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Note

Fractionation of heparin and heparan sulfate as barium salts: high-field, n.m.r.-spectral observations on heterogeneity

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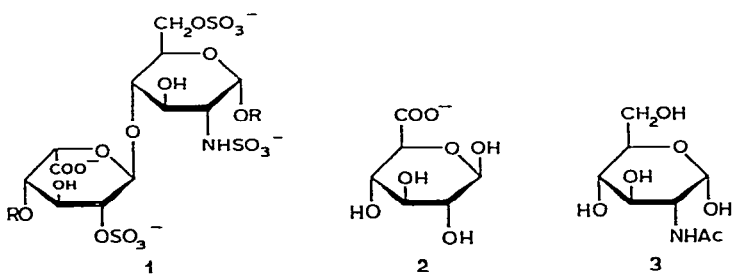
Although heparins are constituted mainly of alternating residues of α-L-idopyranuronic acid 2-sulfate and 2-deoxy-2-sulfamino-α-D-glucopyranose 6-sulfate linked through C-1 of the former and O-4 of the latter (as in **1**), there are notable differences in their contents of minor residues [*i.e.*, β-D-glucopyranuronic acid (**2**) and 2-acetamido-2-deoxy-α-D-glucopyranose (**3**)], as well as in degrees of *O*-sulfation[1]. Heparin from beef lung (B-type) is more fully represented[2] by sequence **1** than is that from hog mucosa (A-type). Some of the latter types, in fact, are relatively close in composition to heparan sulfate, in which residues of **2** and **3** are major constituents[1].

It has been shown[3,4] that heparan sulfate isolated from beef-lung tissue may be fractionated by gel electrophoresis into several components which, at one extreme, consist largely of **1**, and, at the other, primarily of **2** and **3**. With the object of acquiring similar fractions on a much larger scale than is practical by use of gel electrophoresis, we have effected a closely analogous separation of gram quantities of heparan sulfate, from the same source, by controlled sedimentation of its barium salt from water–ethanol mixtures in a preparative ultracentrifuge. Applied to hog-mucosal heparin (A-type), this procedure also afforded fractions that differed widely in their contents of **1**, **2**, **3**, and other residues.



1 2 3

R = glycosyl residue(s)

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RESULTS AND DISCUSSION

The fractional sedimentation of barium heparan sulfate, at an initial concentration of 5% in water, afforded 15 fractions over an ethanol concentration range of 7–35%. ^{1}H-N.m.r. spectra of the first 3 fractions (~25% of the total) were essentially superimposable on the spectrum (shown in ref. 4) of the material having the highest mobility in gel electrophoresis, a spectrum which, in turn, closely resembles the 220-MHz spectra of heparins[5]. Similarly, the spectra of the last 4 fractions were akin to that[4] of the material having the lowest mobility in electrophoresis, whereas intermediate fractions corresponded, as previously[4], to composites of the two extremes.

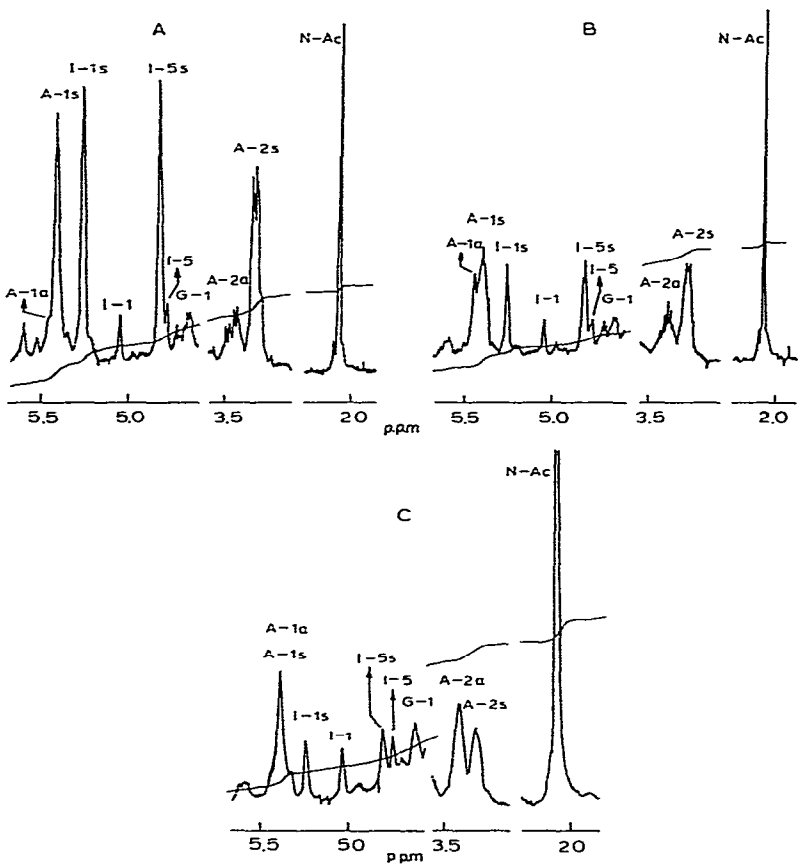
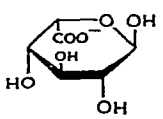


Fig. 1. Partial, 400-MHz, ^{1}H-n.m.r. spectra in D_2O at 70°. (A) and (B) are spectra of sodium heparin (hog mucosal) corresponding to barium salts sedimented by ultracentrifugation at 4% ethanol and 21% ethanol, respectively; (C) is the spectrum of sodium heparan sulfate corresponding to a barium salt sedimented at 12% ethanol. [For clarity, complex clusters of signals between δ 3.5 and 4.5 have been omitted; all signals between δ 2.0 and 3.5 are shown. A-1s and A-2s: H-1 and H-2 of 2-deoxy-2-sulfamino-D-glucose 6-sulfate (in **1**); I-1s and I-5s: H-1 and H-5 of L-iduronic acid 2-sulfate (in **1**); G-1: H-1 of D-glucuronic acid (**2**); A-1a, A-2a, and N-Ac: H-1, H-2, and CH_3 of 2-acetamido-2-deoxy-D-glucose (**3**); I-1 and I-5: H-1 and H-5 of L-iduronic acid (**4**).]

Hence, this progressive increase in the importance of **2** and **3** at the expense of **1**, representing corresponding decreases in the degree of sulfation and content of barium, is consistent with the variations in solubility observed.

In view of these findings, the barium salt of hog-mucosal heparin was subjected to fractional sedimentation in an analogous way, in order to examine the possibility that molecules richer in sequence **1** might thereby be separated from those containing higher proportions of **2** and **3**. Recrystallization of its (sparingly soluble) barium salt is a long-established[6] procedure for the purification of heparin, and the low solubility of the salt proved advantageous for our purposes, in that relatively low concentrations of precipitant were needed, as already mentioned. Consequently, the volumes of liquid handled in the ultracentrifuge were correspondingly small, and gram quantities of heparin were processed with great facility. As described in the Experimental section, nine fractions of the mucosal heparin were obtained.

^{1}H-N.m.r. spectroscopy clearly showed that the compositions of these fractions differed substantially, in a manner consistent with the observations already described for heparan sulfate. Figs. 1A and 1B, which are portions of ^{1}H-n.m.r. spectra of the sodium salts measured at 400 MHz, illustrate the differences between a fraction sedimented at 4% ethanol and one at 21% ethanol, respectively. Using the acetamido CH_3 signal (N-Ac) of each as a reference for comparing signal-intensities, it is apparent that there is a far higher content of sequence **1** (represented by signals I-1s, I-5s, A-1s, and A-2s) in the less-soluble fraction, whereas the more-soluble material contains much more of **2** and **3** (represented by signals G-1, A-1a, A-2a, and N-Ac). For example, a comparison of the integrals for I-1s and N-Ac indicated that the ratios of **1** to **3** are 6:1 (see Fig. 1A) and 2:1 (see Fig. 1B), respectively. Also observed in both spectra are signals (I-1 and I-5) attributable to an iduronic acid residue devoid of a sulfate group at C-2 (*i.e.*, **4**). There is obviously a far greater proportion of **4** in the more-soluble fraction; from relative intensities, the ratio of **4** to iduronic acid 2-sulfate (in **1**) is 1:3 (see Fig. 1B) as compared to a ratio of only 1:8 (see Fig. 1A) in the less-soluble fraction.



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Because of the superior resolution obtainable at 400 MHz, a direct estimate is afforded of the relative proportions of all five types of uronic acid and aminodeoxyhexose residues that, to date, are known to occur in heparin. We have also found that ^{13}C-n.m.r. spectra of the barium salts at 50 MHz give analogous information whereas, as shown earlier, a less-complete set of data was obtained from measurements of ^{1}H-n.m.r. spectra at 220 MHz (ref. 5) or of ^{13}C-n.m.r. spectra at 22–25 MHz (ref. 7). Among other differences is the appearance of the signal attributed to H-2 of **3**. Although, at 220 MHz, this signal exhibits[5] no fine structure, its multiplet appearance

in the 400-MHz spectrum suggests that residues of type 3 occur in more than one environment or, perhaps, that some are *O*-sulfated. Noteworthy also in Fig. 1 are a number of undesignated, weak signals whose origin has yet to be determined. Possible candidates in the region of 5.6 p.p.m. are anomeric-proton signals of pentose and hexose residues in the segment linking heparin chains to protein[1]. Fig. 1C shows corresponding regions of the 400-MHz, ^{1}H-n.m.r. spectrum of a fraction of heparan sulfate sedimented out in the intermediate range of ethanol concentration. It demonstrates, as expected, the greater prominence of residues of **2**, **3**, and **4** in heparan sulfate than in heparin. In addition, however, because virtually all of the signals in this spectrum are counterparts of signals in Figs. 1A and B (although less well resolved), it may be inferred that there are no substantial *qualitative* differences between heparin and heparan sulfate on the basis of their component sugars, modes of linkage, configurations, and conformations, and patterns of *N*-acetylation and *N*- and *O*-sulfation.

As is well known[8], heparins are polydisperse, consisting of species that vary in molecular weight from a few thousand to >20,000. Fractions obtained from a given, heparin preparation may also exhibit differences in composition*. Recently, solvent extraction in the presence of a phase-transfer catalyst was used[10] to separate hog-mucosal heparin on the basis of anionic density, which gave a series of fractions differing in the ratio of sulfate to uronic acid by a factor of 2. The present results reflect an even broader and systematic spread in the degree of sulfation of molecules within a heparin sample and, through the use of high-field, n.m.r. spectroscopy, the nature of this structural heterogeneity has been elucidated in terms of specific, constituent residues (*i.e.*, **1–4**). Moreover, the fractionation procedure described here facilitates access to relatively large quantities of differently constituted components of heparin.

EXPERIMENTAL

Materials and methods. — Barium heparan sulfate (beef lung) and sodium heparin (hog mucosal) were obtained from Upjohn International Inc., Kalamazoo, Mich. The heparin was converted into the barium salt by treatment in water with Amberlite IR-120 (H^+) ion-exchange resin, followed by neutralization with barium hydroxide; the solution of the barium salt was then freeze-dried. Spectra at 220 MHz were recorded by A. A. Gray, Canadian 220-MHz Centre, Sheridan Park, Ont., and those at 400 MHz by D. L. Rabenstein, University of Alberta, Edmonton, Alta.

Fractionation of heparin. — The following is representative of the fractionation procedures used. Barium heparin (2 g) was dispersed in water (40 mL), and material in suspension was removed by centrifugation at 10,000 r.p.m. in a preparative

*It is noteworthy that fractions obtained[9] by ethanol precipitation of the *sodium* salt of hog-mucosal heparin differed little in chemical composition, in contrast to our findings, although their molecular weights differed.

ultracentrifuge (Beckman, Model L). The supernatant solution was titrated with ethanol to incipient turbidity, and centrifugation then gave a second sediment; this process was repeated seven times, with the successive, supernatant solutions, to a final ethanol concentration of 26%. For examination by ^{1}H-n.m.r. spectroscopy, fractions were converted into the sodium salts by ion exchange.

ACKNOWLEDGMENTS

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