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# Note

## Occurrence of 2-*O*-sulphated D-glucuronic acid in rat liver heparan sulphate

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Heparan sulphates (HS) are a complex and heterogeneous family of glycosaminoglycans with a polysaccharide chain composed of alternating units of D-glucosamine and uronic acid (D-glucuronic or L-iduronic) with a (1 → 4) linkage. Sulphate substituents occur as *N*-sulphate (at C-2 of the D-glucosamine residues) or as *O*-sulphate groups (at C-6 of the D-glucosamine or at C-2 of the L-iduronic acid units).

In our previous studies[1–3] we have isolated and characterized a highly sulphated HS from rat liver tissues, L1. In spite of its high degree of sulphation (2.0 sulphate groups/disaccharide), only 50% of the hexosamine residues bear *N*-sulphate groups, and L-iduronic acid accounts for 25% of the total uronic acids.

The high sulphate content of L1 could not be explained on the basis of the sulphation pattern normally observed in heparin-like polysaccharides. As the polymer is only partially *N*-sulphated, the high degree of sulphation is presumably due to the *O*-sulphate groups. The low proportion of L-iduronic acid suggests that positions other than C-2 or C-6 of D-glucosamine are substituted.

In order to determine the location of the remaining *O*-sulphate groups, we continued our structural studies on L1 through $^{13}C$ NMR and methylation analysis, which allowed us to detect the presence of 2-*O*-sulphated D-glucuronic acid residues in L1.

### RESULTS AND DISCUSSION

Methylation analysis by GLC–MS was reported in detail elsewhere[3]. Briefly, from the mass spectra, it is possible to assign the peaks arising from nonsulphated

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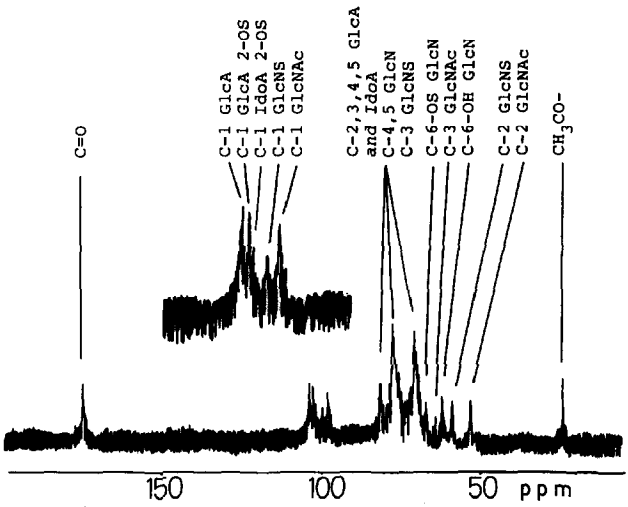


Fig. 1. $^{13}C$ NMR spectrum of highly sulphated heparan sulphate L1 from rat liver.

or 2-*O*-sulphated uronic acids, and nonsulphated, 6-*O*-sulphated, or 3,6-di-*O*-sulphated glucosamine residues. 3,6-Di-*O*-sulphated hexosamine, which was shown[4] to be essential for binding to antithrombin III, was detected as a heparin component by methylation, but it was not observed in the GLC–MS pattern of L1, in spite of its high degree of sulphation[3]. The sulphated-to-nonsulphated uronic acid ratio was found to be 1.7:1.

Partially methylated alditol acetates arising from uronic acid units of L1 were further analyzed by GLC with a capillary column, showing two different peaks (retention times 23.14 and 23.97 min) with identical mass spectra (see Experimental), assigned to a 2-*O*-sulphated residue.

This finding implies that L1 contains two types of uronic acid units bearing a sulphate group at C-2. In addition to the known 2-*O*-sulphated L-iduronic acid, D-glucuronic acid should be partially sulphated at O-2, an unusual position of substitution in heparin and heparan sulphates.

The $^{13}C$ NMR spectrum of L1 (Fig. 1 and Table I) showed five different signals corresponding to anomeric carbons. Resonances at 98.4, 99.9, 101.8, and 104.3 ppm were assigned to C-1 of *N*-acetylated D-glucosamine, *N*-sulphated D-glucosamine, 2-*O*-sulphated L-iduronic acid, and D-glucuronic acid, respectively.

TABLE I

$^{13}C$ NMR resonance assignments for anomeric carbon atoms in heparan sulphates (in ppm).

| | GlcNAc | GlcNS | IdoA 2-OS | GlcA 2-OS | GlcA |
|---|---|---|---|---|---|
| L1 | 98.4 | 99.9 | 101.8 | 103.0 | 104.3 |
| HSm | 97.8 | 99.9 | 101.7 | | 104.4 |
| HSm *O*-sulphated | 98.2 | 99.9 | 101.7 | 103.0 | 104.4 |

One additional peak was observed at 103.0 ppm. This chemical shift could be attributed to the influence of 2-*O*-sulphation on the anomeric resonance of D-glucuronic acid[5].

To confirm this assignment, a low sulphated HS from bovine mucosa (HSm) was *O*-sulphated with sulphur trioxide–triethylamine complex. On comparison of the $^{13}C$ NMR spectra of HSm and of the sulphated product, the appearance of the anomeric resonance at 103.0 ppm was detected in the latter. These results show that D-glucuronic acid residues in heparan sulphates could be readily sulphated at O-2, as a consequence of either in vivo or in vitro polymer modifications.

Previously, Bienkowski and Conrad[6] identified, as minor components, 2- or 3-*O*-sulphated D-glucuronic acid-containing disaccharides among the deamination products of commercial heparin preparations. Fedarko and Conrad[7] found a high proportion of these disaccharides in the nuclear HS pool of cultured rat hepatocytes. Biosynthetic studies on mouse mastocytoma microsomal heparin-like polysaccharides led Kusche and Lindahl[8] to confirm that sulphated glucuronic acid are readily formed by the sulphation of D-glucuronic acid residues and not by back epimerization of sulphated L-iduronic acid units.

Although Bienkowski and Conrad[6] proposed that the sulphate group was located at C-2 of the glucuronic acid units, this assignment remained tentative and required confirmation by other methods[8]. Our results provide further evidence about the occurrence of 2-*O*-sulphated glucuronic acid in rat liver HS. Both $^{13}C$ NMR and methylation analysis are consistent with sulphation at C-2, yielding similar ratios of sulphated to nonsulphated uronic acid residues (1.6:1 and 1.7:1, respectively). Moreover, from the resonance intensities in the anomeric region of the $^{13}C$ NMR spectrum of L1, the proportion of 2-*O*-sulphated residues could be estimated in $\sim 50\%$ of the total glucuronic acid units.

Substitution at C-3 cannot be completely ruled out from the $^{13}C$ NMR spectrum. However, methylation analysis of L1 by GLC–MS did not result in partially methylated alditol acetates that could be derived from a 3-*O*-sulphated D-glucuronic acid unit (thus, this residue would be methylated at C-2 and should give a peak of $m/z$ 117).

It has been suggested[7,9] that polysaccharide chains having a high content of sulphated glucuronic acid units may be involved in the control of cell growth, but their actual functional role is still unknown. On the other hand, the sequential biosynthetic pathway normally accepted for heparin[8] may not be the main operating mechanism for the biosynthesis of L1 since it cannot explain the presence of the high proportion of 2-*O*-sulphated D-glucuronic acid in this heparan sulphate.

## EXPERIMENTAL

*General.* — High sulphated heparan sulphate (L1) was isolated from rat liver[1,2]. Heparan sulphate from bovine mucosa (HSm) was obtained from commercial heparin by-products[2]. The sulphate/disaccharide ratios were calculated from their

total sulphate (rhodizonate test[10]), uronic acid (carbazole–borate test[11]), and hexosamine contents (Elson–Morgan test[12]). *N*-Sulphated glucosamine was determined by the indole test[13]. The iduronic/glucuronic acid ratio was estimated by GLC of the corresponding aldonolactones[14].

GLC–MS was performed with a Varian Mat CH7A mass spectrometer at 70 eV combined with a Varian 1400 gas chromatograph; a glass column (2 mm × 1.2 m) packed with 2% OV-101 on Chromosorb WAW (60–80 mesh) was used at a temperature of 120 to 290°C (rate 6°C/min, gas flow 28 mL He/min).

Capillary GLC was performed with a Hewlett–Packard 5890 gas chromatograph equipped with a flame-ionization detector; an SP 2330 fused silica column (0.25 mm × 15 m) was used at 190°C (gas flow rate 1 mL $N_2$/min).

$^{13}C$ NMR spectra were recorded at room temperature with a Varian XL-100 spectrometer operating at 25.2 MHz; samples were dissolved in 1:1 $D_2O$–$H_2O$; internal standard 1,4-dioxane ($\delta$ 67.30).

*Methylation of rat liver heparan sulphate L1.* — The methylation was performed as described[3]. Briefly, the pyridinium salt of L1 (10 mg) was dissolved in anhyd $Me_2SO$ (2 mL) at room temperature and BuLi (0.9 M in hexane; 2 mL) was added. After 2 h at 40°C, the mixture was cooled and MeI (1.5 mL) was added. Stirring was continued at room temperature until a clear solution was obtained, and the excess MeI was removed. Desulphation was accomplished by the method of Nagasawa et al.[15]. Hydrolysis of the desulphated product was performed with 0.25 M $H_2SO_4$ in AcOH (18 h, 80°C)[16]. This was followed by reduction with $NaBH_4$ and acetylation of the mixture of partially methylated alditols. Uronic acids were analyzed as the corresponding methyl aldonates. Uronate 2-OS showed the following MS: *m/z* 319 (6.6,[M-$CH_2OAc$]$^+$), 247 (8.8, [$CO_2Me \cdot (CHOAc)_2 \cdot CH{=}O^+Me$]), 217 (1.2), 215 (1.0), 187 (5.4), 173 (2.3), 155 (59.5,[$C_7H_7O_4$]$^+$), 131 (1.6), 129 (5.8,[$CH_2{=}COAc \cdot CH{=}O^+Me$]), 113 (3.2), 43 (100,[$CH_3CO^+$]).

*$^{13}C$ NMR spectrum of L1.* — $\delta$ 175.9 (C=O), 104.3 (C-1, GlcA), 103.0 (C-1, GlcA 2-OS), 101.8 (C-1, IdoA 2-OS), 99.9 (C-1, GlcNS), 98.4 (C-1, GlcNAc), 79.8–70.1 (C-2, C-3, C-4, C-5, GlcA, and IdoA; C-4, C-5, GlcN; C-3, GlcNS), 67.3 (C-6-OS, GlcN), 65.0 (C-3, GlcNAc), 62.0 (C-6-OH, GlcN), 58.9 (C-2, GlcNS), 53.0 (C-2, GlcNAc), and 23.7 ($CH_3CO$–).

*O-Sulphation of HSm.* — The pyridinium salt of HS (200.0 mg) was dissolved in 5:1 DMF–$Me_2SO$ and 1:1 $SO_3$–$Et_3N$ complex[17] (2 g) was added. After stirring overnight at room temperature the mixture was poured over water, and the pH was brought to 8.0 using M NaOH. After dialysis and solvent evaporation under diminished pressure, a sulphated product (236.7 mg) was obtained (sulphate/disaccharide ratio 1.8, as compared to 0.7 for the starting product).

*$^{13}C$ NMR spectrum of HSm.* — $\delta$ 176.7 (C=O), 104.4 (C-1, GlcA), 101.7 (C-1, IdoA 2-OS), 99.9 (C-1, GlcNS), 97.8 (C-1, GlcNAc), 79.1–71.6 (C-2, C-3, C-4, C-5, GlcA, and IdoA; C-4, C-5, GlcN; C-3, GlcNS), 67.8 (C-6-OS, GlcN), 65.1 (C-3, GlcNAc), 62.7 (C-6-OH, GlcN), 60.0 (C-2, GlcNS), 53.6 (C-2, GlcNAc), and 24.0 ($CH_3CO$–).

*$^{13}C$ NMR spectrum of O-sulphated HSm.* — $\delta$ 176.8 (C=O), 104.4 (C-1, GlcA), 103.0 (C-1, GlcA 2-OS), 101.7 (C-1, IdoA 2-OS), 99.9 (C-1, GlcNS), 98.2 (C-1, GlcNAc), 79.2–71.6 (C-2, C-3, C-4, C-5, GlcA, and IdoA; C-4, C-5, GlcN; C-3, GlcNS), 68.0 (C-6-OS, GlcN), 65.1 (C-3, GlcNAc), 60.0 (C-2, GlcNS), 53.6 (C-2, GlcNAc), and 24.1 ($CH_3CO$–).

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