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Note

Proton chemical-shift assignments in the n.m.r spectra of heparan and heparin

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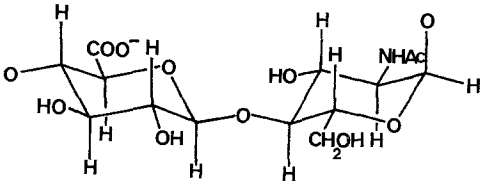
The heparan sulphate and heparin families of polysaccharides are related structurally[1] and display various side-group modifications overlaid on an otherwise, regularly alternating, copolymeric structure. Specific sequences of such substituted sugar residues act as recognition sites for either self-interaction or binding to other biological macromolecules. Thus, characteristic saccharide sequences have been described for heparan sulphate self-association[2] and binding of heparin to antithrombin[3,4]. We now report ^{1}H-n.m.r. data for heparan, a polymer involving alternating 2-acetamido-2-deoxy-α-D-glucose and β-D-glucuronate residues (**1**), and a pig-mucosal heparin, which also contains such sequences.



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The significance of minor structural components in these macromolecules is now appreciated. Thus, the heparin-binding site for antithrombin contains several residues which are not characteristic of the major part of the heparin structure. These include unsulphated L-iduronate, 2-acetamido-2-deoxy-D-glucose, D-glucuronate, and trisulphated 2-amino-2-deoxy-D-glucose residues. Identification of the resonances from such residues is important and includes recognition of the C-2 resonance from the trisulphated 2-amino-2-deoxy-D-glucose[5]. However, the published proton-resonance assignments[6] for D-glucuronate and 2-amino-2-deoxy-D-glucose minor

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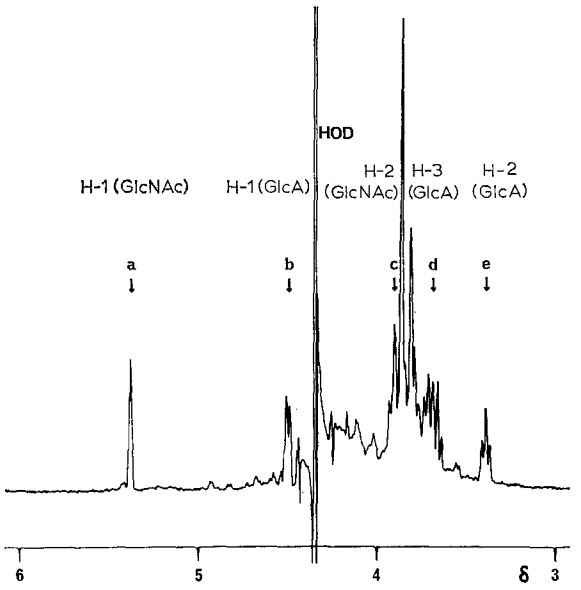

Fig. 1. Partial, 400-MHz, ^{1}H-n.m.r. spectrum (D_2O at 75°) for sodium heparan. Chemical shifts are given in p.p.m. from internal TSP-d_4.

components within heparin and the heparan sulphates require further examination.

The 400-MHz, ^{1}H-n.m.r. spectrum for heparan is shown in Fig. 1. A methyl resonance (not shown) occurs at δ 2.045. Even at this frequency, the spectrum is complex, but three ring resonances are clearly resolved. Two are at a relatively low field, namely, a ~3.5-Hz doublet at δ 5.382, and a 7.6-Hz doublet at δ 4.496 [(a) and (b) in Fig. 1] and are assigned[6] to H-1 of GlcNAc and GlcA, respectively, in **1**. The third is the apparent triplet (e) at δ 3.382 which was previously assigned[6] to H-2 of GlcNAc.

Spin decoupling at (a) caused a slight sharpening at (c), δ 3.896. This relationship was confirmed by double-resonance difference spectroscopy which gave a response only at (c). Irradiation at (b) reduced (e) to a doublet (J 9.15 Hz), and when (e) was decoupled in turn, (b) became a singlet and a simplification also appeared at (d), δ ~3.71. Decoupling at (d) reduced (e) to a doublet. These experiments confirm that (e) must be the resonance from H-2 of GlcA (rather than GlcNAc), and it exhibits couplings of 7.6 ($J_{1,2}$) and 9.15 Hz ($J_{2,3}$) typical of a β-D-glucuronate residue in the $^4C_1$ conformation.

The resonance for H-2 of GlcNAc should actually be assigned to a position ~0.5 p.p.m. downfield, and H-2 is more deshielded than might have been expected for a proton attached to a carbon bearing nitrogen. These findings are confirmed by data for the appropriate monosaccharides. For sodium β-D-glucuronate, H-2 resonates[7] at δ 3.496, whereas, for 2-acetamido-2-deoxy-α-D-glucose, H-2 (as shown by double-resonance difference spectroscopy at 100 MHz) resonates at δ 3.80. These

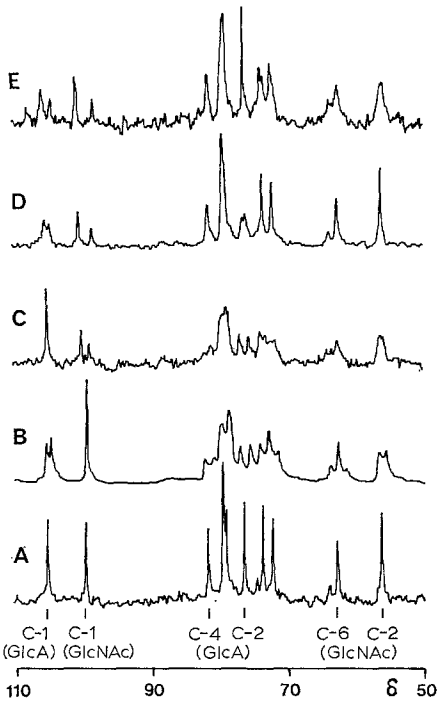

Fig. 2. Partial, 25.1-MHz, ^{13}C-n.m.r. spectra (D_2O at 75°) for sodium heparan: A is the proton-noise-decoupled spectrum. The other traces represent selective proton-decoupling experiments at the chemical shifts indicated: B, δ 5.382; C, δ 4.496; D, δ 3.896; E, δ 3.382. Chemical shifts are given in p.p.m. from internal TSP-d_4.

chemical shifts are probably determined by the ring conformations and the anomeric configuration.

These findings may be correlated with carbon chemical-shift data for heparan, obtained by using selective proton decoupling. In Fig. 2, trace A is the 25.1-MHz, ^{13}C proton-noise-decoupled spectrum for the ring-carbon resonances of heparan. In traces B–E, selective decoupling has been applied at points (a), (b), (c), and (e), respectively, on the ^{1}H spectrum (Fig. 1). B and C confirm that the low-field anomeric resonance at δ 105.32 arises from C-1 of GlcA, whereas the other (δ 99.57) is due to C-1 of GlcNAc. In trace D, the resonance at δ 56.26, known to arise from C-2 of GlcNAc, sharpens (together with other resonances) on irradiation at point (c) (δ 3.896) in the ^{1}H spectrum. However, when, as in E, the decoupling is at (e) (δ 3.382), only one signal sharpens. This is not at δ 56.26, as would be expected for irradiation of H-2 of GlcNAc, but at δ 76.35, which, therefore, can now be assigned to C-2 of GlcA.

In pig-mucosal heparin, the 400-MHz ^{1}H spectrum (not shown) shows a small, composite signal composed of two overlapped triplets at δ 3.392 and 3.434, which is similar in appearance to the resonance for C-2 of GlcNAc in heparan at δ 3.382. The major resonance from H-2 in $GlcNSO_3^-$ residues[6] is seen as a doublet of doublets

($J_{1,2}$ 2.5 Hz, $J_{2,3}$ ~9 Hz) at δ 3.292. Irradiation of the pair of triplets at δ ~3.4 simplifies two small doublets at δ 4.525 and 4.615 (H-1 of GlcA in heparan at δ 4.496). These two resonances may be assigned to H-1 of glucuronate residues, presumably in slightly different environments. Therefore, for heparin also, the previous assignment of the resonances at δ ~3.4 to H-2 of GlcNAc is incorrect. They must be assigned to H-2 of GlcA.

EXPERIMENTAL

Materials and methods. — The sodium salts of heparin (pig mucosal) and by-products from heparin (bovine lung) were provided by Glaxo Biologicals Ltd. The heparin by-products were fractionated according to the methods of Rodén *et al.*[8]. Crude material was treated with alkaline copper sulphate to remove dermatan sulphate. The soluble material, as the calcium salt, was fractionated with ethanol, and glycans precipitating between 18 and 36% of ethanol were recovered. Finally, the heparan sulphates were precipitated as cetylpyridinium complexes[9] and fractionated according to their solubility in M NaCl. The soluble material was deaminatively cleaved by nitrous acid and reduced with sodium borohydride at an alkaline pH, and the degradation products were fractionated on a column of Sephadex G-25 superfine, as previously described[10]. The void-volume fraction was dialysed free of excess salts and lyophilised. This "heparan sulphate" fraction is almost entirely *unsulphated* and has been referred to above as heparan.

Samples for n.m.r. spectroscopy were buffered to pH 7 with phosphate, and the materials for the ^1H study were then triply exchanged with D_2O. 100-MHz ^1H and 25.1-MHz ^{13}C measurements were performed on a JEOL FX100 instrument. 400-MHz ^1H and 100-MHz ^{13}C spectra were obtained from the S.R.C. Very High Field N.M.R. Service unit at the University of Sheffield. Spectra were recorded at 75°, using 3-trimethylsilylpropionic acid-d_4 sodium salt (TSP-d_4) as internal reference (5 mg was sufficient to produce an adequate ^{13}C signal under the 90°-pulse conditions employed).

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