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N.M.R. STUDIES OF OLIGOSACCHARIDES OBTAINED BY DEGRADATION OF BOVINE LUNG HEPARIN WITH NITROUS ACID

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ABSTRACT

The oligosaccharides IdoA($2SO_3$)–[GlcNSO$_3$($6SO_3$)–IdoA($2SO_3$)]$_n$–anManOH($6SO_3$) (n = 1–4) have been prepared from bovine lung heparin by treatment with nitrous acid followed by borohydride reduction and then fractionation by gel filtration. The major resonances of the ^{1}H- (400 MHz) and ^{13}C-n.m.r. (100 MHz) spectra for each oligosaccharide fraction have been assigned using two-dimensional COSY, NOESY, and ^{13}C–^{1}H correlation experiments. The ^{1}H resonances, representing the three distinct molecular environments of IdoA($2SO_3$), namely, at the non-reducing terminus, adjacent to the reducing terminal anManOH($6SO_3$), and in the [GlcNSO$_3$($6SO_3$)–IdoA($2SO_3$)] repeat sequence, have been assigned.

INTRODUCTION

Heparin, a highly sulphated glycosaminoglycuronan of mammalian origin, is widely used as a blood anticoagulant[1]. The preponderant structural unit of bovine lung heparin is [IdoA($2SO_3$)–GlcNSO$_3$($6SO_3$)] although other residues, notably IdoA, GlcA, GlcNSO$_3$, GlcNAc, and GlcNAc($6SO_3$), may occur occasionally[2]. In addition, GlcNSO$_3$(3,6-diSO_3)[3] and GlcA($2SO_3$)[4] residues have been identified recently. Several of these rarer residues are found in a specific oligosaccharide sequence[5] which occurs in approximately one-third[6] of commercial heparin chains and can bind to the natural protease inhibitor antithrombin, thereby mediating the anticoagulant activity of heparin[7].

Investigations of the major components of heparin by n.m.r. spectroscopy include both ^{1}H-[8] and ^{13}C-analyses[9] together with ^{13}C-studies of heparin derivatives[10]. Antithrombin-binding oligosaccharides have been studied by ^{13}C-[11–14] and ^{1}H-n.m.r.[11,12] spectroscopy, and ^{1}H-n.m.r. spectra of a synthetic pentasaccharide having a high affinity for antithrombin have also been published[15,16],

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leading to the assignment of resonances from minor, but important, components. Recently, ^{1}H-[17,18] and ^{13}C-n.m.r.[18] techniques have been utilised in the analysis of tetrasaccharides prepared by the enzymic degradation of heparin.

Deaminative cleavage with nitrous acid has been used extensively as a structural probe for excising oligosaccharides from heparin because of its specificity for the cleavage of 2-deoxy-2-sulphoaminoglucosidic linkages to produce oligosaccharides having, as the reducing terminal, an anhydromannose residue[19] which may be reduced to anhydromannitol. Assignment of the n.m.r. signals from this residue and from the residues of the repeat sequence in various oligosaccharide environments will provide a reference point for further studies of more complex sequences. Complete assignment of the ^{1}H- and ^{13}C-n.m.r. spectra of the disulphated disaccharide from bovine lung heparin[20] has been achieved and we now report the assignment of the complex spectra for larger oligosaccharides.

EXPERIMENTAL

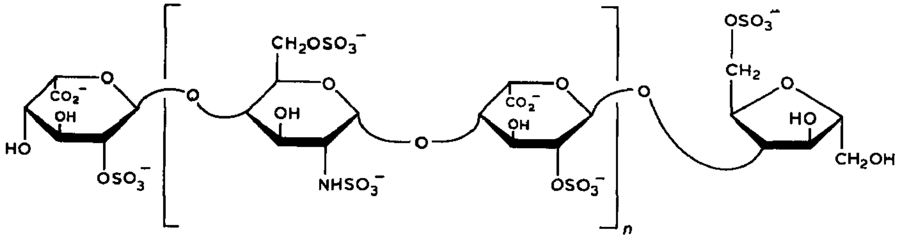
General. — The samples, chromatographic media, the procedure for the degradation of heparin, and the fractionation of oligosaccharides by gel filtration have been described[20]. Analytical high-voltage paper electrophoresis of di- and tetra-saccharides was performed as described previously[21].

N.m.r. spectroscopy. — Spectra were recorded[20] at 60°. Samples (50–100 mg) were prepared[20] in phosphate buffer (pH 7) and, after several exchanges with D_2O, were finally dissolved in 100.0% D_2O using sodium 3-trimethylsilyl[$^2H_4$]propionate (TSP-d_4) as the internal reference[22] for both ^{13}C and ^{1}H.

Two-dimensional homonuclear correlations and n.O.e. determinations were performed for each oligosaccharide using a combined COSY–NOESY–45° method[23]. The ^{13}C–^{1}H two-dimensional correlation experiments for the tetra- and hexa-saccharides were performed according to the method of Bax[24] with broadband homonuclear decoupling, using the delays Δ_1 1/2J s and Δ_2 0.3/J s[25] with an average $^1J_{C,H}$ of 160 Hz.

RESULTS AND DISCUSSION

Structural homogeneity of oligosaccharide fractions. — As bovine lung heparin contains a greater proportion of [IdoA(2SO$_3$)–GlcNSO$_3$(6SO$_3$)] units than



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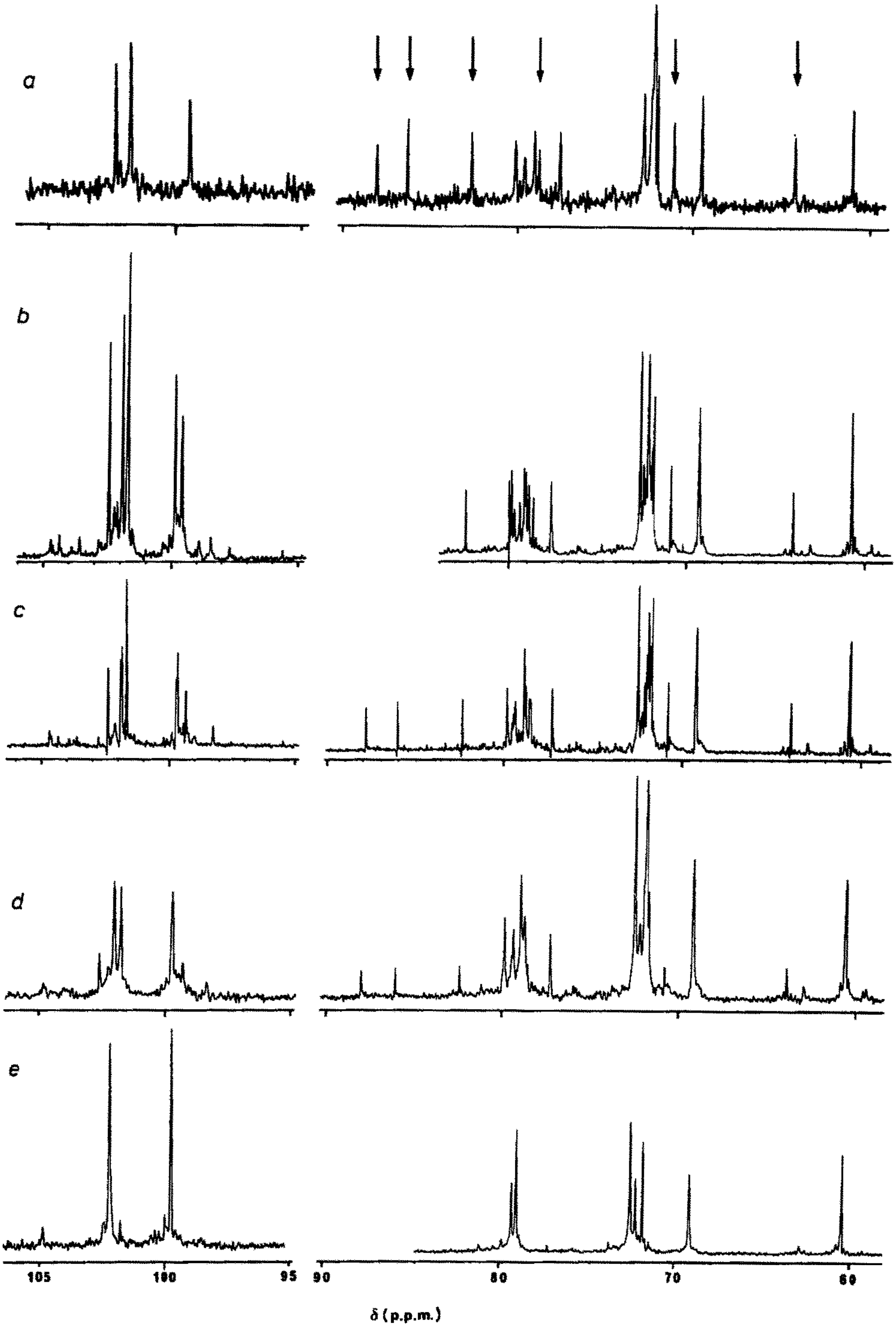

Fig. 1. ^{13}C-N.m.r. spectra (100 MHz) (δ 60–90 and 95–105) for heparin oligosaccharides at 60°: (*a*) tetramer, (*b*) hexamer, (*c*) octamer, (*d*) decamer, and (*e*) heparin polymer. Arrows indicate the positions of the anManOH(6SO$_3$) resonances. The chemical shift scale for the anomeric region is different from that of the remainder of the spectrum. Carboxyl resonances (δ ~180) are omitted.

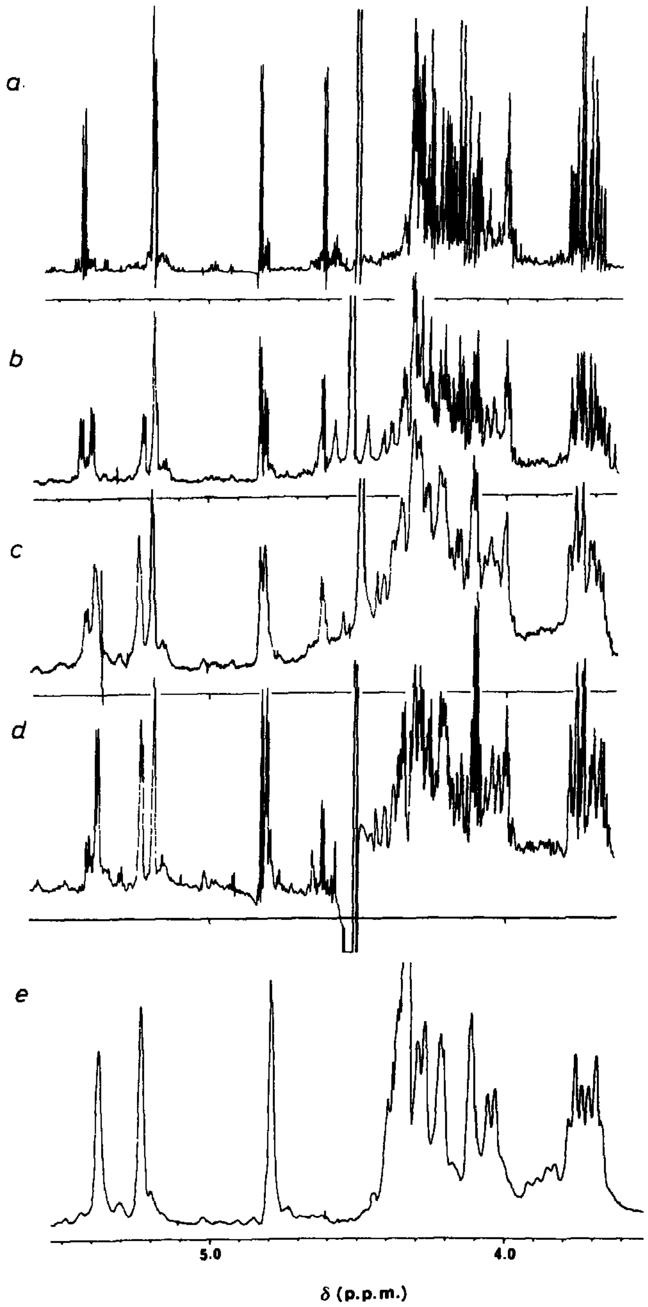

Fig. 2. ^{1}H-N.m.r. spectra (400 MHz) (δ 3.6–5.5) for heparin oligosaccharides at 60°: (*a*) tetramer, (*b*) hexamer, (*c*) octamer, (*d*) decamer, and (*e*) heparin polymer.

mucosal heparin, the majority of oligosaccharides produced by a partial digestion of the former with nitrous acid are represented by structure **1**. Evidence for the structural homogeneity of bovine lung heparin comes from the ^{13}C-n.m.r. spectrum (Fig. 1*e*) in which contributions from minor components are small.

The di- and tetra-saccharide fractions of this series of oligosaccharides from bovine lung heparin ran essentially as single species in analytical high-voltage paper electrophoresis[26]. The tetramer migrated further than the dimer, indicating that the former contained the expected complement of five sulphate groups. Larger oligosaccharides were assumed to have the basic structure shown in **1**, but a few minor signals were observed in the n.m.r. spectra (see below). The major resonances in the ^{13}C- (Fig. 1) and ^{1}H-n.m.r. (Fig. 2) spectra of the oligosaccharides therefore represent the different molecular environments of the GlcNSO$_3$(6SO$_3$) and IdoA(2SO$_3$) residues.

Assignment of ^{1}*H- and* ^{13}*C-resonances for the major components of heparin oligosaccharides.* — (*a*) *IdoA(2SO$_3$)*. The COSY spectra of the tetramer and hexamer show, respectively, two and three distinct connectivities between H-5 and H-4 of the IdoA(2SO$_3$) residues (see Fig. 3). These responses represent the different locations of the IdoA(2SO$_3$) residues and were assigned by reference to the ^{13}C–^{1}H correlation spectra. In the ^{13}C–^{1}H correlation plot of the tetramer, two distinct ^{13}C-shifts, separated by ~6.5 p.p.m. (indicated by arrows in Fig. 4), represent C-4 of the two IdoA(2SO$_3$) residues. The upfield resonance (δ ~72.2) represents C-4 of the terminal IdoA(2SO$_3$), *i.e.*, the C-4 *not* involved in a glycosidic linkage as seen for the disaccharide[20], whereas the downfield resonance (δ 78.79) represents a glycosidically linked C-4 as observed in heparin[9]. Thus, the signals for H-4 were assigned from the ^{13}C–^{1}H correlation plot for the tetramer (Fig. 4) and allowed identification, from the COSY spectrum (Fig. 3*a*), of both H-5 signals for IdoA(2SO$_3$) in the tetramer. The COSY spectrum for the hexamer (Fig. 3*c*), and for larger oligosaccharides, shows an additional response which connects H-4 and H-5 of those IdoA(2SO$_3$) residues flanked by two GlcNSO$_3$(6SO$_3$) residues. This H-5 resonance (δ 4.81) increased in relative intensity in the ^{1}H-n.m.r. spectra of larger oligosaccharides, to become the only H-5 signal in heparin (Fig. 2).

Each of the three H-4 signals connect to three distinct H-3 sites in the COSY spectra of the hexamer and larger oligosaccharides. These H-3 assignments were confirmed by weak long-range 1,3-correlations in the COSY spectra of the tetramer (Fig. 3*a*) and octamer.

The anomeric protons of IdoA(2SO$_3$) gave rise to two signals (Fig. 2). The downfield signal (δ 5.22), first observed for the hexamer, increased in relative intensity for the larger oligosaccharides, and predominated in the heparin spectrum. Therefore, it was assigned to the IdoA(2SO$_3$) residues of the repeat sequence. The signal for H-2 of these residues also had a distinct chemical shift, as identified from the COSY spectra. The anomeric protons of the IdoA(2SO$_3$) residues not in the repeating unit had similar chemical shifts. However, in the NOESY spectrum (Fig. 3*b*) of the tetramer, the response from H-1 of IdoA(2SO$_3$)

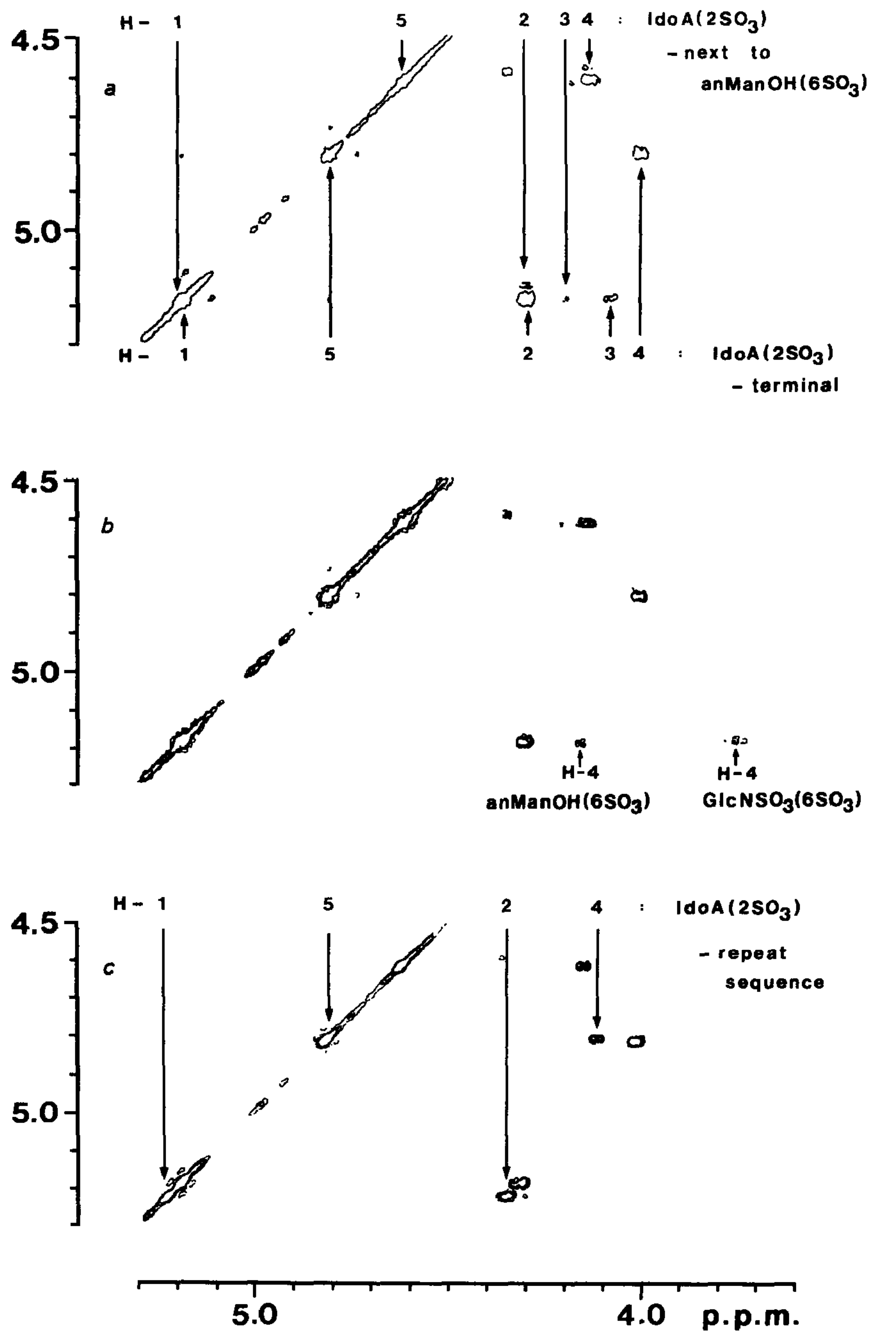

Fig. 3. Sections (δ 3.6–5.3 by 4.5–5.3) of two-dimensional plots: (*a*) tetramer COSY showing proton shifts for IdoA(2SO$_3$) next to anManOH(6SO$_3$) and at the non-reducing terminus, (*b*) tetramer NOESY showing the responses of H-4 of GlcNSO$_3$(6SO$_3$) and H-4 of anManOH(6SO$_3$) to H-1 of IdoA(2SO$_3$) across the respective glycosidic linkages, (*c*) hexamer COSY showing additional responses identifying the protons of IdoA(2SO$_3$) in the repeat sequence. Arrows indicate the responses which identify individual proton chemical shifts.

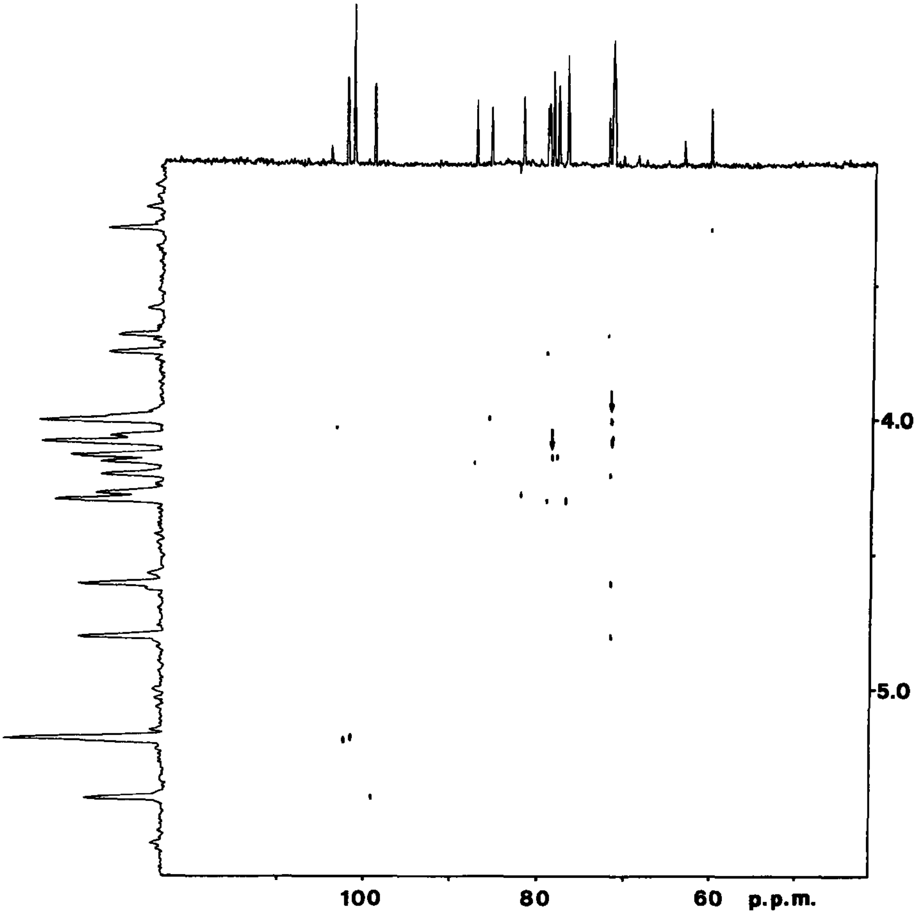

Fig. 4. ^{13}C–^{1}H correlation plot for the tetramer at 60° showing projections of the spectra along each axis. Arrows indicate connections between C-4 and H-4 of the IdoA($2SO_3$) residues.

to H-4 of GlcNSO$_3$(6SO$_3$) was marginally upfield of that to H-4 of anManOH-(6SO$_3$). Thus, the signal of H-1 of the terminal IdoA(2SO$_3$) was assigned upfield (by <0.01 p.p.m.) of that of H-1 from the IdoA(2SO$_3$) next to anManOH(6SO$_3$). The signal of H-2 of the terminal IdoA(2SO$_3$) was identified from the COSY spectrum for the hexamer through connectivities to both H-1 and H-3 which showed the signal of this H-2 to be ~0.01 p.p.m. upfield of that of H-2 from the IdoA(2SO$_3$) next to anManOH(6SO$_3$).

The chemical shifts of the ^{1}H-n.m.r. signals for IdoA(2SO$_3$) in the three distinct environments within these oligosaccharides are listed in Table I. These assignments enabled the dominant molecular influences governing the proton-shifts of IdoA(2SO$_3$) in the disaccharide[20] to be determined. Thus, the signals of H-3 and H-4 in the disaccharide had chemical shifts similar to those of terminal IdoA(2SO$_3$) residues in the oligosaccharides, whereas the major influence on the chemical shift

TABLE I

^{1}H CHEMICAL SHIFTS (δ) FOR IdoA(2SO$_3$): (A) AT THE NON-REDUCING TERMINUS, (B) IN THE REPEAT SEQUENCE, AND (C) NEXT TO anManOH(6SO$_3$) IN HEPARIN OLIGOSACCHARIDES

Proton	*Location*	*Tetramer*	*Hexamer*	*Octamer*	*Decamer*	*Heparin*
H-1	A	5.19	5.18	5.18	5.19	—
	B	—	5.22	5.22	5.23	5.23
	C	5.19	5.18	5.18	5.19	—
H-2	A	4.31	4.31	4.31	4.31	—
	B	—	4.35	4.34	4.35	~4.35[a]
	C	4.31	4.32	4.31	4.31	—
H-3	A	4.10	4.10	4.09	4.10	—
	B	—	4.22	4.22	4.21	~4.21[a]
	C	4.22	4.21	4.20	~4.2[b]	—
H-4	A	4.03	4.02	4.01	4.00	—
	B	—	4.11	4.12	4.11	~4.11[a]
	C	4.15	4.15	4.15	4.16	—
H-5	A	4.83	4.83	4.82	4.82	—
	B	—	4.81	4.80	4.81	4.79
	C	4.62	4.61	4.61	4.62	—

[a] Approximate value from broad resonance. [b] Only approximate value as a confirmatory COSY 1,3 correlation is not observed.

of the signal for H-5 in the disaccharide arose from the presence of a neighbouring anManOH(6SO$_3$). However, the chemical shifts of the signals for H-1 and H-2 of IdoA(2SO$_3$), whether terminal or adjacent to anManOH(6SO$_3$), did not show significant differences.

The chemical shifts for the ^{13}C-n.m.r. signals of the IdoA(2SO$_3$) residues are listed in Table II. The signals for the anomeric carbons of the tetramer and hexamer were assigned from the ^{13}C–^{1}H correlation spectra and provided the basis for identifying the corresponding signals from the larger oligosaccharides (Fig. 1). The signal of C-2 of the terminal IdoA(2SO$_3$) residue was assigned *via* the hexamer ^{13}C–^{1}H correlation plot. The group of signals at δ ~79 comprised those of C-2 and C-4 of the non-terminal IdoA(2SO$_3$) residues and C-4 of GlcNSO$_3$(6SO$_3$). These signals were assigned for the tetramer and hexamer *via* the ^{13}C–^{1}H correlations, but definitive assignments were not possible for the octamer and decamer because of spectral crowding. Similarly, only approximate chemical shifts could be given for the signals of C-3 and C-5 at δ ~72. The carboxylate ^{13}C-resonances were readily assigned and were well downfield at δ ~180.

(*b*) *GlcNSO$_3$(6SO$_3$)*. The ^{13}C (Fig. 1) and ^{1}H (Fig. 2) resonances for the anomeric position in GlcN fall into two groups. The upfield ^{1}H resonance was correlated with the downfield ^{13}C signal in the ^{13}C–^{1}H correlation plot for the hexamer. These signals grew in relative intensity as the size of the oligosaccharide

TABLE II

^{13}C CHEMICAL SHIFTS (p.p.m.) FOR IdoA(2SO$_3$): (A) AT THE NON-REDUCING TERMINUS, (B) IN THE REPEAT SEQUENCE, AND (C) NEXT TO anManOH(6SO$_3$) IN HEPARIN OLIGOSACCHARIDES

Carbon	*Location*	*Tetramer*	*Hexamer*	*Octamer*	*Decamer*	*Heparin*
C-1	A	101.74	101.73	101.70	101.76	—
	B	—	101.94	101.88/101.93^a	102.03	102.20
	C	102.35	102.47	102.44	102.61	—
C-2	A	77.35	77.66	77.36	77.40	—
	B	—	78.93	~79^c	~79^c	79.26^d
	C	79.36	79.88	~79^c	~79^c	—
C-3	A	~72.0^b	~72^c	~72^c	~72^c	—
	B	—	~72^c	~72^c	~72^c	72.75^e
	C	~72.2^b	~72^c	~72^c	~72^c	—
C-4	A	~72.2^b	~72^c	~72^c	~72^c	—
	B	—	79.43	~79^c	~79^c	79.26^d
	C	78.79	79.18	~79^c	~79^c	—
C-5	A	~72.0^b	~72^c	~72^c	~72^c	—
	B	—	~72^c	~72^c	~72^c	72.46/72.07^f
	C	~72.2^b	~72^c	~72^c	~72^c	
C-6	A	178.41	178.36	178.43	178.45	—
	B	—	176.85	176.95	176.93	176.85
	C	176.63	176.59	176.61	176.56	—

[a] Two resonances identified. [b] From ^{13}C–^{1}H correlation, signals are assigned to groups at either δ ~72.0 or ~72.2, as the resonances were incompletely resolved. [c] Approximate chemical shifts due to spectral crowding. [d] The signals for C-2 and C-4 of IdoA(2SO$_3$) in heparin were coincident. [e] The signal for C-3 of IdoA(2SO$_3$) was coincident with that of C-3 of GlcNSO$_3$(6SO$_3$) in heparin. [f] These signals represent C-5 of IdoA(2SO$_3$) and C-5 of GlcNSO$_3$(6SO$_3$).

increased (Figs. 1 and 2) and were assigned to GlcNSO$_3$(6SO$_3$) residues in the repeat sequence. The remaining anomeric resonances, which showed a ^{13}C–^{1}H correlation for the hexamer, decreased in relative intensity as the size of the oligosaccharide increased and therefore represented a GlcNSO$_3$(6SO$_3$) in an atypical environment.

Proton assignments for the GlcNSO$_3$(6SO$_3$) residue in the tetramer were obtained from connectivities within the COSY plot; however, only a mean shift for H-6 and H-6′ was obtained. All GlcNSO$_3$(6SO$_3$) protons were therefore assigned for the tetramer and the data are given in Table III. A mean shift value for the H-6,6′ signals in the hexamer was determined from the ^{13}C–^{1}H correlation, but these proton shifts were not obtained for larger oligosaccharides. Assignments of the signals of other GlcNSO$_3$(6SO$_3$) protons in the larger oligosaccharides were confirmed *via* COSY spectra, and the data are listed in Table III. Thus, all

TABLE III

^{1}H CHEMICAL SHIFTS (δ) FOR $GlcNSO_3(6SO_3)$ IN HEPARIN OLIGOSACCHARIDES

Proton	*Tetramer*	*Hexamer*	*Octamer*	*Decamer*	*Heparin*
H-1[a]	5.42	5.43	5.42	5.41	—
H-1[b]	—	5.39	5.38	5.38	5.38
H-2	3.30	3.30/3.29[c]	~3.29	~3.29	3.29
H-3	3.70	3.70/3.66[c]	~3.69	~3.70	~3.69[d]
H-4	3.78	3.76	~3.75	~3.77	~3.76[d]
H-5	4.02	~4.0	~4.0	~4.0	~4.04[d]
H-6,6′	~4.32[e]	~4.30[f]	—	—	~4.3[d]

[a]In an atypical oligosaccharide environment. [b]In the repeat sequence. [c]Two separate shift positions were observed. [d]Approximate value obtained from a broad resonance. [e]Mean value obtained from COSY plot. [f]Mean value obtained from the ^{13}C–^1H correlation.

$GlcNSO_3(6SO_3)$ proton signals, with the exception of H-1, had chemical shifts which were independent of residue location within the oligosaccharides.

Assignment of the signals for C-2 and C-6 for $GlcNSO_3(6SO_3)$ was possible by comparison with published values for heparin[9]. The signal for C-3 was identified from the ^{13}C–^1H correlations for the tetramer and hexamer and by its position in the spectrum of heparin[9]. The ^{13}C–^1H correlation for the hexamer showed two signals for C-4. The downfield signal at δ 79.73 was assigned to the residue adjacent to the non-reducing terminal $IdoA(2SO_3)$ by comparison with the ^{13}C–^1H correlation plot for the tetramer. For the larger oligosaccharides, individual shifts for the signal of C-4 at δ ~79 (Fig. 1) were not obtained. The lowest field signal of the group at δ ~79 was assigned to C-4 of $GlcNSO_3(6SO_3)$ in heparin[9]. The signal for C-5 of $GlcNSO_3(6SO_3)$ occurred at δ ~72; however, the complexity of this group of signals precluded definitive assignments. The ^{13}C data for $GlcNSO_3(6SO_3)$ in the oligosaccharides are listed in Table IV.

(*c*) *anManOH*(*6SO*$_3$). The signals for the methylene carbons of anManOH-$(6SO_3)$ were assigned readily; C-1 and C-6 were unsulphated and sulphated, respectively, and, as for all $anManOH(6SO_3)$ resonances, they decreased in relative intensity as the size of the oligosaccharide increased. The signals for the methylene protons were not observed easily. The signals for H-1 and H-1′ were overlapped by those of H-3,4 of the $GlcNSO_3(6SO_3)$ but could be identified, for the tetramer, from an expanded plot. A mean chemical shift for the signals of each of the other pairs of methylene protons in the tetramer and hexamer was obtained from the broad response observed in the ^{13}C–^1H correlation plots.

The signals of C-2,4,5 of $anManOH(6SO_3)$ occurred in a convenient "window" and were assigned by comparison with the spectrum of the di-

TABLE IV

^{13}C CHEMICAL SHIFTS (p.p.m.) FOR GlcNSO$_3$(6SO$_3$) IN HEPARIN OLIGOSACCHARIDES

Carbon	*Tetramer*	*Hexamer*	*Octamer*	*Decamer*	*Heparin*
C-1[a]	99.42	99.59	99.36	99.32	—
C-1[b]	—	99.88	99.68/99.73[c]	99.73	99.76
C-2	60.68	60.80	60.67/60.72[c]	60.75	60.80
C-3	72.57	72.67	72.55	72.64	72.75[f]
C-4	79.87	79.73/79.09[c]	~79[e]	~79[e]	79.50
C-5	~72.2[d]	~72[e]	~72[e]	~72[e]	72.07/72.46[g]
C-6	69.25	69.34	69.26	69.33	69.40

[a] In an atypical oligosaccharide environment. [b] In the repeat sequence. [c] Two distinct resonances were observed. [d] See note *b*, Table II. [e] See note *c*, Table II. [f] See note *e*, Table II. [g] See note *f*, Table II.

saccharide[20]. The signals of the protons attached to these carbons were identified *via* ^{13}C–^{1}H correlations. The tetramer NOESY plot (Fig. 3*b*) confirmed the assignment of the signal for H-4. The location of the signal for H-3 was determined *via* the COSY spectra through connectivities to both H-2 and H-4. The signal for C-3 was identified by comparison of its position with that in the spectrum of the disaccharide[20].

Thus, ^{13}C resonances for anManOH(6SO$_3$) were fully assigned and the data are listed in Table V. However, assignments of the proton signals were made only for the tetramer and hexamer (see Table VI).

Evidence for the presence of minor components. — The oligosaccharides contained small amounts of some of the less frequently occurring saccharides despite the parent polymer being amongst the most structurally homogeneous of the heparins.

Variations in the degree of sulphation provide the most common source of structural heterogeneity. Thus, the small ^{13}C signal at δ ~63 throughout the series

TABLE V

^{13}C CHEMICAL SHIFTS (p.p.m.) FOR anManOH(6SO$_3$) IN HEPARIN OLIGOSACCHARIDES

Carbon	*Tetramer*	*Hexamer*	*Octamer*	*Decamer*
C-1	63.97	64.08	63.96	64.04
C-2	86.05	86.18	86.06	86.14
C-3	78.52	78.67	78.54	78.63
C-4	87.80	87.93	87.86	88.05
C-5	82.32	82.45	82.35	82.47
C-6	70.85	70.93	70.88	70.96

TABLE VI

^{1}H CHEMICAL SHIFTS (δ) FOR anManOH(6SO_3) IN HEPARIN OLIGOSACCHARIDES

Proton	*Tetramer*	*Hexamer*
H-1	3.77	~3.73[a]
H-1′	3.71	~3.73[a]
H-2	4.01	3.98
H-3	4.16	4.15
H-4	4.18	4.16
H-5	4.29	4.27
H-6,6′	~4.26[a]	~4.24[a]

[a] Values represent the mean of the chemical shifts of the signals of the two methylene protons obtained from the ^{13}C–^1H correlations.

(Fig. 1) represents C-6 of unsulphated GlcN. For the hexamer, these small signals were due to GlcNSO_3, but, additionally, may be due to GlcNAc for larger oligosaccharides because, for these larger species, signals representing C-1 (δ ~98.3), C-2 (δ ~56), and the methyl carbon (δ ~24) of GlcNAc[10] were observed. These GlcNAc resonances were not readily observed in the spectrum of the parent heparin, so it appears that the degradation procedure concentrated the few GlcNAc residues present into the octamer and decamer fractions.

The trisulphated GlcN of the antithrombin-binding sequence in heparin[3] was present to a small extent in these oligosaccharides as indicated by a small signal ~1.5 p.p.m. upfield of the resonance for C-1 of GlcNSO_3(6SO_3) which represents C-1 of the trisulphated residue[11]. Likewise, the signal for C-2 appeared ~1 p.p.m. upfield (δ ~59.5) when the C-3 was sulphated[11]. These resonances were also visible in the ^{13}C-n.m.r. spectrum of the parent heparin.

The small signals at δ >103 (Fig. 1) provided an indication of the low level of unsulphated uronates. There was no evidence for any sulphated glucuronate; however, the presence of such residues could not be discounted as a few signals remained unidentified and there may have been others underneath the larger resonances. Galactose and xylose of the proteoglycan linkage region may also have been present. The only "window" in which signals from these species may be observed is on the proton spectra at ~3.5 p.p.m. where the signals for H-2 and H-5*a* of xylose occur[27]; indeed, some minor signals were observed in this region.

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