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**Note**

## Studies, by $^{13}$C-n.m.r. and $^{1}$H-n.m.r. spectroscopy of polysaccharide structure following sulfation

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As with $^{1}$H-n.m.r. spectroscopy, the $^{13}$C-n.m.r. technique has the potential for non-degradative detection of the changes caused by introduction of such substituents as sulfate groups and thereby locating their positions. Proton and $^{13}$C-n.m.r. spectroscopy have been used by several investigators to study the structural characteristics of heparins and other naturally occurring sulfated glycosaminoglycans[1–5]. This note describes the $^{1}$H-n.m.r. and $^{13}$C-n.m.r. spectra of simple and complex polysaccharides before and after sulfation. The results show that changes in the displacements of resonances provide a convenient method for detection of *O*-sulfation at specific sites.

A comparison was made between the spectra of native and sulfated polysaccharides from asafaetida and from those of larchwood arabinogalactan. Sulfation introduced large downfield shifts in the primary hydroxymethyl signal (C-6) of 62.03 p.p.m. for the asafaetida polysaccharide, whereas the signal (C-6) at 62.2 p.p.m. for native arabinogalactan was not present after sulfation, indicating that the primary hydroxyl groups were extensively substituted. Identification of shifts in resonances of other carbon atoms (C-2–C-5) was much more difficult. The $^{13}$C-n.m.r. spectrum of the D-arabino-D-galactan of cells of the insect protozoan *Crithidia fascicula* was found to be complex, as shown by the C-1 region, which showed 8 signals[7].

Comparison of the $^{13}$C-n.m.r. spectra of sulfated chitin and amylopectin showed that the C-6 signals at 61.8 p.p.m. in both polysaccharides were shifted considerably downfield, to 66.9 and 67.6 p.p.m., respectively. Jennings and coworkers[8,9] have rationalized the $^{13}$C-n.m.r. spectrum of amylose in $D_2O$ at pD 14. The resonance of $\alpha$ and $\beta$ anomers of glucose on sulfation at O-3 or O-6 was reported[10] to show a strong downfield ( +6.5 or +6.6) shift of the resonance at the position of substitution, accompanied by smaller upfield displacements ( −0.1 to −2.0) of the signals of adjacent carbon atoms.

Inulin has two hydroxymethyl groups, and its $^{13}$C-n.m.r. spectrum showed that the signals at 62.2 p.p.m. (C-1) and 63.4 p.p.m. (C-6) are both influenced by sulfation. The signal at 62.2 p.p.m. was considerably weaker, indicating preferen-

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tial sulfation at this position. The $^{13}$C-n.m.r. spectrum of inulin has been reported[11], signal assignments were C-1 (62.2), C-2 (104.5), C-3 (78.5), C-4 (76.6), C-5 (82.4), and C-6 (63.4) at pD 13.

In $^{13}$C-n.m.r. studies, the C-1 and C-6 resonances are readily identified as the farthest downfield and the farthest upfield peaks, respectively. However the C-2–5 resonances fall in the chemical-shift range of 68–78 p.p.m., and positive identification is much more difficult. The task is even more complex when dealing with heteropolysaccharides. Analysis for sulfate in many of the compounds studied suggested the introduction of more than one sulfate groups per sugar residue. In these instances, $^{1}$H-n.m.r. spectra were useful for determining the chemical shifts caused by sulfation of secondary alcohol groups at C-2–C-5. Comparison of the $^{1}$H-n.m.r. spectra of native and the sulfated asafaetida polysaccharides showed that the signal at 3.7 p.p.m., attributed to protons adjacent to the primary alcohol group, had moved downfield, whereas the signal for protons adjacent to the secondary alcohol group was of lower intensity. Asafaetida polysaccharide has been reported[6] to contain 4-*O*-methylglucuronic acid residues. This presumption was evidenced by a high-field doublet at 1.2 p.p.m., assignable to the methyl protons. The splitting may be caused by the adjacent (secondary) hydroxyl proton. Comparison of the spectra of native and sulfated larchwood xylan showed that the intense signal at 3.4 p.p.m. attributable to the proton adjacent to the secondary alcohol groups present in the native polysaccharide resonated at significantly lower field in the sulfated polysaccharide, because of sulfation at C-2 and -3 of the (1→4)-$\beta$-D-linked D-xylopyranan backbone of the larchwood polysaccharide.

It may be concluded that sulfation occurred preferentially at the most accessible of the primary hydroxymethyl groups, except in polysaccharides where the primary hydroxymethyl groups were involved in glycosidic linkages. In the latter group, the signal at 3.4 p.p.m. assigned to the secondary-alcohol proton ($^{1}$H-n.m.r.) showed a downfield shift in the sulfated material, indicating *O*-sulfation.

## EXPERIMENTAL

*$^{1}$H-N.m.r. spectra.* Polysaccharides were dissolved in $D_2O$ and 0.1–0.2 mL of solutions (50 mg/mL) were used in 5-mm tubes. Spectra were recorded with Varian FT-80A or XL 200 spectrometers with Fourier transform, with tetramethylsilane as the external reference.

*$^{13}$C-N.m.r. spectra.* — Spectra were recorded with a Varian FT-80A spectrometer. Polysaccharide solutions (50–100 mg/mL) in $D_2O$ of 0.8–1.0 mL volume in 10-mm tubes were used. Tetramethylsilane was used as the standard, with a coaxial 5-mm tube.

*Preparation of the polysaccharide sulfates.* — Asafaetida polysaccharide was partially purified by the method of Jones and Thomas[6]. Larchwood xylan and arabinogalactan were purchased commercially (Sigma). All three polysaccharides were further purified by chromatography on *O*-(diethylaminoethylamino)-

(DEAE)-cellulose. The DEAE-cellulose (200 g) was mixed with 500 mL of 5mM potassium phosphate buffer (pH 7.1) and transferred as a slurry to a column (4.5 cm × 100 cm). After equilibration with the phosphate buffer, the polysaccharide (0.5 g) dissolved in 50 mL of the same buffer was transferred to the column, which was again equilibrated with the buffer. The column was then eluted with 0.1M sodium chloride. The eluates from the equilibration buffer were discarded, whereas the eluates from 0.1M sodium chloride were collected in 7-mL fractions and analyzed for polysaccharide by the phenol–sulfuric acid method[12]. The results showed one sharp peak, and the fractions from this area were pooled, dialyzed, and lyophilized. The specific rotations of the polysaccharides were $[\alpha]_D^{20}$ −61° for asafaetida, $[\alpha]_D^{20}$ +14° for the arabinogalactan, and $[\alpha]_D^{20}$ −68° for the xylan polysaccharides. The purified polysaccharides and commercial samples of inulin, amylopectin, and chitin (Sigma) were sulfated by using chlorosulfonic acid–pyridine complex by the procedure of Ricketts[13]. The sulfated polysaccharides were further purified by chromatography on DEAE-cellulose as already described and isolated as the sodium salts. The specific rotations of the sulfated polysaccharides were: xylan $[\alpha]_D^{20}$ −70°, asafaetida $[\alpha]_D^{20}$ −26°, inulin $[\alpha]_D^{20}$ −20°, amylopectin $[\alpha]_D^{20}$ +110°, chitin $[\alpha]_D^{20}$ −24°, and arabinogalactan $[\alpha]_D^{20}$ +12°. The percent of sulfur after acid hydrolysis was measured gravimetrically as barium sulfate. The values were 15.9 for arabinogalactan; 18.3 for asafaetida; 13.9 for xylan; 16.0 for inulin; 20.5 for amylopectin, and 11.0 for chitin. Molecular weights were measured for the sulfated polysaccharides by using a Knaur Membrane Osmometer Type 01.00. Three different concentrations (0.2, 0.5, and 1%) were dissolved in 1% sodium chloride, and osmotic pressures were compared against 1% sodium chloride in the lower compartment. The molecular weights were: arabinogalactan 23,500, asafaetida 37,500, xylan 25,000, inulin 16,000, amylopectin 49,000, and chitin 25,000.

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