Vol. 85, No. 1, 1978
November 14, 1978

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 14-20

CONFORMATIONS OF THE MAJOR RESIDUES IN HEPARIN. ^{1}H-NMR SPECTROSCOPIC STUDIES

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Received July 21,1978

ABSTRACT

A detailed analysis is presented of the 270 MHz ^{1}H-NMR spectrum of heparin in D_2O solution at 35°. All signals due to the α-L-iduronic acid 2-sulfate (I) and 2-deoxy-2-sulfamino-D-glucose 6-sulfate (A) residues, that together comprise the major disaccharide repeating sequence of the polymer, were resolved by use of the "convolution difference" technique. Interproton coupling constants calculated for the computer simulated spectrum indicate that "I" residues possess the $^1C_4$ (L) conformation, and "A" residues the $^4C_1$ (D) conformation. The preferred orientation of the 6-sulfate group of "A" residues was also established.

INTRODUCTION

The well known biological functions of heparin are attributed to interaction of this polysaccharide with specific plasma proteins, i.e., with antithrombin III and lipoprotein lipase for anticoagulant and antilipemic (fat-clearing) activities, respectively. These interactions involve the anionic substituents of heparin, although a subtle combination of structural features may be required (1) for high antithrombin III activity.

Heparin preparations from different sources show variations in composition. However, all heparins consist mainly of repeating disaccharide sequences in which α-L-idopyranuronic acid 2-sulfate (residue I) and 2-deoxy-2-sulfamino-α-D-glucopyranose 6-sulfate (residue A) are linked through positions 1 and 4. Generally, this sequence accounts for > 85% of beeflung heparins and > 70% of hog-mucosal heparins. The minor sugar constituents are D-glucuronic acid and 2-acetamido-2-deoxy-glucose, which usually are dispersed along the heparin chains, possibly in block arrangements (2).

Established criteria for conformational analysis of monosaccharides (3)

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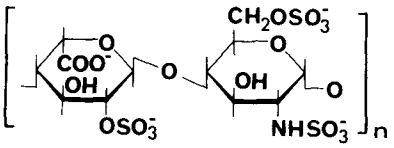


strongly favor the $^4C_1$ (D) conformation for the A moiety, but fail to make a clear prediction as to the conformation of the I moiety because of the small energy difference expected between alternate chair forms of L-idopyranose. An assessment of the conformation of the L-iduronic acid residues is of obvious relevance to the shape of the heparin chain, because alternate conformations of these residues imply a reversal in the orientation of all substituents, including the glycosidic bonds. Previous studies by ^{1}H-NMR spectroscopy at 220 MHz in D_2O solution, have suggested (4) that the A and I residues have the $^4C_1$ (D) and $^1C_4$ (L) conformations, respectively. However, this was based on only a partial analysis, because the spectra were incompletely resolved.

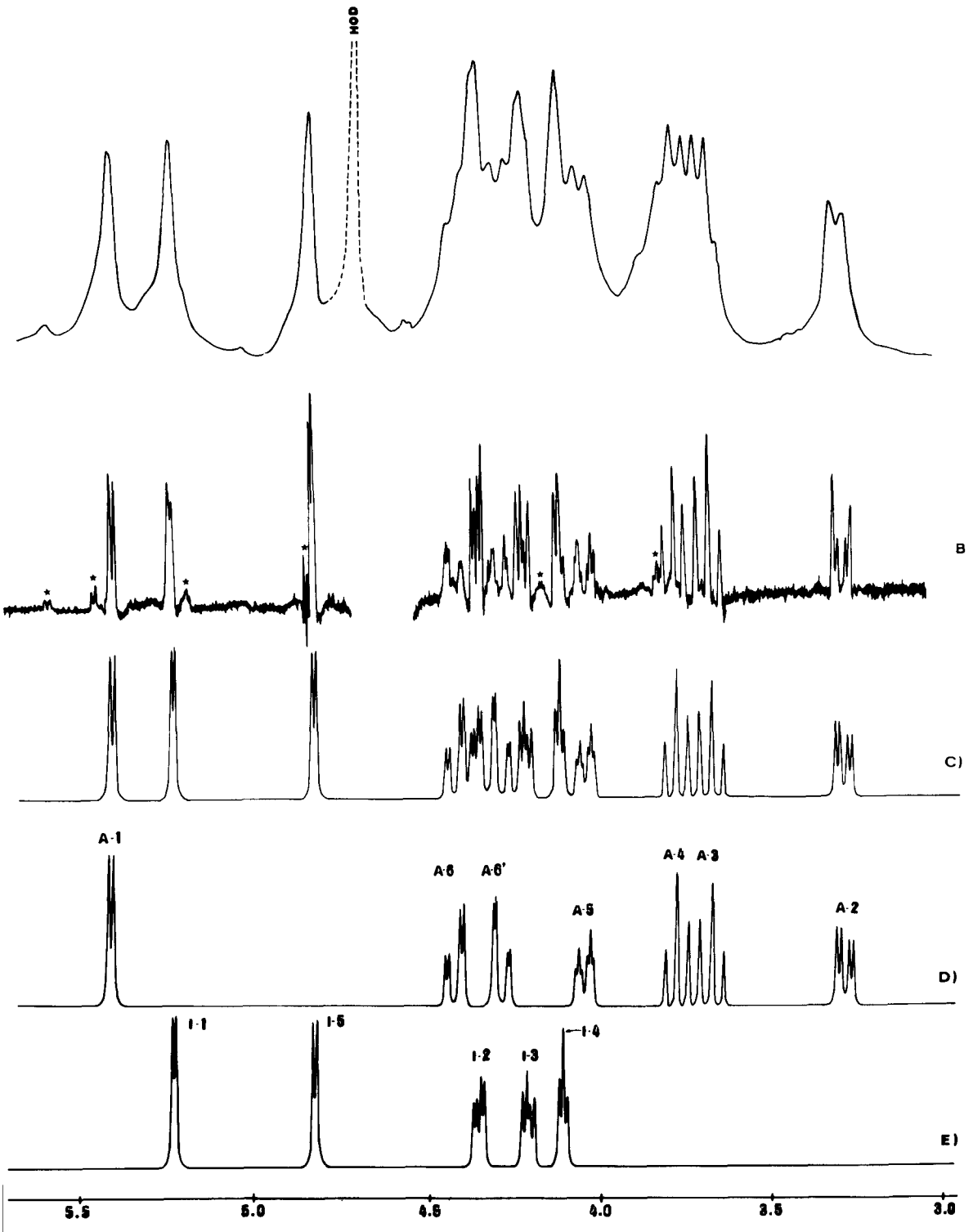
We now report a full analysis of the 270 MHz ^{1}H-NMR spectrum of the major sugar residues of heparin at 35°. This has been made possible by the fact that all of the pertinent signals have been resolved, and their characteristics cross-checked by computer-simulation. The calculated interproton coupling constants are evaluated in terms of the conformations of the A and I moieties, as well as the orientation of the 6-sulfate group on the hexosamine moiety.

MATERIALS AND METHODS

Heparin (Na salt from the Upjohn Co., Kalamazoo, Mich., 155 USP units/mg) was a preparation from beef lung, already characterized in previous work (4,5). A preparation from pig intestinal mucosa used for comparison purpose was a reference standard from the University of Chicago (Dr.s. A.J.Cifonelli and A.S.Mathews). Most of the NMR measurements were performed on a 10% (w/v) solution of heparin in D_2O (99.9%, Merck, Darmstadt), following several exchanges with D_2O 99.7% (Merck). The spectra were measured on a Bruker 270 MHz spectrometer at different temperatures. Chemical shifts were measured with reference to internal TSP. Simulations of the spectra and refinement of the spectral parameters were performed with the LAOOCOON computer program.

RESULTS AND DISCUSSION

Figure 1 shows the ^{1}H-NMR spectrum of heparin, "normal" (A) and after "convolution difference" (B), the latter being acquired through computer-processing of the normal spectrum by subtraction of the broad linewidth components as described (6) for resolution enhancement of protein spectra. The figure also shows the computer-simulated spectra for residues A and I (C and D).


HOD
B
C)
A-1
A-6
A-6'
A-5
A-4
A-3
A-2
D)
I-1
I-5
I-2
I-3
I-4
E)
5.5
5.0
4.5
4.0
3.5
3.0

Chemical shifts and coupling constants for the ring protons are given in Table I. (Data for minor signals, labelled with asterisks in Figure 1 and clearly not attributable to units A and I, are not included). The signal assignment were made with the aid of homonuclear spin-decoupling. Data for $J_{4,5}$, $J_{5,6}$ and $J_{5,6'}$ of A residues were obtained from the better resolved spectrum at 90°C.

The coupling constants found for the ring protons of A residues, which are close to those measured recently at high field for 2-acetamido-2-deoxy--α-D-glucose (7), confirm the expected $^4C_1$ (D) conformation. That is, $J_{1,2}$ is small (3.66 Hz) in accord with a gauche relationship between H-1 and H-2, whereas the other constants are large (9-10 Hz), as required for trans-diaxal orientations.

By contrast, the coupling constants for protons of the I residues are all < 6 Hz which, therefore, rules out the $^4C_1$ (L) conformation. In the alternate $^1C_4$ (L) conformation, as shown readily by Newman projections, the vicinal protons (all gauche) occur in three environments with respect to their anti-periplanar atoms (Fig. 2): (a) H-1/H-2 are opposite to a carbon and an oxygen; (b) H-2/H-3 and also (c) H-3/H-4, to two carbons; and (d) H-4/H-5, to two oxygens. According to recently-established criteria for gauche coupling in carbohydrates (8), the constants to be expected are 2.0-3.5 Hz for case (a), 3.5-5.5 Hz for (b) and (c), and 0.8-3.5 Hz for case (d). The observed values are in good agreement for H-1/H-2 (2.64 Hz) and H-3/H-4 (3.44 Hz), although somewhat higher than predicted for H-2/H-3 (5.90 Hz) and H-4/H-5 (3.09 Hz).

The small deviations of the two latter J values might reflect a slight distortion of the $^1C_4$ (L) conformation, which is not entirely unexpected since some flattening of the ring could relieve interactions between the 1,3-syn-diaxial C-O bonds. Alternatively, since the above-mentioned generalizations (8) have been derived from neutral carbohydrates only, it is possible that $J_{2,3}$ and $J_{4,5}$ are influenced additionally by the charged groups at C-2 ($-OSO_3^-$) and C-5 ($-COO^-$). It is improbable, however, that the sulfate group at O-2 affects the above couplings. In fact, the interproton coupling constants recently observed for the (non-sulfated) α-L-idopyranuronic residues of dermatan sulfate are essentially the same as those observed for the sulfated residues of heparin (9).

According to the present data, therefore, most of the heparin molecule can be depicted by the following disaccharide repeating unit, in which the A

Fig. 1 - ^{1}H-NMR spectrum of heparin, at 270 MHz, 35°C. (c = 10% w/v in D_2O). A) Normal. B) After "convolution-difference". C) Computer simulated. D) and E) are the computer-simulated spectra for residues A and I.

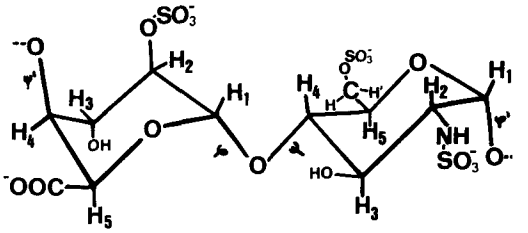
TABLE I

Spectral parameters (*) for heparin

	Iduronic acid unit			Glucosamine unit	
	35°C	90°C		35°C	90°C
δ_1	5.225	5.218	δ_1	5.401	5.369
δ_2	4.347	4.350	δ_2	3.284	3.298
δ_3	4.205	4.213	δ_3	3.673	3.691
δ_4	4.106	4.120	δ_4	3.771	3.750
δ_5	4.820	4.772	δ_5	4.035	4.049
J_{12}	2.64	3.29	δ_6	4.407	4.378
J_{23}	5.90	6.10	$\delta_{6'}$	4.278	4.284
J_{34}	3.44	3.60	J_{12}	3.66	3.57
J_{45}	3.09	3.14	J_{23}	9.98	9.88
			J_{34}	9.09	8.91
			J_{45}	9.23	9.23
			J_{56}	2.92	2.92
			$J_{56'}$	2.15	2.15
			$J_{66'}$	-11.23	-11.23

(*) Chemical shifts in ppm from internal TSP, coupling constants in Hz.

residue has the $^4C_1$ (D) conformation, and the I residue the $^1C_4$ (L) conformation.



These findings substantiate earlier proposals on structure advanced on the basis on a partial analysis of ^{1}H-NMR spectra (4), and are compatible as well with ^{13}C-NMR spectral (9,10) and X-ray fiber (11) data.

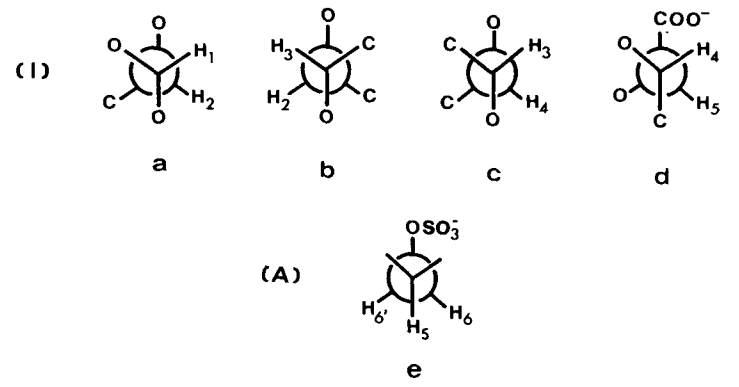


Fig. 2 - Newman projections for ring protons of iduronic acid (I:a,b,c,d) and protons at C_6 of aminosugar (A,e).

Other information obtained from the present data concerns the rotamer population of the $-CH_2-O-S$ fragment at C-6 of residue A. The equally small values for $J_{5,6}$ and $J_{5,6'}$ indicate that conformer (e) (Fig. 2) is preponderant. This differs notably from the data for 2-acetamido-2-deoxy-D-glucose (7) and D-glucose (13) where substantial proportions of the other two staggered conformers are implicated. It appears probable that (e) is favored in heparin because it minimizes polar, as well as steric, interactions of the $-OSO_3^-$ group.

If the energy difference between the $^1C_4$ and $^4C_1$ conformations of the I residue were small, as might have been anticipated from calculations on the idopyranose ring (3), changes in geometry may well occur as the environment is altered. However, neither the ^1H- nor ^{13}C-NMR spectrum of heparin exhibits a substantial change in (^1H-^1H or ^{13}C-^1H) coupling as a function of temperature (30-90°), concentration (0.1-20%), or pH (1.5-10) (11). This strongly suggests that the $^1C_4$ (L) conformation of the iduronic acid, as well as the $^4C_1$ (D) conformation of the aminosugar, residue is readily maintained and hence, is highly stable. Some displacements in chemical shift occur over the ranges cited above as well as in the presence of metal ion, but these may reasonably be attributed to changes in overall molecular conformation, arising from rotations about the inter-residue glycosidic bonds (11).

Although the data reported here deal with beef-lung-heparin, the conclusions reached can be extended to include heparins from hog mucosa. In fact, the most prominent ^1H-NMR signals of the mucosal heparins are superimposable on those of Fig. 1(a). Work is in progress to clarify the structural significance of the D-glucuronic acid and 2-acetamido-2-deoxy-D-glucose residues in both types of heparin.

ACKNOWLEDGMENTS

The authors gratefully acknowledge helpful discussions with Drs. G.K. Hamer and J.R. Vercellotti. Drs. A.J. Cifonelli and A.S. Mathews, and the Upjohn Co., very generously furnished samples of heparin.

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