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## Note

# The heterogeneity of heparan sulfate from beef-lung tissue; p.m.r.-spectral evidence

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Different preparations of the glycosaminoglycan heparan sulfate (heparitin sulfate) differ substantially in composition, as shown by differences in their ratios of acetamido sulfoamino groups[1,2] and of D-glucuronic acid L-iduronic acid[2,3] Most probably, this is related to the fact that individual batches from commercial sources can be fractionated[1,4,5] into a number of components which, themselves, differ in composition as well as in physical properties The widest variations have been found[5] among fractions isolated by gel electrophoresis, and the results of the investigation suggest that heparan sulfate from beef-lung tissue contains at least four distinct polysaccharides

Examination of the electrophoretically distinct fractions by p m r spectroscopy graphically demonstrated some of the differences to be found between these materials The spectra of three fractions, selected to emphasize specific features, are reproduced in Figs 1a–c. These materials are of high (I), intermediate (II), or low (III) electrophoretic mobility, and are the same as (or similar to) fractions D, B, and A of ref 5 At one extreme (fraction I), the spectrum (Fig 1a) is virtually indistinguishable from that of a B-type heparin[6]; *i e*, the various signals that have been used[6,7] to characterize the 2-deoxy-2-sulfoamino-α-D-glucose 6-sulfate and α-L-iduronic acid 2-sulfate residues of heparin are clearly evident here also Weak signals at 2 1 and 3 5 p p m, attributable, respectively, to the $CH_3$ and H-2 of an acetamidodeoxyhexose residue, are the only evidence of a significant content (about 5%) of a third component, as has also been found for the spectra of some heparins[6,7]. When these spectral observations are assessed together with the earlier analytical, rotatory, and other data for the material[4,5], fraction I (D) may reasonably be designated a heparin The fact that its average molecular weight is only about one-third that of a "typical" heparin[5] renders its presence in the batches of heparan sulfate understandable, as the latter are isolated from the mother liquors of heparin extracts

The spectrum of fraction III (Fig 1c) illustrates the other extreme Features most typical of the heparin type of spectrum are now absent By contrast, there are

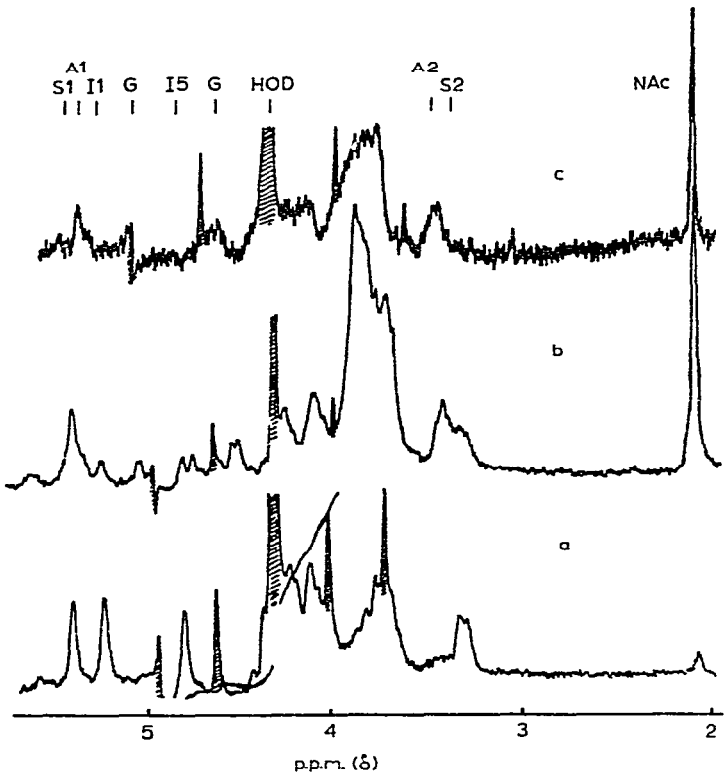


Fig 1 Proton magnetic resonance spectra at 220 MHz (solvent $D_2O$, temp 70°) of fractions prepared from heparan sulfate of beef-lung tissue [a, fraction I, b, fraction II, c, fraction III A1 (or A2), H-1 (or H-2) signal of acetamidodeoxyhexose residue, S1 (or S2), H-1 (or H-2) signal of deoxysulfoaminohexose residue, I1 (or I5), H-1 (or H-5) signal of iduronic acid residue, G, signals H-1 and H-5 of glycuronic acid residues (tentative), NAc, acetamido $CH_3$ signal Shaded areas represent the residual deuterium hydroxide (HOD) peak, or spinning side bands ]

prominent signals at 2 1, 3 5, and 5 3 p p m that may be acsribed to $CH_3$, H-2, and H-1, respectively, of residues of 2-acetamido-2-deoxy-α-D-glucopyranose Other signals are found at 5 1 and 4 7 p p m, undoubtedly related to the uronic acid component(s) known to be present[5] Another feature that characterizes spectrum 1c is the fact that the group of signals in the region 3 7–4 0 p p m is relatively much stronger (by 1 5–2) than the corresponding signals in spectrum 1a That is, there are fewer deshielded protons in III, a finding that accords well with the lower content of *O*-sulfate (deshielding) groups in this fraction as compared[5] with* I. As an overall comment, spectrum 1c is notably different in several respects from those of all of the other types of glycosaminoglycan that have been examined[7], and this suggests that fraction III represents a distinct, new, member of this class of polymers

*It is noteworthy that several disaccharides differing widely in *O*-sulfate content have been found as products of the enzymic breakdown of heparan sulfate[8]

A fraction of intermediate electrophoretic mobility, namely, fraction II, had the spectrum shown in Fig 1b This spectrum strongly resembles that of a preparation of heparan sulfate examined earlier[7], and exhibits several features that relate it to both Figs 1a and 1c For example, the acetamido $CH_3$ signal is of a relative intensity intermediate between those of spectra 1a and 1c Similarly, H-2 signals associated with both the acetamido (1c) and sulfoamino (1a) types of deoxyhexose residue are almost equally prominent Moderately strong signals ascribed to H-1 and H-5 of α-L-iduronic acid residues (1a) are present, as are two signals that, for Fig 1c, were assigned tentatively to residues of the uronic acid component of III In several ways, therefore, spectrum 1b appears to be a composite of spectra 1a and 1c, this was true of other intermediate fractions that were isolated, in close agreement with the comparative analysis of chemical composition reported previously[5]

Hence, it is possible that fraction II and similar fractions are unresolved mixtures of heparin of low molecular weight with the polysaccharide represented by III Alternatively, of course, they might consist of a range of copolymers made up of segments analogous in structure to heparin and to III, rather than being simple mixtures, but the latter possibility has been shown to be the more probable[9] Thus, when II (av mol wt 25,000) was incubated with a purified heparanase from *Flavobacterium heparinum*, it yielded disaccharides that accounted mainly for the acetamidodeoxyhexose residues of II, together with a polymer (av mol wt 6,000) enriched in deoxysulfoaminohexose residues This result suggests that the fraction II macromolecule contains regions akin to III, and also heparin-like regions

The suggestion has already been made[7] that the relatively strong acetamido $CH_3$ signals in the spectra of some heparins (A type) may be due to the presence of heparan sulfate In the light of the current findings and preceding discussion there is, indeed, a strong possibility that these heparins incorporate such polymers as III or II in proportion to their content of acetamidodeoxyhexose residues

*Some additional comments on the p m r spectra* — Other aspects of the p m r spectra examined in this study merit comment Because the quantity of individual fractions isolable by preparative electrophoresis was limited by practical considerations, relatively small samples (usually about 5 mg) were used for the spectroscopic study Part of the accompanying problem of high dilution was offset by multiple scanning (*e g*, as in Figs 1a and 1b), but most of the spectra suffered interference from strong, spinning side-bands associated with the HOD peak

In some instances, line broadening was a more serious problem For example, consider the spectrum (Fig 2a) of a rapidly migrating material (equivalent to I) isolated in one experiment By comparison with Fig 1a (and the spectra of ref 7) over the region 3–5 5 p p m, this spectrum is clearly recognizable as of the heparin type (the 1 5–2 5 p p m region is described in the succeeding paragraph) A remarkable feature of spectrum 2a, however, is the unusual broadness of the signals attributable to H-1 and H-5 of residues of iduronic acid, by comparison, the appearance of the H-1 and H-2 signals of the hexosamine residues is essentially normal Most probably, this line broadening was caused by paramagnetic ions in the sample, although, as

shown in Fig 1a, such contamination did not always occur Attempts to reproduce this striking effect by adding salts of various paramagnetic ions ($Fe^{3+}$, $Cr^{3+}$) to solutions of heparin in $D_2O$ gave rise only to a general broadening of all signals*. Nevertheless, because of the high selectivity observed for the uronic acid, as compared with that for the hexosamine residues, it may prove worth while to pursue further a search for paramagnetic species that can differentiate so clearly between such residues

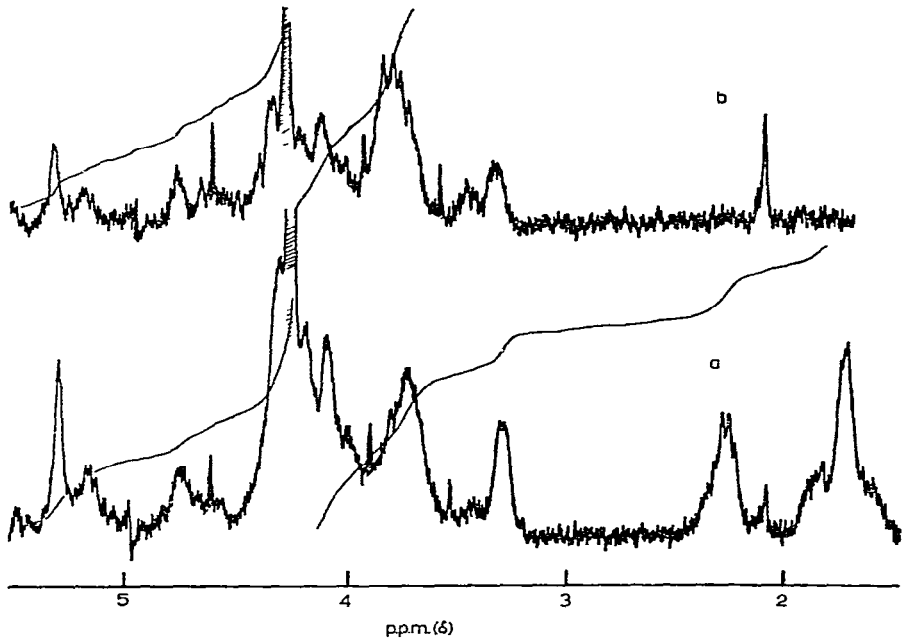


Fig 2 Proton magnetic resonance spectra at 220 MHz (solvent $D_2O$, temp 70°) of heparan sulfate fractions exhibiting extreme line-broadening in the region 4 8–5 2 p p m [a, Fraction corresponding to fraction I (represented by Fig 1a), b, fraction intermediate in composition between I and II (Figs 1a and 1b)]

Fig 2 also demonstrates another advantage of a p m r -spectroscopic examination in this area of study As may be seen from the two strong bands centered at 1 7 and 2 3 p p m , this fraction contained a substantial quantity of impurity that undoubtedly was derived from the poly(acrylamide) support used in the gel electrophoresis The presence of such a contaminant was not indicated by the other analyses performed[5], analyses that are almost universally used in studies on glycosaminoglycans It is important to note also that this impurity was *not* the cause of line broadening, because yet another fraction from the same experiment gave a spectrum (Fig 2b) closely similar to spectrum 2a but contained no poly(acrylamide) In agreement with the fact that this fraction had a slightly lower rate of migration, the material

*Dr M Vincendon very kindly conducted this study

is notably richer in its content of acetamidodeoxyhexose, as shown by the relatively strong signals at 2 1 and 3 5 p p m (attributable to $CH_3$ and H-2) in Fig. 2b as compared with 2a, placing it intermediate in composition between fractions I and II.

*Conclusions* — The p m r spectra of fractions obtained from heparan sulfate of beef-lung tissue support earlier chemical analyses showing that this heparan sulfate is highly heterogeneous One extreme fraction is indistinguishable from heparin of low molecular weight, whereas another, yet to be fully characterized, is sufficiently different from other glycosaminoglycans to be regarded as a new type Intermediate fractions correspond to copolymers and/or mixtures of these other two polysaccharides

## EXPERIMENTAL

The barium salt of heparan sulfate from beef-lung tissue was a gift from the Upjohn Company, Kalamazoo, Michigan This material was converted into its ammonium salt, and fractionated essentially as described previously[5]. the polysaccharide was subjected to electrophoresis (2V/cm, 24 h) in barbital buffer (pH 8 6) containing 1% of agarose on a column of poly(acrylamide) gel The gel was then sectioned, and the material contained in individual sections was extracted into water and recovered by precipitation with ethanol Further purification of the fractions isolated was effected by electrophoresis on an agarose-gel block, either at[4] pH 8 6 (fraction I) or at[5] pH 2 0 (fractions II and III)

For examination by p m r spectroscopy, the individual factions were repeatedly subjected to deuterium exchange with deuterium oxide, and then dissolved in 99 95% deuterium oxide The spectra were recorded at the Canadian 220-MHz Centre, Sheridan Park, Ontario

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