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ASSIGNMENT OF THE ^{1}H-N.M.R. SPECTRA OF HEPARIN AND HEPARAN SULPHATE

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ABSTRACT

Resonances from the main repeating unit of heparan, →4)-β-D-GlcA-(1→4)-α-D-GlcNAc-(1→, have been assigned by using a sample of the capsular polysaccharide of *E. coli* K5. Comparison of the spectra of heparan sulphate samples before and after *O*- and/or *N*-desulphation, with re-*N*-acetylation or re-*N*-sulphation, allowed assignment of some of the H-1 doublets in terms of sequence effects. Chemical shifts for H-1 of unsulphated uronic acid residues are influenced by 6-sulphation of the nearest neighbour GlcN on the reducing side; those of GlcN residues vary according to whether they have IdoA or GlcA as the nearest neighbour on the reducing side. The H-1 doublets due to residues in the binding sequence for antithrombin have been assigned by comparison of the spectra of heparins having high and low affinities for immobilised antithrombin.

INTRODUCTION

The sulphated glycosaminoglycans (GAGs) heparin and heparan sulphate are structurally related compounds with a wide variety of biological activities. Heparin is found in mast cells in some tissues and species, whereas heparan sulphate is widely distributed, if not ubiquitous, in vertebrates[1]. Heparin is an important anticoagulant and antithrombic agent, its interactions with lipoproteins and their lipases are of interest with respect to the regulation of atherosclerosis[2], and it has attracted attention as a potential anti-tumour agent through its effect on angiogenesis[3]. Heparan sulphate occurs in proteoglycans on the surface of many types of cell and may have a role in cell recognition and growth control. These GAGs are heterogeneous with respect to composition, sequence, and molecular weight. The biological activities of commercially prepared samples of heparin vary according to tissue of origin and method of preparation and should be explicable in terms of structural differences. Heparan sulphate prepared from the by-products of heparin manufacture is even more heterogeneous.

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The major resonances have previously been assigned in the ^{1}H-n.m.r. spectra of lung heparin[4], other GAGs[5–8], oligosaccharides derived from heparin and heparan sulphate[9–13], heparan sulphate[14,15], and chemically modified heparins[16].

We have been investigating the use of high-field ^{1}H-n.m.r. spectroscopy as an aid to the characterisation and standardisation of commercial samples of heparin. The assignments reported in the literature are not adequate to describe the spectra in sufficient detail for our purposes; in seeking to extend and clarify these partial and sometimes contradictory assignments, the spectra of native heparin and heparan sulphate have been compared with those of GAGs of simpler structure, some of which are chemically modified heparin or heparan sulphate samples.

RESULTS

In the following, GAGs were studied in which all IdoA is α-L, all GlcA is β-D, all GlcN is α-D, and all linkages are (1→4): (*a*) bovine lung heparin, mainly $-[IdoA(2SO_3)-GlcNSO_3(6SO_3)]_n-$ (**1**); (*b*) the capsular polysaccharide from *E. coli* K5, mainly $-(GlcA-GlcNAc)_n-$ (**2**); (*c*) *O*- and *N*-desulphated, re-*N*-acetylated heparan sulphate (HSIIA), containing **2** and $-(IdoA-GlcNAc)_n-$ (**3**); (*d*) *N*-desulphated, re-*N*-acetylated heparan sulphate (HSIIB), containing **2** and $-[IdoA(2SO_3)-GlcNAc(6SO_3)]_n-$ (**4**); (*e*) *O*- and *N*-desulphated, then re-*N*-sulphated heparan sulphate (HSIIC), containing **2**, $-(IdoA-GlcNSO_3)_n-$ (**5**), and $-(GlcA-GlcNSO_3)_n-$ (**6**); (*f*) *O*- and *N*-desulphated heparan sulphate (HSIIC), containing **2**, $-(IdoA-GlcN)_n-$ (**7**), and $-(GlcA-GlcN)_n-$ (**8**).

Each of these GAGs also contains other sequences in small proportions. HSII-type heparan sulphates are intermediate in sulphate content between HSI (sulphate/carboxylate ratio ~1) and heparins (sulphate/carboxylate ratio >2)[17]. Structures **1** and **2** represent the main repeating disaccharide units of heparin and low-sulphated heparan sulphate, respectively. Structures **3–6** represent the repeating disaccharides produced from **1** by the chemical modifications indicated.

The biosynthesis of heparin and heparan sulphate produces other sequences with intermediate degrees of sulphation, the only combination known to be forbidden being GlcNAc linked at C-1 to IdoA or $IdoA(2SO_3)$ (ref. 18). Comparison of the spectra of (*c*)–(*f*) and their precursors led to the assignments of some minor resonances in the spectra of native heparins and heparan sulphates due to these other sequences, thereby allowing a better degree of characterisation than was possible hitherto.

The information so obtained was used in the examination of the spectra of samples of heparin having high and low affinities for immobilised antithrombin prepared from both bovine lung and porcine mucosal heparins.

The chemical shifts of signals from protons in polysaccharides are often reported to four significant figures. This may be valid for neutral polysaccharides, but presents difficulties with the strongly anionic GAGs for which small changes of ionic strength and temperature have a greater effect on the spectrum. Several

strategies (see Experimental) were used to remove unwanted divalent and paramagnetic counter-ions in order to reduce both variations in chemical shift and line broadening (particularly for IdoA resonances), but variations between different spectra still did not allow the chemical shifts to be given to more than three significant figures. An extra consequence of ion-exchange treatment is a reduction in the number of doublets in the region for anomeric protons (H-1), indicating that some of the unwanted counter-ions were exchanged only slowly. Another source of minor variation is related to the reference signal. Internal trimethylsilylpropanesulphonic acid (TSP) was not satisfactory as its chemical shift varied according to the degree of sulphation of the sample. All the figures reported are therefore relative to external TSP in a solution in D_2O of a heparan sulphate of intermediate sulphate content.

TABLE I

^{1}H-N.M.R. DATA FOR (*a*) AND (*b*)

Atom *Chemical shifts (p.p.m.)*	(a)	(b)
GlcN[a]		
H-1	5.39	5.39
H-2	3.31	3.92
H-3	3.70	3.78
H-4	3.78	3.67
H-5	4.06	3.83–3.85
H-6	4.37	3.83–3.85
H-6′	4.30	3.83–3.85
Uronic acid[b]		
H-1	5.24	4.51
H-2	4.37	3.39
H-3	4.23	3.71
H-4	4.13	3.77
H-5	4.81	3.80
J *values (Hz)*		
GlcN[a]		
$J_{1,2}$	3.6	4.1
$J_{2,3}$	10.3	9.6
$J_{3,4}$	10.0	9.4
$J_{4,5}$	9.6	9.0
$J_{5,6}$	2.3	[c]
$J_{5,6'}$	[c]	[c]
$J_{6,6'}$	−11.6	[c]
Uronic acid[b]		
$J_{1,2}$	3.0	8.3
$J_{2,3}$	6.2	8.7
$J_{3,4}$	3.5	8.8
$J_{4,5}$	3.0	9.5

[a]$GlcNSO_3(6SO_3)$ in (*a*), GlcNAc in (*b*). [b]$IdoA(2SO_3)$ in (*a*), GlcA in (*b*). [c]Not measurable.

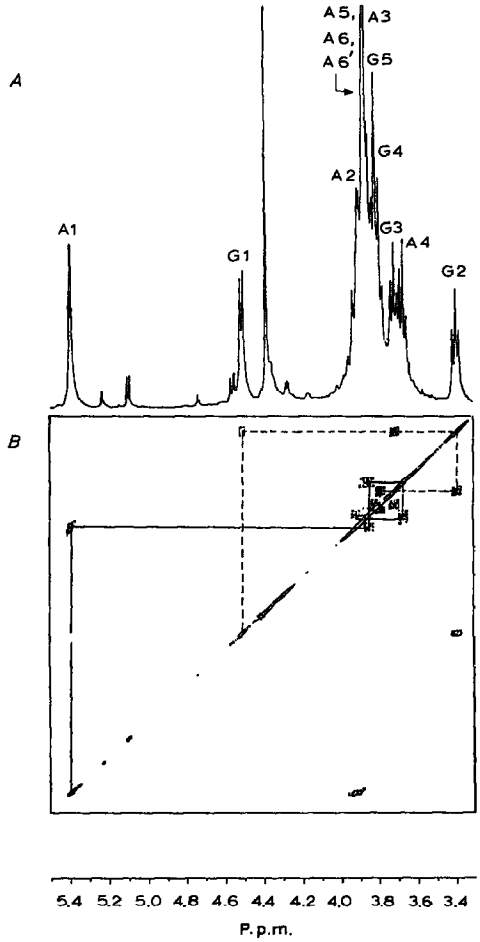


Fig. 1. *A*, 1D ^{1}H-n.m.r. spectrum; and *B*, 2D COSY spectrum at 500 MHz (343 K) of a sample of the capsular polysaccharide from *E. coli* K5. Signals from GlcNAc and GlcA are labelled A1–6′ and G1–5, respectively. Cross-peaks in the COSY spectrum show spin–spin connectivities for the GlcNAc (——) and GlcA (------) systems. The singlet for NAc of GlcNAc occurs at 2.06 p.p.m.

Assignment of the resonances arising from the principal repeating-disaccharide sequences. — The GAGs (*a*) and (*b*) contain, in structures **1** and **2**, the most frequently occurring disaccharide repeating-units in heparin and in heparan sulphate, respectively, and the chemical shifts and coupling constants for the major resonances are given in Table I. The spectrum of (*a*), bovine lung heparin, was assigned from the values in ref. 4, with which it is in good agreement. The spectrum of (*b*), the capsular polysaccharide of *E. coli* K5, was assigned with the aid of a COSY spectrum (Fig. 1). Structure **2** contains one β-D-GlcA residue and one α-D-GlcNAc residue, and the signals at 4.51 (d, *J* 8.3 Hz) and 5.39 p.p.m. (d, *J* 4.1 Hz) were assigned to the respective anomeric protons in accordance with the Karplus re-

lationship[19] on the assumption that each sugar ring was in the $^4C_1$ chair form. Cross-peaks, indicating spin–spin coupling, connected these H-1 doublets to the H-2 signals at 3.39 p.p.m. for GlcA and 3.92 p.p.m. for GlcNAc, and so on around the sugar ring as shown in Fig. 1. The spectrum is in good general agreement with that[15] of desulphated re-*N*-acetylated heparan.

Assignment of doublets of the anomeric protons in the spectra in terms of sequence, using modified heparan sulphates. — Assignments of the resonances of the ring protons of the minor component in heparins and heparan sulphate were often difficult due to overlap with signals from the major components, even when 2D-n.m.r. techniques were used. However, much compositional and sequence information could be extracted from the region of the spectrum which contained the H-1 doublets. The nature of the residue giving rise to any particular H-1 doublet

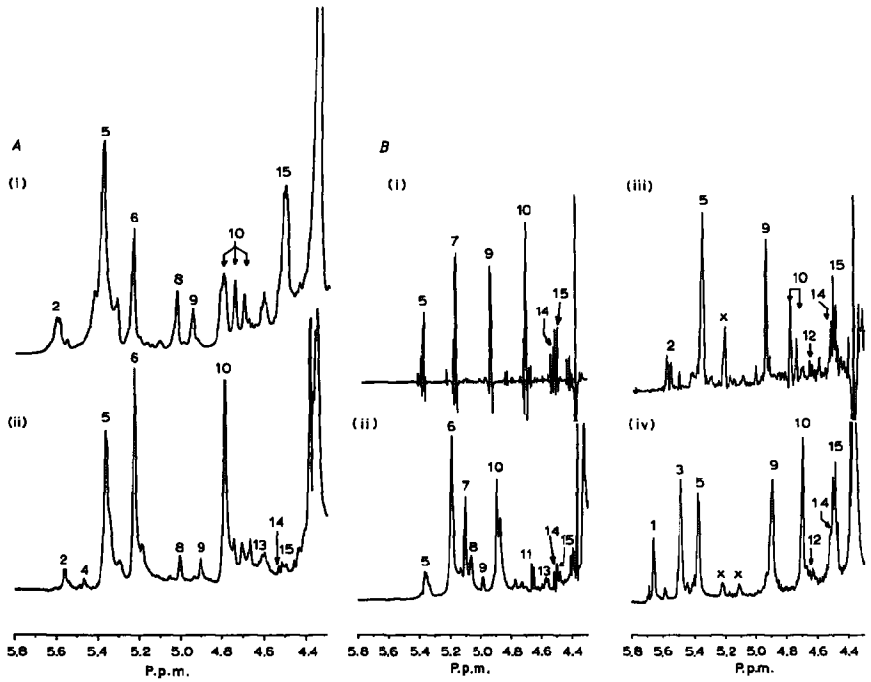


Fig. 2. Partial ^{1}H-n.m.r. spectra (resolution enhanced) at 500 MHz (343 K): *A*, (i) heparan sulphate HSl and (ii) porcine mucosal heparin Hep M; *B*, (i) (*c*), (ii) (*d*), (iii) (*e*), and (iv) (*f*). The H-1 doublets (or groups of doublets) are numbered as follows: 1, GlcN linked to GlcA as in **8**; 2, $GlcNSO_3$, including that linked to GlcA as in **6**; 3, GlcN linked to IdoA as in **7**; 4, $GlcNSO_3$, including $GlcNSO_3(3,6diSO_3)$ in the antithrombin binding sequence; 5, $GlcNSO_3$ linked to $IdoA2SO_3$ as in **1** *and* GlcNAc linked to GlcA as in **2**; 6, $IdoA2SO_3$ as in **1** and **4**; 7, GlcNAc linked to IdoA as in **3**, and to $IdoA2SO_3$ as in **4**; 8, IdoA linked to $GlcN(6SO_3)$; 9, IdoA linked to GlcN not 6-sulphated as in **3**, **5**, and **7**; 10, H-5 of IdoA and $IdoA2SO_3$; 11, GlcA (possibly including $GlcA2SO_3$); 12, GalNAc in desulphated dermatan sulphate impurity; 13, GlcA linked to $GlcN(6SO_3)$; 14, possibly Gal in the linkage region; 15, GlcA linked to GlcN not 6-sulphated as in **2**, **6**, and **8**.

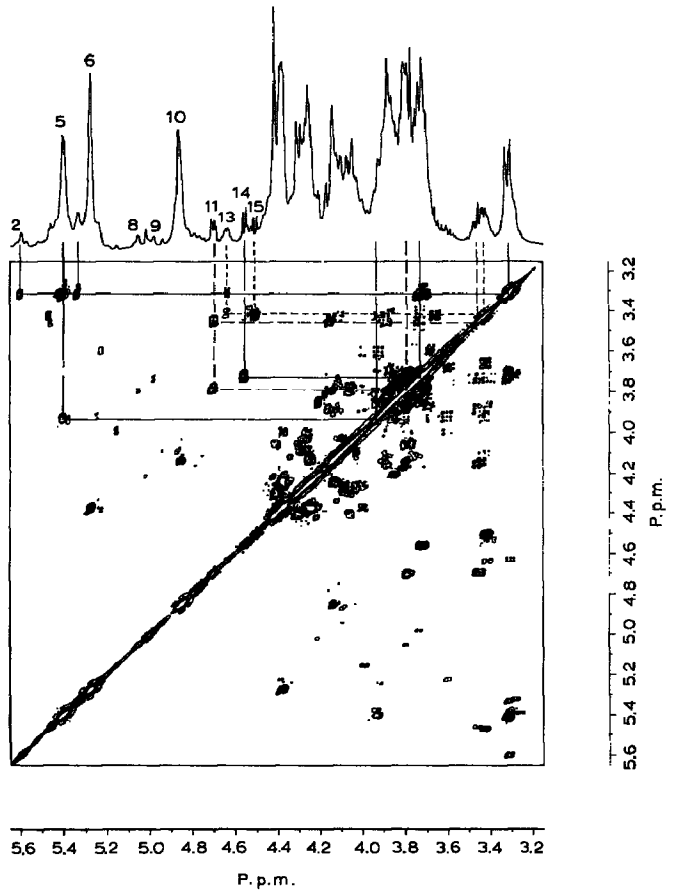


Fig. 3. 1D and 2D COSY ^{1}H-n.m.r. spectra of HSIIB. The numbering of the H-1 doublets is as in Fig. 2. Some of the cross-peaks indicating H-1,2 connectivities are indicated as follows: $GlcNSO_3$, ——; GlcA, ------; H-1β not GlcA, —— and — —.

could usually be ascertained from the chemical shift, the coupling constant, and the chemical shift of the multiplet from the corresponding H-2 as revealed by cross-peaks in the COSY spectrum. In some instances, comparison of the spectra of native heparan sulphates with those of specifically modified forms allowed sequence effects on the chemical shifts of the H-1 doublets to be identified.

Partial spectra of the modified heparan sulphates (*c*)–(*f*) are shown in Fig. 2, with the corresponding regions for samples of a commercial porcine-mucosal heparin and a heparan sulphate having a relatively low degree of sulphation. Fig. 3 shows the 1D and 2D COSY spectra of one of the heparan sulphates used as starting material, and the spectra of the other two samples did not differ thereform significantly except as mentioned in the text.

Doublets for the anomeric protons of the GlcN residues. — The H-1 doublets

from *N*-sulphated GlcN in **1** and **5**, and from GlcNAc in **2**, were grouped together near 5.4 p.p.m. so that it was not possible by inspection to assess their relative contributions to a 1D spectrum.

The 2D COSY spectrum of HSIIB (Fig. 3) showed cross-peaks connecting a doublet at 5.39 p.p.m. with an H-2 signal at 3.93 p.p.m. (as in **2**). Two other doublets in the group at 5.30–5.45 p.p.m. were connected similarly to an H-2 resonance at 3.30 p.p.m. (as in **1**), and a third at 5.46 p.p.m. was connected to a multiplet at 3.43 p.p.m.

A group of minor H-1 doublets was found at ~5.6 p.p.m., down-field of the main GlcN resonance, in the native GAGs and in some of the modified GAGs. *N*-Desulphation and re-*N*-acetylation abolished the doublets [(*c*) and (*d*)], whereas they were retained after *N*- and *O*-desulphation followed by re-*N*-sulphation [(*e*)]. In the COSY spectrum of HSIIB, a cross-peak connected these doublets to multiplets at 3.30 p.p.m. Therefore, this group of doublets could be assigned to *N*-sulphated GlcN residues.

In structures **3** and **4**, *N*-acetylated GlcN is linked to IdoA rather than GlcA. This change has a shielding effect on H-1 of GlcNAc, which gave a doublet at 5.17 p.p.m. for **3** and 5.11 p.p.m. for **4**. The spectra of (*c*), (*d*), and (*f*) also contained H-1 doublets from GlcNAc attached to GlcA, at ~5.37 p.p.m.

The spectrum of (*f*) had two other prominent GlcN H-1 doublets at 5.50 and 5.67 p.p.m. connected to double doublets (from the COSY spectrum, not shown) at 3.35 p.p.m. Each arose from H-1 of unsubstituted GlcN, originally sulphated. The difference in chemical shift was of the same order (~0.2 p.p.m.) as that between the H-1 resonances of GlcNAc linked to IdoA and GlcA [(*c*) and (*d*), respectively], and suggested corresponding differences between GlcN linkages in (*f*). If this situation applies also to *N*-sulphated GlcN, the H-1 doublet from $GlcNSO_3$ linked to GlcA should be found ~0.2 p.p.m. down-field of that linked to IdoA, which is near 5.4 p.p.m. Therefore, the H-1 doublets near 5.6 p.p.m. in the original GAGs probably included that arising from $GlcNSO_3$ linked to GlcA.

Doublets of the anomeric protons of IdoA residues. — Several doublets (J ~3 Hz) between 4.90 and 5.30 p.p.m. could be assigned to H-1 of IdoA and $IdoA(2SO_3)$. The main doublet due to $IdoA(2SO_3)$ occurred at 5.20–5.29 p.p.m. with a minor doublet slightly up-field in the spectra of the native heparins and heparan sulphates.

Unsulphated IdoA gave H-1 doublets in the range 4.90–5.05 p.p.m., which were identified as such from the COSY spectra of (*c*) (not shown) and the HSIIB (Fig. 3). A doublet at 4.97 p.p.m. for (*c*) was connected to the H-2 multiplet at 3.68 p.p.m. Another doublet, seen in the spectrum of HSIIB at 5.04 p.p.m., was connected to an H-2 multiplet at 3.76 p.p.m. Two doublets for unsulphated IdoA were also seen in the spectra of Hep M and HSl. The lower-field doublet did not occur in the spectra of the *O*-desulphated compounds (*c*) and (*f*) [an unidentified doublet at 5.09 p.p.m. in the spectrum of (*e*) was an artefact arising from its preparation from (*f*)] and was assigned therefore to IdoA residues linked to 6-sulphated GlcN.

The doublets at 4.90–5.00 p.p.m. were assigned to IdoA residues linked to GlcN residues not 6-sulphated, by comparison with the corresponding doublets in the spectra of the *O*-desulphated samples (*c*), (*e*), and (*f*).

Doublets of the anomeric protons of the β-D residues. — The only residue in the main part of the chain of heparin and heparan sulphate in the β-D configuration is GlcA. There were several H-1 doublets, with the expected *J* value of 7–8 Hz, between 4.4 and 4.8 p.p.m. in the relevant spectra. The COSY spectra of HSIIB (Fig. 3) displayed cross-peaks showing coupling between most of these doublets and multiplets near 3.4 p.p.m., consistent with assignment to GlcA residues by comparison with the spectrum of (*b*).

Two doublets in this spectrum, at 4.52 and 4.67 p.p.m., were connected to multiplets at 3.70 and 3.77 p.p.m., respectively. There are several possible explanations for this, namely, the presence in the sample of dermatan sulphate impurities (containing β-D-GalNAc), or of the protein linkage sequence (containing β-D-Gal), or of a small proportion of GlcA2SO_3 (ref. 20). Dermatan sulphate is a common impurity in heparin and heparan sulphate, and contains β-D-GalN. HSIIC contains a small proportion of this impurity, and its β-D-GalN content is ~10% of the total hexosamine (compared with <1% in HSI, HSIIA, and HSIIB)[21]. The *O*-desulphated product of this impurity is the source of a doublet (*J* 8 Hz) in the COSY spectrum of (*f*) at 4.63 p.p.m., which was coupled to a multiplet at 4.00 p.p.m. These chemical shifts agree with those reported by Gatti *et al.*[5] and with our unpublished measurements on dermatan sulphate, the *O*-desulphation of which does not affect the chemical shift of resonances for H-1 or H-2 of GalNAc to any significant extent. Therefore, the two anomalous doublets cannot be explained in this way.

Another possible source of these H-1 doublets is the linkage sequence through which the glycosaminoglycan chain is attached to its original protein core:

[β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Xyl*p*-L-Ser].
3 2 1

9

The proton spectrum at 500 MHz of this sequence has been published[22]. The H-1 doublets from β-D-Gal residues 2 and 3 are at 4.534 and 4.615 p.p.m., with the H-2 multiplets at 3.789 and 3.606 p.p.m., respectively. It is tempting to assign the doublets at 4.67 and 4.52 p.p.m. to the two D-Gal residues in this sequence, although the shifts of the H-2 multiplets did not correspond well to those in the published spectra (differences between the spectra of the free oligosaccharide and that bound to GAG are to be expected). Unfortunately, the recommended structural reporter-group[22] for β-D-Gal substituted at C-3 (the H-4 multiplet at 4.19 p.p.m.) could not be distinguished in any of our spectra, and the Xyl H-1 doublet (at 4.470 p.p.m. with H-2 at 3.381 p.p.m.), if present, is obscured in both the 1D and 2D spectra by the H-1 doublet of GlcA in **2**.

Of the H-1 doublets attributable to β-GlcA, a group at 4.60–4.66 p.p.m. was present in the spectra of heparan sulphate and heparin, which disappeared on *O*-desulphation, *i.e.*, in (*c*), but survived *N*-desulphation, *i.e.*, in (*d*) [the much smaller residual H-1 doublets in the spectra of (*e*) and (*f*) were a consequence of incomplete desulphation]. The COSY spectrum of HSIIB indicated coupling to H-2 multiplets near 3.4 p.p.m., as would be the case for GlcA. It is unlikely that the H-2 multiplet of 2-sulphated GlcA would be seen at this chemical shift since the sulphate group would be expected to have a considerable deshielding effect. Therefore, it is suggested that these H-1 doublets were due, at least in part, to GlcA residues linked to 6-sulphated GlcN residues. In the spectra of the lung heparins studied, these were the only H-1β doublets observed.

The above assignments are illustrated and summarised for a commercial porcine-mucosal heparin (Hep M) and a sample of heparan sulphate (HSI) in Fig. 2.

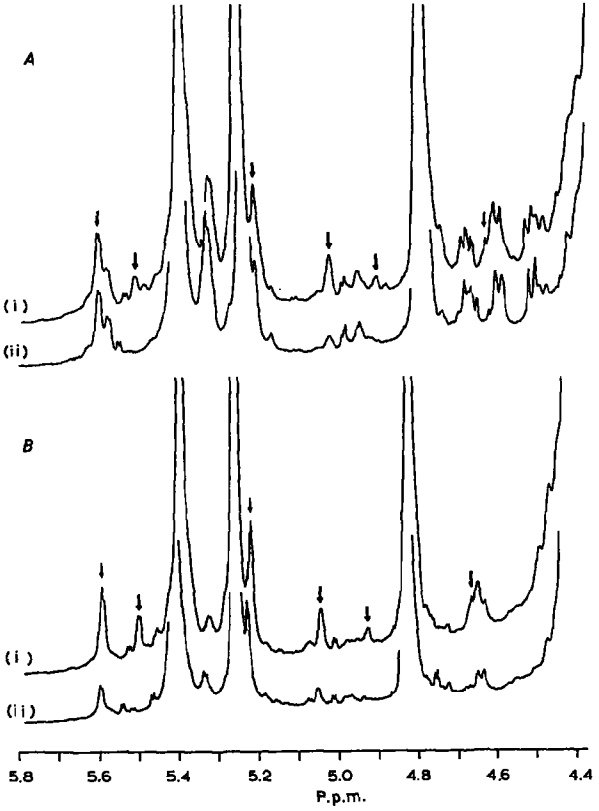
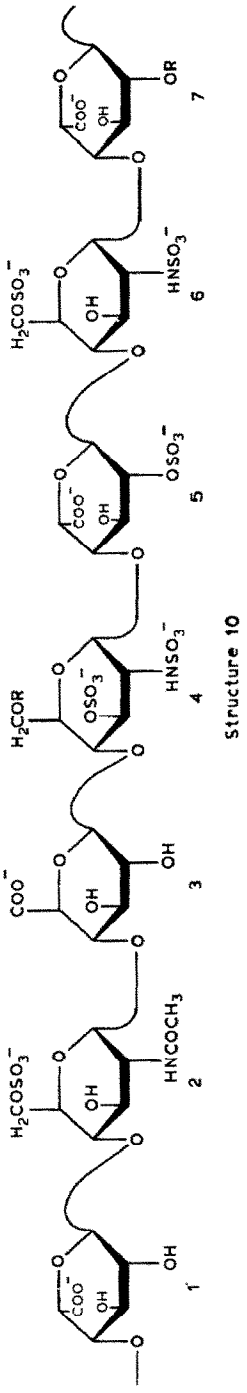
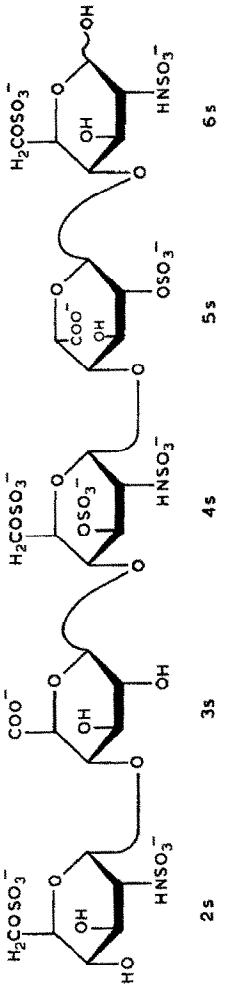


Fig. 4. Partial ^{1}H-n.m.r. spectra at 500 MHz (343 K) of *A*, porcine mucosal heparin fractions of (i) high and (ii) low affinity for antithrombin; *B*, bovine lung heparin fractions of (i) high and (ii) low affinity for antithrombin. The arrows indicate the H-1 doublets enhanced in both mucosal and lung high-affinity fractions.



Structure 10



Structure 11

TABLE II

CHEMICAL SHIFTS (p.p.m.) OF THE H-1 DOUBLETS WITH INCREASED INTENSITY IN THE SPECTRA OF HEPARINS HAVING HIGH AFFINITY FOR ANTITHROMBIN

Residue (see **10***)*	*Lung heparin*	*Mucosal heparin*	*Synthetic pentasaccharide*[24]	*Residue (see* **11***)*
1	5.05	5.04		
2(6)	5.60	5.59	5.624	2s
3(7)	4.66	4.65	4.625	3s
4	5.51	5.50	5.508	4s
5	5.22	5.22	5.192	5s
			5.441	6s
?	4.94	4.93		

Comparison of spectra of heparins of high and low affinities for antithrombin. — Fractions of high affinity for immobilised antithrombin, prepared from bovine lung and porcine mucosal heparins, had anticoagulant activities (anti-Xa assay) of 260 and 310 u/mg, respectively, and each low-affinity fraction had an activity of <1 u/mg. Certain resonances were enhanced in the spectra of each high-affinity sample. Six of these were H-1 doublets (Fig. 4). The enhancements were generally clearer in the simpler spectra of lung heparin. The chemical shifts of these signals and their suggested assignments to the specific sequence of sugars involved in binding of heparin to antithrombin[23] (**10**) are shown in Table II. The corresponding chemical shifts of the H-1 doublets from sugars in a synthetic pentasaccharide closely resembling part of this sequence[24] (**11**) are given for comparison; the numbering of residues is taken from ref. 23.

The H-1 doublet at 5.05 p.p.m. was assigned to residue 1 in **10**, in accordance with the assignment above for IdoA linked to 6-sulphated GlcN. The enhanced doublet at 5.60 p.p.m. might be accounted for by residue 2 or residue 6 (each of which may be *N*-sulphated, as in **11**, in structural variants[23]) in accord with the reasoning above concerning *N*-sulphated GlcN linked to GlcA. In the spectrum of the synthetic pentasaccharide, this residue is at the non-reducing terminus and so may not be a satisfactory model. Residue 3 is the only GlcA in the binding sequence; therefore, the enhanced signal at 4.66 p.p.m. was assigned thereto. Residue 7 may also be GlcA[23] and some contribution to the enhancement in the 4.66-p.p.m. region may arise therefrom. This is in accord with the assignment above for the anomeric proton of GlcA linked to 6-sulphated GlcN; no enhancement was observed for resonances assigned to GlcA linked to GlcNAc. For residue 4, the unusual 3-sulphated GlcN, H-1 may be assumed to have given rise to the enhanced doublet at 5.51 p.p.m. by comparison with the corresponding signal in the spectrum of the synthetic pentasaccharide. Residue 5, IdoA($2SO_3$), is linked to GlcNSO_3($6SO_3$), and its H-1 should therefore give a resonance close to the main H-1 doublet of IdoA($2SO_3$); the enhanced peak at 5.22 p.p.m. has therefore been assigned thereto.

Residue 6, *N*-sulphated GlcN linked to IdoA, would be expected to give a signal not resolved from the main GlcN signal at 5.37–5.40 p.p.m. A doublet at 4.94 p.p.m. was also enhanced in the spectra of each high-affinity fraction. The above assignments indicate that this resulted from an unsulphated IdoA residue linked to a GlcN residue unsulphated at C-6. No such disaccharide unit was reported[23] as being present in the antithrombin binding region, at least up to the dodecasaccharide level.

A weak doublet at 5.55 p.p.m. in each low-affinity fraction did not occur in the high-affinity fractions, but, in the latter, a doublet similar in appearance occurred at 5.53 p.p.m. This may represent a change in chemical shift due to the effects of counter-ions arising from differences in the preparation of high-affinity as compared with low-affinity samples. Similar changes in chemical shift are seen for the main GlcN and IdoA H-1 doublets and also the H-5 of IdoA. The difference in relative intensity of the NAc signal at 2.08 p.p.m. between high- and low-affinity fractions of lung heparin is striking; almost all of the *N*-acetyl content of the original sample was found in the high-affinity fraction.

DISCUSSION

Attempts have been made to use high-field ^{1}H-n.m.r. spectra to assess the composition of samples of heparin and heparan sulphate. Ayotte *et al.*[14] used the H-1 doublets in 400-MHz spectra of a series of heparins and heparan sulphates to measure the proportions of different residues, but they did not identify any sequence effects. Their assignment of these spectra differs from ours in that the double doublet from H-2 of GlcNAc was placed at 3.4 p.p.m.; Huckerby and Nieduszynski[15], using double-resonance spectroscopy, showed the H-2 signal to be at 3.9 p.p.m. in the 400-MHz spectrum of a sample of heparan sulphate with low sulphate content. This finding has been confirmed by the COSY spectra of (*b*) and of samples of heparan sulphate. In a more recent publication, Ayotte and Perlin[16] have repeated their earlier assignment.

The increased resolution of 500-MHz spectra, together with resolution enhancement by Lorentzian–Gaussian transformation, allows more detailed spectra to be obtained. The availability of 2D techniques simplifies assignment, especially in crowded areas of the spectrum. Even so, and with careful preparation of samples in order to eliminate unwanted counter-ion effects as far as possible, there are more signals in the spectrum than can be accounted for by sequence effects due to known variations in the constituent monosaccharide residues of heparin and heparan sulphate. However, the samples studied here show a moderately consistent picture with respect to both assigned and unassigned signals. The conclusion of Ayotte *et al.*[14] that the distinction between heparin and heparan sulphate is qualitative, consisting of differences in proportions of different constituent residues, is supported by our work, at least for heparan sulphates prepared from the by-products of heparin manufacture. This conclusion applies also to the

species and tissue differences between our heparin samples. The spectra of mucosal heparin and of both heparan sulphate samples (Figs. 2 and 3) can be described as superpositions of the spectra of (*a*) and (*b*) (representing the repeating disaccharides most common in these compounds) in varying proportions, with minor contributions arising from less abundant sequences.

Sequence effects on the chemical shifts of the H-1 doublets (due to variations in the C-1-attached nearest neighbour) can be seen for both GlcN and uronic acid residues. The chemical shifts of the H-1 doublets of GlcN (whether *N*-sulphated or *N*-acetylated) vary by ~0.2 p.p.m., according to whether they are attached to IdoA or GlcA residues. GlcNAc attached to an IdoA residue gives a distinctive H-1 doublet at 5.1–5.2 p.p.m. which is not seen in more than trace intensity in heparin or heparan sulphate, as would be expected from the biosynthesis of these compounds in which the epimerisation of a GlcA to an IdoA residue is dependent[18] on the prior *N*-sulphation of the GlcN residue attached at C-4. The H-1 doublet of $GlcNSO_3(3,6diSO_3)$ has been identified, which leaves several doublets (with chemical shifts in the range of 5.29–5.62 p.p.m., with *J* 4 Hz, and showing coupling to multiplets near 3.3 p.p.m.) unaccounted for.

Unsulphated IdoA residues give H-1 doublets differing by ~0.1 p.p.m. according to whether or not the GlcN attached is 6-sulphated or not; it was not possible to demonstrate a similar effect for 2-sulphated IdoA by using the above series of modified heparan sulphates. Again, more doublets are present in this region (4.9–5.3 p.p.m.) than could be assigned.

GlcA residues also give a variety of H-1 doublets; those attached to 6-sulphated GlcN give signals ~0.15 p.p.m. down-field of those not so attached. The H-1 doublet of β-D-GalNAc of a desulphated dermatan sulphate impurity in one of the samples was readily identified by a COSY spectrum. A doublet at 4.52 p.p.m., connected to an H-2 multiplet at 3.70 p.p.m. in the COSY spectrum of HSIIB, might be assigned tentatively to H-1 of β-D-Gal in the proteoglycan-linkage region, although there is no corroboratory evidence. Another doublet, at 4.67 p.p.m. with H-2 at 3.77, may arise from H-1 of $GlcA2SO_3$. Further work is being undertaken to improve these assignments.

The contribution from the antithrombin binding sequence to the region for H-1 doublets in the spectrum of heparin is readily identifiable in the spectra of fractions with high antithrombin affinity and can be assigned in accordance with the sequence effects described above. One doublet, with a chemical shift typical of H-1 of IdoA linked to a non-6-sulphated GlcN residue and quite clearly enhanced in the spectra of the high-affinity fractions, cannot be assigned to any residue as yet identified as being associated with the binding sequence.

Some of the remaining unassigned H-1 doublets may be due to residues at or very near the reducing and non-reducing ends of polysaccharide chains and others to the consistent presence of various counter-ions undergoing slow exchange, or to monosaccharide residues (and therefore sequence effects) not yet identified.

Ayotte and Perlin[14] have published the spectra of a series of modified

heparins prepared in the same way as our modified heparan sulphates. Our results are not reconcilable with theirs, however, especially with respect to their compound **4**, which should be similar to (*d*). In the spectrum they presented, a strong resonance at 5.42 p.p.m. was assigned to H-1 of GlcNAc. We find this resonance, for GlcNAc linked to IdoA or IdoA($2SO_3$), near 5.15 p.p.m., and we attribute major doublets down-field of 5.35 p.p.m. to H-1 of GlcN which, if acetylated, is linked to GlcA. Otherwise, the GlcN is *N*-sulphated or in free base form.

EXPERIMENTAL

HSI, HSIIA, and HSIIB were samples of porcine mucosal heparan sulphate essentially identical, respectively, to HSI (ref. 17) [also HeS A (ref. 25)], HSIIA (ref. 17) [also HeS E (ref. 25)], and HSIIB (ref. 17) [also HeS G (ref. 25)] previously described. HSIIC was a mixture of heparan sulphates, mainly type HSIIA, with some dermatan sulphate impurity. Hep M was a commercial porcine-mucosal heparin (Leo Pharmaceuticals batch 184055), (*a*) was heparin from bovine lung (Upjohn batch 070ES), (*b*) was a sample of capsular polysaccharide from *E. coli* O10:K5:H4 (ref. 26), (*c*) was prepared from HSIIA by solvolytic *O*- and *N*-desulphation[27] followed by re-*N*-acetylation, (*d*) was prepared from HSIIB by *N*-desulphation[28] and re-*N*-acetylation, (*e*) was prepared from HSIIC by *O*- and *N*-desulphation and then re-*N*-sulphation[29], (*f*) was *O*- and *N*-desulphated HSIIC. Heparin fractions with high and low affinities for antithrombin were prepared by affinity chromatography on a column of immobilised antithrombin.

A solution of each sample of GAG in water (2 mL) was washed through a column (1.5 × 8 cm) of Chelex 100 (Biorad) or Amberlite CG-120, or a 1-mL Bond-Elut SCX column (Analytichem International), each in the Na^+ form, with water (10 mL), and the eluate was concentrated at 40° under reduced pressure. A solution of the residue in D_2O (99.8%) was lyophilised and the process was repeated thrice. A solution of each GAG (50–100 mg) in D_2O (100%, 0.5 mL) was used for spectroscopy.

The ^{1}H-n.m.r. spectra were recorded with a Bruker AM 500 spectrometer at a nominal probe temperature of 343 K. 1000–1600 Transients of 16k data points were accumulated over a spectral width of 5000 Hz. Resolution enhancement was performed by Lorentzian–Gaussian peak transformation. Chemical shifts were referenced externally to trimethylsilylpropanesulphonic acid (TSP) in an aqueous ~10% solution of heparan sulphate.

2D Correlation spectroscopy (COSY)[30] was performed at 343 K, using the standard Bruker microprogram. The data were transformed with a sine-bell window and zero-filling in the F1 dimension. All the spectra incorporated a 45° mixing pulse and no fixed delay. For HSIIB, 256 spectra with a spectral width (SW) of 1269 Hz were collected. The corresponding figures for (*b*) were 256 and 1171 Hz, for (*c*) 512 and 2000 Hz, and for (*f*) 256 and 1250 Hz.

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