of the spectrum are resolved to 0.8 Hz and rounded to ± 1 Hz. n.d., not determined. [b] ^3J-values are for residue B within the given oligosaccharide. [c] ^3J-values are for residue D within the given oligosaccharide. [d] Position within the octasaccharide has not been determined. [e] ^3J-values represent more than one resonance. [f] D-glucosamine precedes a D-glucuronic acid.

in the two D-glucuronic acid-containing oligosaccharides examined. These assignments differ from those previously published[23,27], in which the D-glucuronate H-5 is assigned to a resonance in the region of 3.94–3.99 p.p.m., *i.e.*, downfield from the H-3 and H-4 resonances at 3.71–3.87 p.p.m. In the previous studies, the spectra were collected at either an unspecified temperature and pH[23], or at an unspecified pH and ambiguous temperature (both 25 and 70°)[27]. The present assignments are, however, in good agreement with those obtained by 2D n.m.r. analysis of a synthetic heparin pentasaccharide corresponding to the minimal antithrombin binding sequence. This pentasaccharide contains a D-glucuronic acid residue flanked by one di- and one tri-sulfated D-glucosamine[28]. The D-glucuronate H-5 in this fragment is assigned to a resonance at 3.77 p.p.m. In the enlarged region of the COSY spectrum for hexasaccharide-1 shown in Fig. 5, the resonance at 3.99 p.p.m. is assigned to a D-glucosamine H-5 proton, with connectivities clearly visible to both the D-glucosamine H-4 and H-6. There are no other crosspeaks present to suggest connectivity between a D-glucuronate H-5 peak underneath the D-glucosamine H-5 and the D-glucuronic acid H-4 proton at 3.80 p.p.m. In further support of the location of the D-glucuronic acid H-5 proton, the integrated area of this region (3.6–3.9 p.p.m.) of the hexasaccharide-1 spectrum, when compared to that for a single proton, accounts for the presence of the three D-glucuronate protons (H-3, 4,

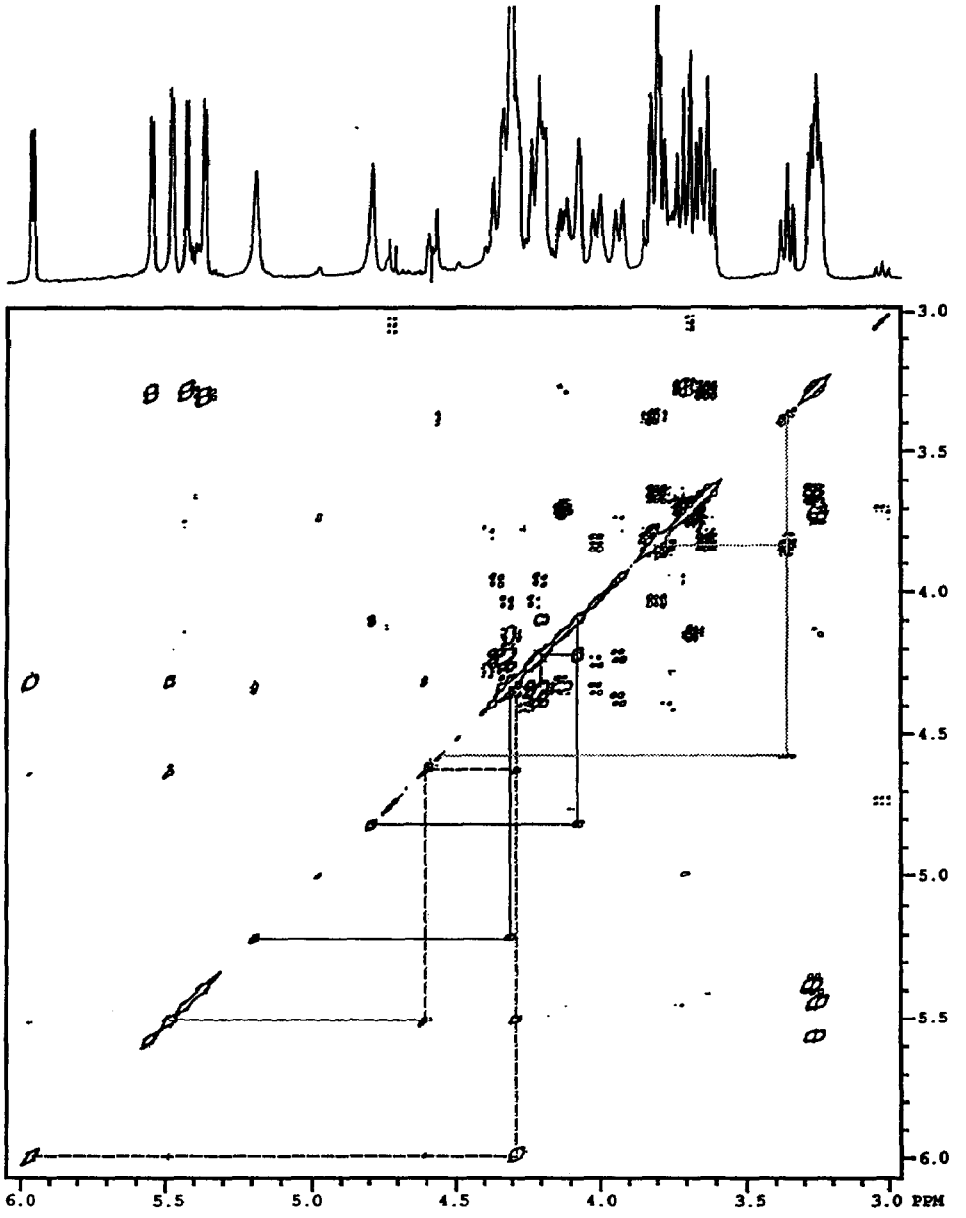

Fig. 4. 400 MHz homonuclear ^{1}H COSY plot of hexasaccharide-1, showing the connectivity among resonances assigned to the 4,5-unsaturated residue (dashed line), L-iduronate 2-sulfate residue (solid line), and D-glucuronate residue (dotted line).

and 5) as well as three sets of D-glucosamine H-3 and H-4 protons. Another minor difference in the published assignments of the D-glucuronic acid resonances is in the position of the H-2 proton (3.38 p.p.m). Merchant *et al.*[27] assign resonances at 3.27–3.30 p.p.m. to this proton, a region shown by the 2D COSY analysis (Figs. 4 and 5) to contain only D-glucosamine H-2 resonances.

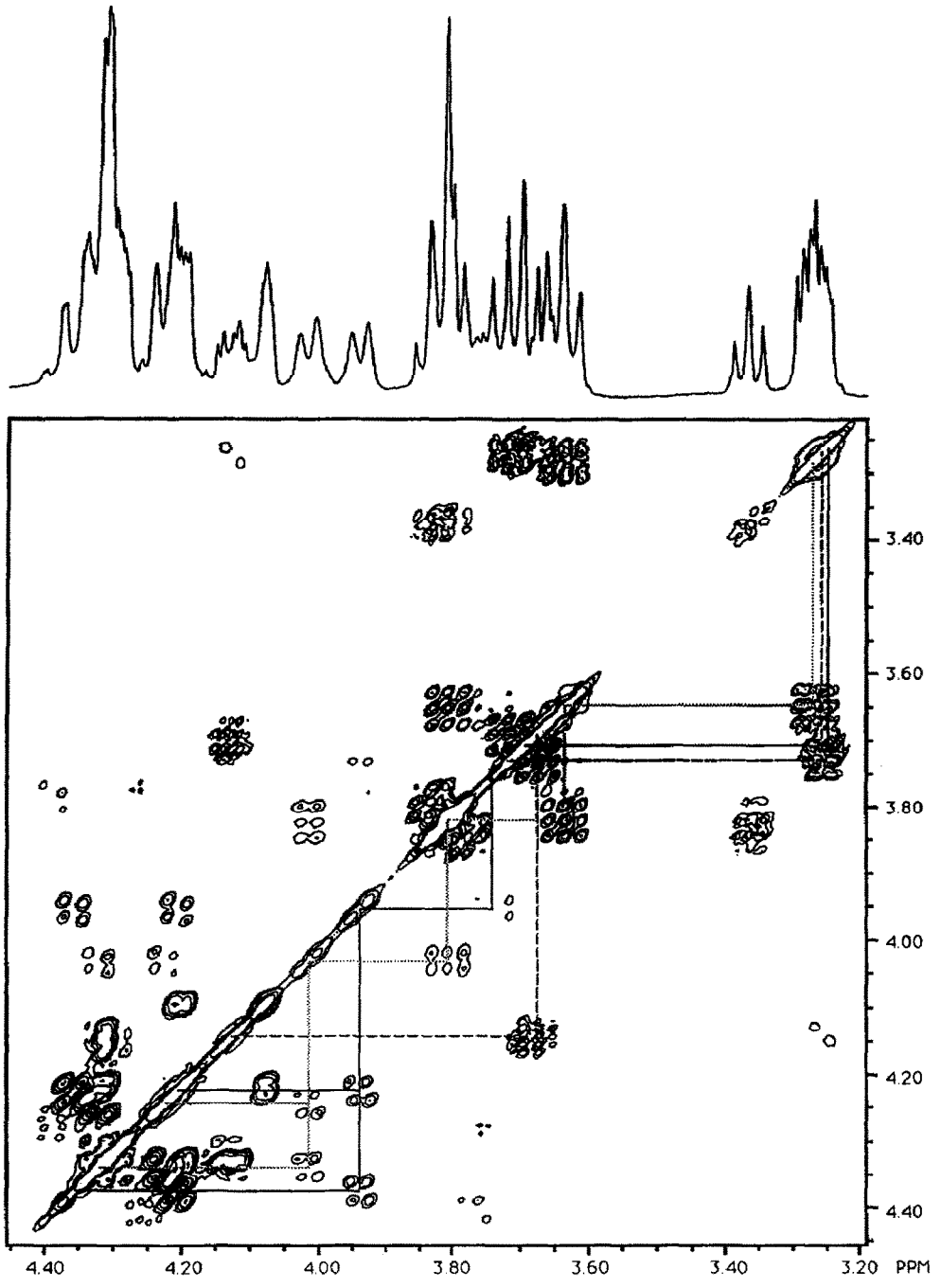

Fig. 5. Enlarged region (3.2–4.1 p.p.m.) of the ^{1}H COSY plot of hexasaccharide-1 showing the connectivity among resonances assigned to the reducing end D-glucosamine (dashed line), internal D-glucosamine preceding a D-glucuronate residue (solid line), and internal D-glucosamine preceding an L-iduronate 2-sulfate residue (dotted line).

Comparison of hexosamine assignments. — Assignment of the reducing-end D-glucosamine is possible by two independent means. One depends upon the equilibrium between α and β anomers for each heparin fragment, giving rise to distinct and detectable resonances for each species. The population distribution of approximately 85% α, 15% β results in less intense resonances for the α anomer reducing-end H-1 and H-2 protons compared to the internal D-glucosamine protons. The second means of assignment comes from the ^{1}H–^{13}C inverse heterocorrelation data for the two tetra- and two hexa-saccharides[25]. The characteristic upfield chemical shift of the reducing-end anomeric carbon together with the crosspeak in the heterocorrelation data provide an unambiguous assignment of the reducing-end D-glucosamine H-1 resonance. The COSY data for the two series of oligosaccharides indicate that the reducing-end D-glucosamine H-2 and H-4 resonances lie upfield of those for the internal D-glucosamine, while the H-3 and H-5 resonances occur further downfield than their counterparts. Another distinction between reducing-end and internal D-glucosamines is seen in the relative positions of the H-6 and H-6′ resonances. Excluding the disaccharide H-6,6′ protons, the difference in chemical shift for this pair of protons is significantly less for a reducing-end D-glucosamine (0.05 p.p.m.) than for an internal residue (0.1–0.15 p.p.m.). This was also seen but not commented upon by Torri *et al.*[28] for their synthetic pentasaccharide. The difference in position of the disaccharide H-6 and H-6′ is 0.13 p.p.m., again reflecting its unusual composition.

The D-glucosamine resonances are sensitive to the neighboring hexuronic acids as well as to position (internal *vs.* reducing-end) within the heparin fragments. The presence of a D-glucuronate residue in the series-1 fragments has an effect on the resonances of the adjacent reducing-end D-glucosamine, most notably the H-3 and H-4 resonances. Due to the downfield shift of the H-3 resonance and a concomitant upfield shift in position of the H-4 resonance, their relative positions are reversed compared to their series-2 counterparts. Even more dramatic is the effect that the D-glucuronate has on the anomeric proton of the preceding D-glucosamine (residue B in tetrasaccharde-1 and residue D in hexasaccharide-1). The ^{1}H-n.m.r. spectra of the series-1 heparin fragments indicate that the position of this anomeric proton (5.56–5.59 p.p.m.) is consistently shifted 0.1–0.2 p.p.m. downfield compared to other internal or reducing-end D-glucosamine H-1 resonances (5.38–5.45 p.p.m.). This is corroborated by ^{1}H-n.m.r. data published for the synthetic, high-affinity pentasaccharide[28] containing a nonreducing-end D-glucosamine followed by a D-glucuronic acid residue. The D-glucosamine anomeric proton resonates at 5.62 p.p.m., which is downfield compared to both the reducing-end D-glucosamine H-1 (5.44 p.p.m.) and the trisulfated internal D-glucosamine H-1 (5.51 p.p.m.). Neither Merchant *et al.*[27] nor Linker and Hovingh[23] described any unusual chemical shift (5.34–5.36 p.p.m.) for the D-glucosamine anomeric proton linked to a D-glucuronic acid in their purified tetrasaccharides. Similar effects are seen in the ^{13}C spectra of these heparin fragments. The chemical shift of the D-glucosamine anomeric carbon differs by 0.5–1.2 p.p.m., depending upon whether it is linked to D-glucuronate or L-iduronate 2-sulfate[25].

Effects of fragment length. — The chemical shifts given in Tables I–IV can be used to make comparisons among the six oligosaccharides which are structurally related according to length. The resonances from the 4,5-unsaturated residue A in each fragment do not differ greatly. The largest differences seen are for the disaccharide (0.03–0.07 p.p.m.), due to this residue's atypical linkage to a reducing-end 2-amino-2-deoxy-D-glucosamine 6,*N*-disulfate. The disaccharide reducing-end resonance assignments also differ from the other reducing-end assignments for the same reason. Positions of the anomeric ^{1}H-n.m.r. resonances for tetrasaccharide-1 residue B and hexasaccharide-1 residue D are perturbed as compared to the corresponding resonances in the series-2 fragments. The difference in position of the D-glucosamine H-1 resonance between tetrasaccharide-1 and tetrasaccharide-2 is 0.141 p.p.m., while the difference in chemical shift for these resonances in the hexasaccharide fragments is 0.181 p.p.m. This difference in chemical shift differences of 0.040 between the tetra- and hexa-saccharides must arise from differences in the overall tertiary structure of the oligosaccharides. Similar conclusions can be drawn from differences in perturbations in the reducing-end D-glucosamine resonances. The difference in position of the tetrasaccharide H-4 resonances is 0.033 p.p.m., compared to a 0.046 p.p.m. difference in position of the hexasaccharide H-4 resonances. The H-3 resonances differ in position as well, by 0.060 p.p.m. for the tetrasaccharides and by 0.014 p.p.m. for the hexasaccharides. Since similar changes (the substitution of D-glucuronic acid for L-iduronate 2-sulfate) are occurring in both lengths of heparin fragment, these differences must also arise from variations in the overall structure of these fragments.

Comparison of coupling constants. — Coupling constants for the hexuronate and D-glucosamine residues were determined from the one-dimensional ^{1}H-n.m.r. spectra (resolution of 0.1 Hz/pt.), and are given in Tables V and VI. For resonances that overlapped severely in the 1D spectra, the coupling constants were determined from crosspeaks in the 2D COSY spectra (resolution of 0.8 Hz/pt.). Values are similar to those reported in the literature for similar heparin tetra- and hexa-saccharides[12,20,23,24,29], as well as for the high-affinity pentasaccharide[28]. The smaller ^3J-values for the L-iduronate 2-sulfate protons of 2–4 Hz (Table V) are indicative of the $^1C_4$ conformation in which the adjacent protons are *gauche* to one another[12,20]. Likewise, the larger ^3J-values of 7–9 Hz for the D-glucuronate ^{1}H resonances are consistent with a $^4C_1$ conformation. Intermediate values for the 4,5-unsaturated residue are probably due to distortion of the ring structure by the unsaturated bond. The D-glucosamine coupling constants are small for $J_{1,2}$ and large for $J_{2,3}$, $J_{3,4}$, and $J_{4,5}$, indicating that these residues are in the $^4C_1$ conformation.

CONCLUSIONS

In summary, this paper describes the ^{1}H-n.m.r. characterization of six related oligosaccharides and compares the chemical shifts of the resonances. 2D N.m.r. methods can be used to assign all of the oligosaccharide resonances. Several important changes to previously published data are described. The D-glucuronic acid H-5 protons

in tetrasaccharide-1 and hexasaccharide-1 are assigned to resonances in the same region as (rather than downfield from) the H-3 and H-4 D-glucuronate protons. The presence of D-glucuronic acid in these fragments affects the positions of neighboring D-glucosamine resonances, in particular shifting the anomeric proton signal in the preceding D-glucosamine 0.1–0.2 p.p.m. downfield and reversing the order of the H-3 and H-4 resonances. The position of the residue within the oligosaccharide and the nature of the neighboring residues affect the chemical shifts of the constituent resonances to a greater extent (0.1–0.2 p.p.m. for the D-glucosamine anomeric proton adjacent to D-glucuronic acid) than the length of the oligosaccharide (0.04 p.p.m. for this same proton). Variations in chemical shift among internal L-iduronate 2-sulfate resonances and differences in chemical shift differences which depend on length both indicate that the overall conformation of the oligosaccharides differs among structurally related fragments.

EXPERIMENTAL PROCEDURES

Materials. — Porcine intestinal mucosal heparin, *Flavobacterium* heparinase (EC 4.2.2.7), and other chemicals were obtained from Sigma Chemical Co. Bio-Gel P6 matrix and QAE-25 strong anion-exchange resin were purchased from BioRad Laboratories. The h.p.l.c. column used in charge separations was a 5 mm × 25 cm Spherisorb strong anion-exchange column from Phase Separations (Norwalk, CT).

Heparin depolymerization. — Porcine intestinal mucosal heparin was cleaved using the enzyme heparinase. One to two grams of heparin were dissolved in 50 mL of a solution containing 0.1M sodium acetate pH 7.0, mM calcium chloride, and 0.02% sodium azide, and combined with one vial of heparinase I (250 units, where one unit is defined as the quantity of enzyme necessary to form 0.1 μmol of unsaturated uronic acid product per hour at pH 7.5 and 25°)[30]. The reaction proceeded at room temperature, and generation of cleaved fragments, concomitant with formation of a 4,5-unsaturated moiety, was monitored by change in absorbance measurements at 232 nm, using an extinction coefficient of 5200 M^{-1} cm^{-1} for all species[31]. The reaction was stopped by lyophilization when a constant absorbance was reached (after ~ 100 h).

Size-exclusion chromatography. — The lyophilized heparinase fragments were dissolved in a small volume of water and applied in aliquots of 100–150 absorbance units to a 125 x 2.5 cm Bio-Gel P6 column equilibrated in 0.5M ammonium hydrogencarbonate. The lengths of the pooled oligosaccharides in each heparin fraction were confirmed by analysis of the ^{1}H-n.m.r. spectra. For a given oligosaccharide, the area of a spectrum is proportional to the number of protons contributing to the spectrum. The lower molecular weight heparin oligosaccharides were pooled according to size, refractionated, and desalted by repeated lyophilization.

H.p.l.c. — Separation by charge of the tetra-, hexa-, and octa-saccharide pools was accomplished by strong anion-exchange h.p.l.c. using a Waters system. Two Model 510 pumps running at a flow rate of 1.5 mL/min were used to generate a linear gradient of NaCl at pH 3.5, as described by Rice *et al.*[32]. Fractions were collected for each peak eluting from the column, as monitored by absorbance at 214 nm (Model 440 Waters u.v.

detector). The disaccharide samples were fractionated over a 25 x 2.5 cm QAE-25 anion-exchange column; 1-mL fractions were collected and monitored at 232 nm. The two major peaks from each h.p.l.c. profile were desalted by either Amicon ultrafiltration through a YC05 membrane (cutoff at 500 daltons) or equilibrium dialysis with Spectra-Por 6 dialysis tubing (cutoff at 1000 daltons). The major disaccharide peak was desalted over a G-10 desalting column.

N.m.r. spectroscopy. — The homogeneous, desalted heparin samples were lyophilized, dissolved in D_2O, and examined with a Bruker AM narrow-bore spectrometer operating at 400 MHz for ^1H. The pH value (uncorrected for deuterium isotope effects) was adjusted to pH 7.0 for each sample using an Ingold 3-mm diameter combination electrode. Experiments were performed at 310 K (37°) on 400-μL samples at 5–10mM concentration. This temperature was chosen to provide chemical shift data at the same temperature (physiological) as that used for separate antithrombin–heparin fragment-binding studies[33]. ^1H-n.m.r. spectra were collected using a block size of 8K and a spectral width of 6 p.p.m. The data were zero-filled to 16K prior to transformation. Correlated spectroscopy (COSY) experiments were performed using a block size of 2K in the F_2 dimension and 1K in the F_1 dimension, with a sweep width of 4 p.p.m. The data were transformed using a sine-shifted bell function. Assignments for the ^1H spectra were made from COSY data for each component sugar. Chemical shifts were determined with reference to external sodium 3-trimethylsilyl-1-propanesulfonate (DSS).

ACKNOWLEDGMENTS

This work was supported by NIH grant HL32595. The n.m.r. facility is supported in part by the Center in Molecular Toxicology through NIH grant ES00267.

REFERENCES

1 S. F. Mohammad, W. H. Anderson, J. B. Smith, Y. K. Chuang, and R. G. Mason, *Am. J. Pathol.*, 104 (1981) 132–141.
2 L. D. Brace and J. Fareed, *Thromb. Res.*, 42 (1986) 769–782.
3 D. E. Smith and L. T. Furch, *J. Biol. Chem.*, 257 (1982) 6518–6524.
4 E. D. Korn, *J. Biol. Chem.*, 215 (1955) 1–14.
5 T. Olivecrona, G. Bengtsson, S.-E. Marklund, U. Lindahl, and M. Höök, *Fed. Proced. Am. Soc. Exp. Biol.*, 36 (1977) 60–65.
6 R. D. Rosenberg, *Triangle*, 23 (1984) 43–48.
7 I. Björk and Ä. Danielsson, in A. J. Barrett and G. Salvesen (Eds.), *Proteinase Inhibitors*, Elsevier, Amsterdam, 1986.
8 R. W. Carrell, P. B. Christey, and D. R. Boswell, in M. Verstraete, J. Vermylen, H. R. Lijen, and J. Arnout (Eds.), *Thrombosis and Haemostasis*, Leuven, Leuven University Press, 1987.
9 M. Höök, U. Lindahl, A. Hallen, and G. Backström, *J. Biol. Chem.*, 250 (1975) 6065–6071.
10 L. Jansoon, M. Höök, A. Wasteson, and U. Lindahl, *Biochem. J.*, 149 (1975) 49–55.
11 M. Kusche, G. Backström, J. Riesenfeld, M. Petitou, J. Choay, and U. Lindahl, *J. Biol. Chem.*, 263 (1988) 15474–15484.
12 D. R. Ferro, A. Provasoli, M. Ragazzi, B. Casu, G. Torri, V. Bossennec, B. Perly, P. Sinaÿ, M. Petitou, and J. Choay, *Carbohydr. Res.*, 195 (1990) 157–167.
13 S. T. Olson, H. R. Halvorson, and I. Björk, *J. Biol. Chem.*, 266 (1991) 6342–6352.
14 J. Choay, M. Petitou, J.-C. Lormeau, P. Sinaÿ, B. Casu, and G. Gatti, *Biochem. Biophys. Res. Comm.*, 116 (1983) 492–499.

15 D. H. Atha, A. W. Stephens, and R. D. Rosenberg, *Proc. Natl. Acad. Sci., U.S.A.*, 81 (1984) 1030–1034.
16 D. H. Atha, J.-C. Lormeau, M. Petitou, R. D. Rosenberg, and J. Choay, *Biochemistry*, 24 (1985) 6723–6729.
17 R. D. Rosenberg and P. S. Damus, *J. Biol. Chem.*, 248 (1973) 6490–6505.
18 J. F. G. Vliegenthart, L. Dorland, and H. van Halbeek, *Adv. Carbohydr. Chem. and Biochem.*, 41 (1983) 209–273.
19 A. S. Perlin, D. M. Mackie, and C. P. Dietrich, *Carbohydr. Res.*, 18 (1971) 185–194.
20 G. Gatti, B. Casu, G. K. Hamer, and A. S. Perlin, *Macromolecules*, 12 (1979) 1001–1007.
21 L. Ayotte, E. Mushayakarara, and A. S. Perlin, *Carbohydr. Res.*, 87 (1980) 297–301.
22 B. Casu, P. Oreste, G. Torri, G. Zoppetti, J. Choay, J.-C. Lormeau, M. Petitou, and P. Sinaÿ, *Biochem. J.*, 197 (1981) 599–609.
23 A. Linker and P. Hovingh, *Carbohydr. Res.*, 127 (1984) 75–94.
24 C. A. A. van Boeckel, S. F. van Aelst, G. N. Wagenaars, J.-R. Mellema, H. Paulsen, T. Peters, A. Pollex, and V. Sinnwell, *Recl. Trav. Chim. Pays-Bas*, 106 (1987) 19–29.
25 P. Gettins and A. P. Horne, *Carbohydr. Res.*, 223 (1992) 000–000.
26 B. Casu, M. Petitou, M. Provasoli, and P. Sinaÿ, *Trends Biochem. Sci.*, 13 (1988) 221–225.
27 Z. M. Merchant, Y. S. Kim, K. G. Rice, and R. J. Linhardt, *Biochem. J.*, 229 (1985) 369–377.
28 G. Torri, B. Casu, G. Gatti, M. Petitou, J. Choay, J. C. Jacquinet, and P. Sinaÿ, *Biochem. Biophys. Res. Comm.*, 128 (1985) 134–140.
29 M. Petitou, J.-C. Lormeau, B. Perly, P. Berthault, V. Bossennec, P. Sié, and J. Choay, *J. Biol. Chem.*, 263 (1988) 8685–8690.
30 A. Linker and P. Hovingh, *Methods Enzymol.*, 28 (1972) 902–911.
31 A. Linker and P. Hovingh, *Anal. Biochem.*, 11 (1972) 563–568.
32 K. G. Rice, Y. S. Kim, A. C. Grant, Z. M. Merchant, and R. J. Linhardt, *Anal. Biochem.*, 150 (1985) 325–331.
33 A. P. Horne and P. Gettins, unpublished work.