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220 MHz spectra of heparin, chondroitins, and other mucopolysaccharides[1]

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Characteristics of proton magnetic resonance spectra of heparins at 220 MHz, coupled with spectral data for model compounds and with information obtained chemically, indicate that heparins are composed principally of (1 → 4)-linked α-L-idopyranosyluronic acid residues (**3**) and 2-amino-2-deoxy-α-D-glucopyranosyl residues (**1**); also, that sulfate groups are located at position -2 and -6 of **1**, and -2 of **3**. D-Glucopyranosyluronic acid residues (**2**) appear to be minor constituents, which is at variance with all structures that have been proposed previously for heparin.

Spectra of chondroitins A, B, and C, which are readily distinguishable, are in close accord with formulae that have been proposed for these mucopolysaccharides; on the same basis, the spectrum of keratan sulfate is less consistent. Hyaluronic acid affords a poorly resolved though, nonetheless, distinctive spectrum. The general view that mucopolysaccharides are constituted basically of residues of hexosamine and uronic acid in equimolar proportion receives support from the current findings. However, heparitin appears to possess a more heterogeneous type of structure than do these other polymers.

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Major structural features of many mucopolysaccharides, heparin, the chondroitins, hyaluronic acid, etc., are well recognized (e.g. see ref. 1). It appears that common to each is a linear polymeric chain in which hexosaminyl and hexosyluronic acid residues alternate, although other types of sequences may occur. Differences among the mucopolysaccharides arise from variations in chain length, in the configuration of residues, their mode of glycosidic bonding, the number and location of *O*-sulfate groups, contents of other kinds of sugar residues and of acetamido and sulfamino groups, and in their association with amino acids and proteins. Structural studies on the mucopolysaccharides frequently are complicated by insufficiency of material, difficulty in assessing purity, and a general dearth of procedures that permit selective, high-yield, degradation of the polymers. Hence it is probably true at present to say that most mucopolysaccharides have only been partially characterized and that details of structure, perhaps even major features in some instances, remain to be determined.

In principle, proton magnetic resonance (p.m.r.) spectroscopy should be a highly useful physical method for examining such polymers, particularly because it can furnish excellent quantitative data. Hence, with suitable resolution, it should be feasible to estimate the relative proportions of the components of a mixed polymer from the relative intensities of resonance signals attributable to the different residues present. An attempt to estimate the compositions of various heparins by p.m.r. spectroscopy at 100 MHz has been described earlier (2). In the current study, possible advantages of measuring the spectra of heparins at a higher frequency, i.e., at 220 MHz are assessed, and the spectra of several other mucopolysaccharides are described as well.

Heparin

The 100 MHz spectra of heparins show notable variations among samples from different sources (2, 3), but all are characterized by three signals (*a*, *b*, and *c*) at 4.7–5.4 p.p.m., chemical shifts commonly associated with anomeric protons (Fig. 1A). Accordingly, each of these signals has been attributed to H-1 of the three main kinds of residues found in heparin, i.e., 2-deoxy-2-(sulfamino)-α-D-glucopyranosyl (**1**),α-D-glucopyranosyluronic acid (**2**), and L-idosyluronic acid (**3**), respectively and, based on the integrals, it has been suggested that heparins are comprised of approximately equimolar proportions of these residues (2). In addition, the total intensity of *a*, *b*,

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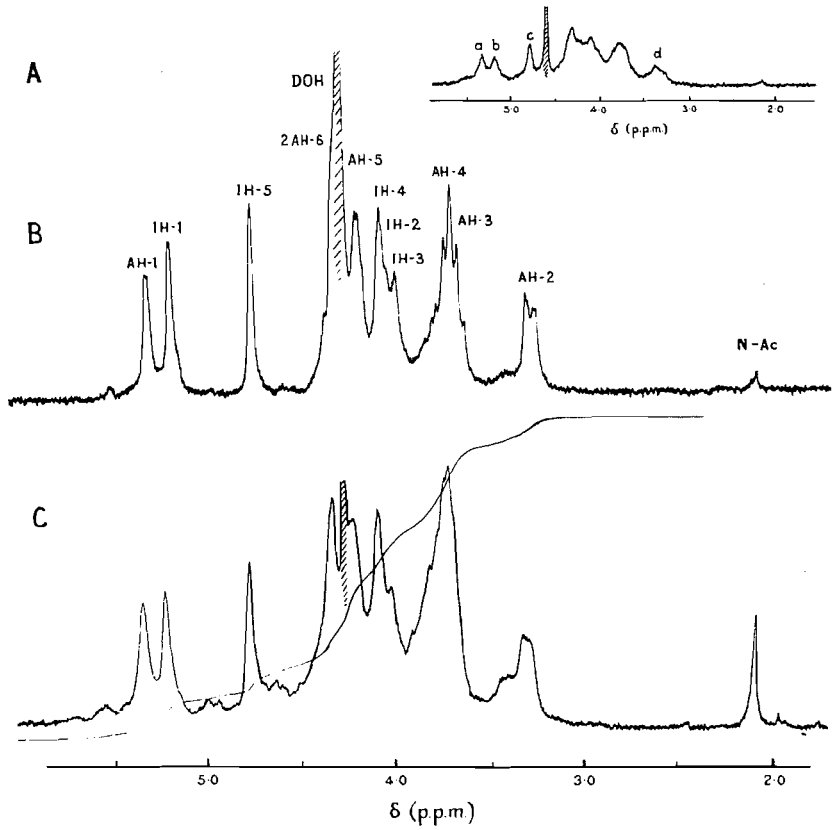
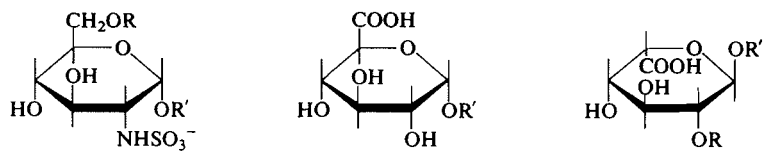


FIG. 1. Proton magnetic resonance spectrum of heparin in deuterium oxide of (A) type B heparin at 35 °C, 100 MHz; (B) type B heparin at 70 °C, 220 MHz; (C) type A heparin at 70 °C, 220 MHz. Peak designations are explained in the text.



1 R = H
7 R = SO_3^-

2

3 R = H
15 R = SO_3^-

and *c* relative to that of the higher field signals (*d–h*) appeared to agree with the ratio expected. Thus, for a total of 17 protons in a trisaccharide basic unit, seven for each hexosaminyl residue, *plus* five for each of the two uronosyl residues, the expected ratio is 3:14, and the value found was about 3:15 (2).

In arriving at this latter value, an attempt was made to correct for overlap by the rather broad signals of the spectra (2). However, measurements at 220 MHz (Fig. 1B) substantially minimize the problem of overlap because they afford a better separation of signals. According to the integrals obtained under these more satisfactory conditions, the ratio of *a–c*:*d–h* is only about 3:10 (3:9.6–9.9 for four heparin samples). Thus, signals *a*, *b*, and *c* have a relatively higher intensity than had been deduced from the 100 MHz spectra, and therefore each can no longer be ascribed to the anomeric proton of a different residue. This finding contradicts our previous suggestion (2) that heparins contain approxi-

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mately equimolar proportions of residues **1**, **2**, and **3**. Hence it is pertinent that a concurrent chemical examination of heparin (4) also does not support a 1:1:1 type of structure.[4] The chemical data in fact suggest a ratio of about 1:0.2:1, i.e., a relatively much lower proportion of residues of D-glucopyranosyluronic acid than would have been expected. In view of these results, it becomes necessary to reassess the p.m.r. spectra of heparins, and the following discussion deals with this question.

Signals *a* and *h* have previously been attributed to H-1 and H-2, respectively, of hexosaminyl residues by reference to chemical shifts and spacings in the spectra of model compounds, and particularly to the upfield displacement that these signals undergo upon hydrolysis of the sulfamino group (2). Since these similarities are even more clearly evident at 220 MHz, the earlier assignments are retained (AH-1 and AH-2, Fig. 1B).

As noted above, it appears no longer valid to assume that both *b* and *c* are produced by anomeric protons. However, an attractive alternative is to attribute signal *c* to H-5 of the L-idopyranosyluronic acid residues (**3**). This follows from an examination of the spectra of some methyl glycosiduronic acids both as salts and as free acids. Thus, H-5 of methyl α-D-idopyranosiduronic acid (**4**) or of its β-anomer (**5**) resonates in the region of signal *c* (see Fig. 1A) and, like the latter, these signals are characterized by a narrow spacing (Fig. 2).[5] Furthermore, all of these signals are shifted downfield by about 0.3 p.p.m. when the solution is acidified (Fig. 2). Although the H-5 signal for methyl α-D-glucopyranosiduronic acid (**6**) is displaced downward in the same way, its relatively low frequency and particularly its broad spacing (ca. 10 Hz) make it highly unlikely that *c* is produced by H-5 of the D-glucopyranosyluronic acid residues (**2**) in heparin. Therefore since H-5 of **3** most probably produces signal *c*, the anomeric proton (IH-1) of the same residue may now be regarded as the source of signal *b* rather than, as before (2), H-1 of residue **2**.

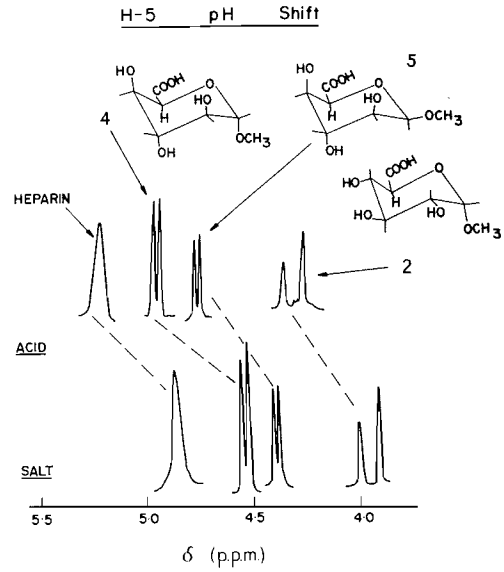
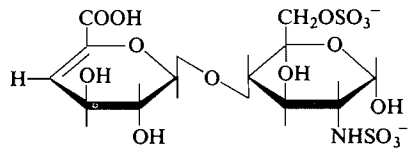


FIG. 2. The effect of p*H* (salt vs. free acid) on the chemical shift for H-5 of some uronosides in deuterium oxide (the acids were prepared by introducing excess of deuterium chloride); data obtained at 30 °C and at 100 MHz.

These revised assignments require that L-idosyluronic acid be a major constituent of heparin, in contrast to earlier views (1, 2, 6–8), and that it be present to about the same extent as the hexosamine constituent, which is consistent with our most recent chemical evidence (4). In addition, the total area of *a* and *b* relative to that of signals *c* to *h*, inclusive, agrees moderately well with expectation. The expected ratio is 1:5 (i.e., two anomeric protons as compared with six other protons of (**1**) *plus* four other protons of **3**, and the found value is 1:5.1–5.5[6] (for four samples of type B (3) represented in Fig. 1B).



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[4]Recent detailed analysis of various heparins also obviates this possibility and supports earlier indications that hexosamine accounts for about half of the carbohydrate portion of the heparin molecule (5).

[5]Representation of **4** and **5** in the conformations shown is based on unpublished p.m.r. data, and the preparation of these compounds (by catalytic oxidation of the corresponding D-idopyranosides) also has yet to be published (14).

[6]The limits of error of such measurements with solutions of polysaccharides in deuterium oxide are uncertain. That is, there are few fully characterized polysaccharides that give well resolved p.m.r. spectra to serve as standards.

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Other signal assignments in Fig. 1B are suggested by reference to the spectra of various model compounds. The tall, narrow, signal at 4.4 p.p.m. (2AH-6) has about the same chemical shift (4.3 p.p.m.) as the two H-6 protons of 2-deoxy-2-(sulfamino)-α-D-glucopyranose-6-sulfate (**7**). Similarly, signals for the other protons of the hexosamine moiety (i.e. AH-3, -4, and -5) are designated on the basis of the spectra of **7** and of disaccharide **8** (Fig. 3A), both of which have been obtained by enzymolysis of heparin (9–11). That is, Fig. 3 illustrates the correspondence in chemical shift between signals (AH) for the hexosamine moiety of **8** and various signals of the heparin spectrum (Fig. 3C) (at 100 MHz). Shown also is the spectrum (Fig. 3B) of a higher oligosaccharide formed in the enzymolysis (9, 10) which, by clearly exhibiting characteristics of these two other spectra, emphasizes the similarities between them. Signals produced by protons -2, -3, and -4 of the L-idosyluronic acid residues are less easily designated because of the lack of suitable reference compounds. There is good evidence from periodate oxidation data (7, 12, 13) that an *O*-sulfate group generally is located at position-2 of the hexosyluronic acid residues in heparin, and this assignment is confirmed by structure **8**. Position-4 of the L-idopyranosyluronic acid residues is involved in glycosidic bonding (4, 11). Hence both H-2 and H-4 of **3** would be expected to resonate at perhaps 0.3–0.4 p.p.m. to low field of the corresponding signals for the related idopyranosyluronic acids (**4** and **5**) (14), i.e. at about 4.0 and 4.3 p.p.m., respectively, which is close to the assignments proposed for IH-2 and IH-4. The assignment for IH-3 is more tentative.

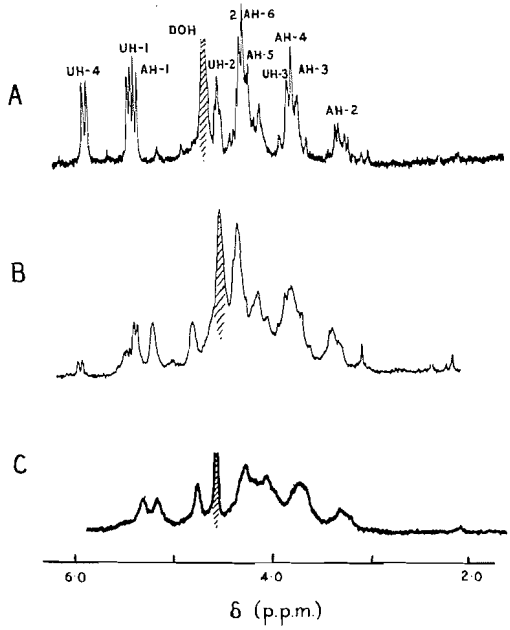

FIG. 3. Proton magnetic resonance spectrum at 100 MHz of (A) disaccharide **8** at 35 °C; (B) an oligosaccharide produced by enzymolysis of heparin (9), at 55 °C; (C) heparin, type B, at 35 °C. Solvent, deuterium oxide.

Missing from Fig. 1B are possible assignments for protons of the D-glucopyranosyluronic acid residues of heparin. Although such residues were formerly thought to account for a major proportion of the polymer, according to our current interpretation of the spectra they produce no easily recognizable signals. Nevertheless, there are some weak signals in the region of *a* and *b* and, probably, others underlying the latter signals. Surely some of these must be associated with type **2** residues, but in any event the proportion of such residues in heparin would have to be relatively low, in conformity with the recent chemical evidence (4), if the spectral assignments are to have general validity.

Relationships between the p.m.r. spectra of heparin and the glycosidic configuration of its component residues merits comment. It has been shown that the hexosaminyl residues **1** possess the α-D-linkage (1, 15, 16), and this designation receives support from the fact that AH-1 gives rise to a narrow signal and resonates at relatively low field (see ref. 17). Based on the structure of disaccharide **8**, the α-L-configuration is assigned for residues of L-idopyranosyluronic acid. Hence the narrow spacing of signal IH-1 and its relatively large chemical shift similarly indicate that IH-1 is equatorially oriented, which suggests further that these uronic acid residues of heparin possess the 1C conformation,[7] the mirror image of **4**.

Fig. 1C illustrates the spectrum of a representative of group A heparins (3). Several of the signals observed are not present, or are very weak, in Fig. 1B: in addition to the signal close to 2.0 p.p.m., attributed to protons of *N*-acetyl groups, there are others at about 3.4, 3.7, 4.6, and 5.0 p.p.m. This latter group of signals may represent residues that are not present in the group B

[7]In the alternate, C1 conformation, the diaxial arrangement of H-1 and H-2 should give rise to a much broader spacing (see ref. 17).

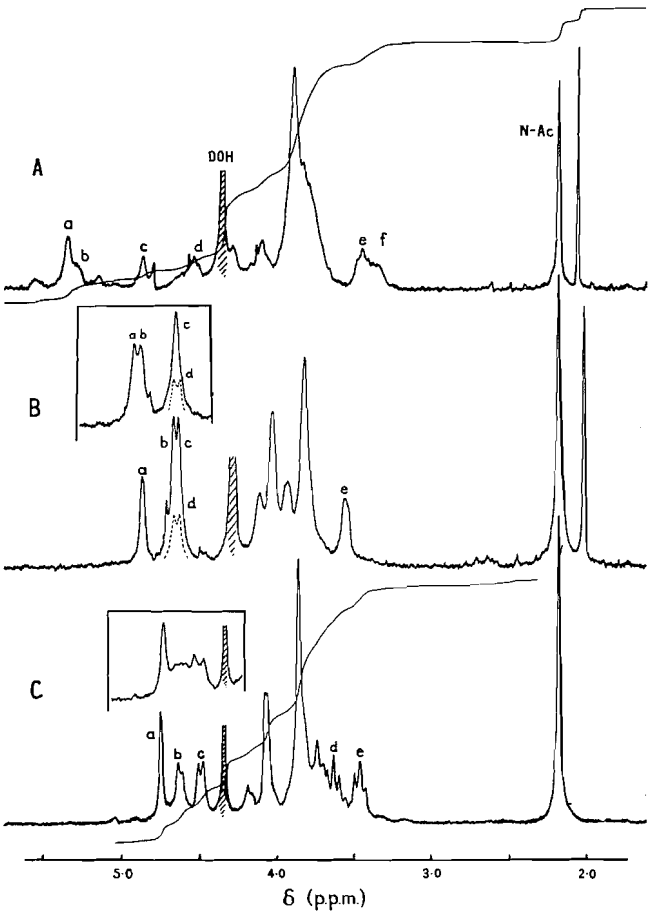

FIG. 4. Proton magnetic resonance spectrum at 220 MHz of (A) heparitin; (B) chondroitin B (inset: low field signals after acidification); (C) chondroitin A (inset: low field signals at 100 MHz). Solvent, deuterium oxide; 70 °C. The sharp singlet at about 2.0 p.p.m. in 4A or 4B is attributed to acetate anion.

heparins, but it is possible also that a small proportion of another polysaccharide is present (about 20% in this sample, based on the relative intensity of the *N*-acetyl signal, although the percentage is variable (Table 1, ref. 2)). Whatever the source of these "extra" signals in spectrum 1C, it is not eliminated by the currently best purification procedures, which favors the possibility that it is an integral part of the molecule, although another possibility is considered below.

Other Mucopolysaccharides

Other mucopolysaccharides afford p.m.r. spectra at 220 MHz that differ notably from those of heparins, and are themselves sufficiently distinctive to possibly serve for characterization purposes. Even at 60 MHz (18) substantial differences are apparent. However, since only one or two preparations of each polymer has as yet been examined, it is not known if variations occur from one source to another. Figs. 4 and 5 show the spectra of specimens that have been furnished by Dr. J. Cifonelli from the Reference Collection at the University of Chicago. Most of the better known mucopolysaccharides are represented, each having been the subject of substantial chemical and enzymic study (1), and the accompanying formulae (**9** to **14**) illustrate the basic structural sequences that are ascribed to them.

A general comment may be made concerning the region of lowest field signals in these spectra, which is associated most readily with anomeric protons. Aside from heparitin (Fig. 4A), these

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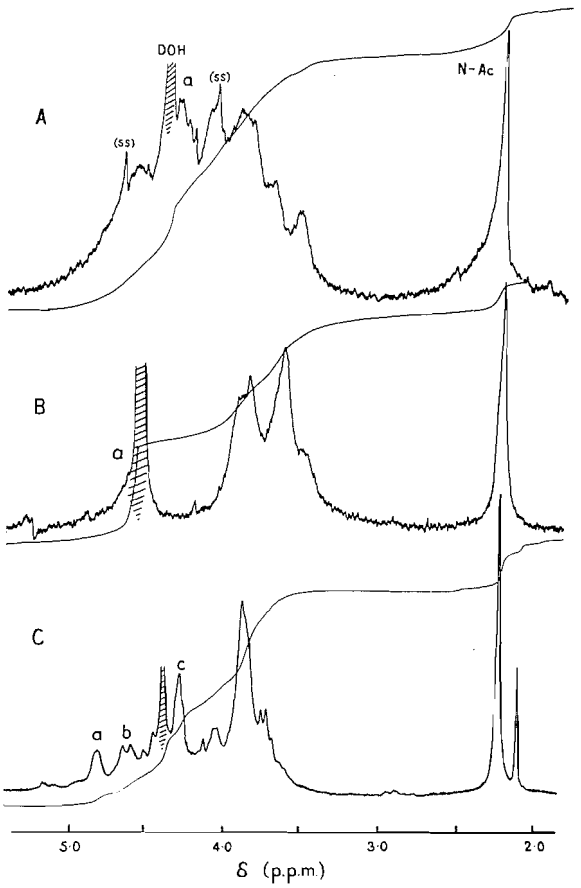
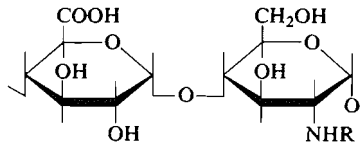


FIG. 5. Proton magnetic resonance spectrum at 220 MHz of (A) chondroitin C; (B) hyaluronic acid; (C) keratan sulfate. Solvent, deuterium oxide; 70 °C, (B) at 50 °C.

polymers do not produce signals comparable in chemical shift to AH-1 and IH-1 of Fig. 1 but, rather, at relatively higher field. In general this accords with the formulated structures in which the anomeric protons usually are axially oriented, and with the evidence that strongly-deshielding *N*- and *O*-sulfate groups usually are not found as 2-substituents of the glycosyl residues.

Heparitin

Heparitin shows several characteristics similar to heparin. Its lowest field signal (*a*, Fig. 4A) has the same chemical shift and shape as signal AH-1 of heparin which suggests that the 2-deoxy-2-sulfamino-D-glucosyl residues known to be present possess the α-D-configuration (as in **9**). Similarly, signal *b* (and an α-D-configuration) may be ascribed to the anomeric center of the



9 R = CH_3CO or SO_3^-
Heparitin

2 - acetamido - 2 - deoxy - D - glucosyl residues of heparitin. The combined intensity of *a* and *b* relative to the intensity of the upfield signal ascribed to *N*-acetyl protons, corresponds to an approximately equimolar ratio for the two types of hexosaminyl residues, in agreement with chemical analyses (1, 19, 20). There appear to be at least two signals at about 3.4 p.p.m.; signal *f* has the same chemical shift as AH-2 of the heparin spectrum and hence probably arises from the proton α to the sulfamino group, whereas *e* could account for H-2 of the 2-acetamido derivative. Signals *c* and *d* might well be associated with the D-glucosyluronic acid residues although, since the specific rotation of heparitin is very close to that of heparin, further examination of the uronoside composition should be pertinent.[8] In contrast to the situation with heparin, relatively few of the heparitin protons resonate in the region of the HOD signal, which is in accord with evidence that heparitin contains few, or no *O*-sulfate groups to cause deshielding of primary and secondary protons. Also, the fact that its spectrum contains a large number of signals of differing relative intensities, indicates that heparitin has a more heterogeneous type of structure than heparins appear to possess.

A comparison of the spectrum of heparitin with that of type A and B heparins (Fig. 1C and B) suggests the possibility that A heparins could contain some of heparitin. There is especially strong correspondence with respect to signal *e*, the *N*-acetyl signal, and the intense grouping of signals at about 3.7 p.p.m. (compare Figs. 1C and 4A), i.e., the signals that mainly differentiate between A and B heparins. Clearly this question merits further examination.

Chondroitin Sulfate B

The spectrum of dermatan (chondroitin sulfate B) (Fig. 4B) is better resolved, and suffers less

[8]Thus, for example, the chemical shift and shape of signal *c* suggest that it could be produced by H-5 of an iduronosyl residue, as for heparin.

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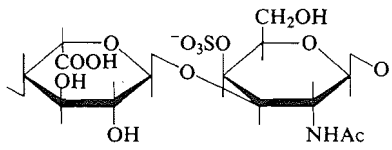
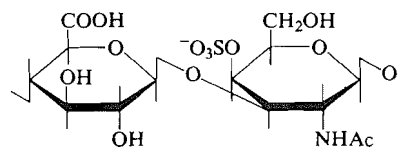


10
Chondroitin B

interference from the HOD signal, at 70 °C than at ambient temperature. Signal *a* and the group of signals *b*, *c*, and *d*, account for a total of about four protons. These protons likely are the anomeric ones of the two different residues (**10**), H-4 of the D-galactosaminyl residue, and H-5 of the L-idosyluronic acid residue. One of the *b*, *c*, *d* group of signals is shifted downfield on acidification by 0.3 p.p.m. and hence is most readily attributed to this H-5 proton (*b* in inset of Fig. 4B). Also, its chemical shift in both media is close to that of IH-5 of the heparin spectra and, like the latter signal, it is narrow. There is a relatively broad signal in this group (*d*). Because of its spacing and upfield position this signal is ascribed to H-1 of the hexosaminyl residue, which agrees with the assigned β-D-configuration for the latter (1, 21, 22). Proton H-1 of the L-idosyluronic acid residue probably produces signal *a*; as discussed for heparin, its narrow spacing is indicative of an α-L-configuration when the residue possesses the 1C chair conformation. The fact that its chemical shift is upfield relative to IH-1 of Fig. 1 is to be expected, in view of the absence of a 2-sulfate group in dermatan. By contrast, the presence of the 4-*O*-sulfate group, as well as its equatorial orientation, undoubtedly contributes to the downfield position of H-4 of the hexosaminyl residue (*c*), and the narrow spacing of this signal concurs with its gauche relationship to both H-3 and H-5.

From the relative intensities of these low field signals and the *N*-acetyl signal (Fig. 4B) (i.e. 4:27), the content of 2-acetamido-2-deoxy-hexosamine in the polymer is estimated to be about 45%, i.e., close to the expected value. However, the overall integral for this spectrum suggests that the largest group of signals (between 3.5 and 4.4 p.p.m.) account for a total of closer to nine protons rather than the value of eight required by formula **10**. Hence, as found with heparins, the relative intensities of the various signals do not furnish a simple numerical basis suitable for critically evaluating a given structural possibility. Whether this represents a problem of measurement[9] or one of structure has yet to be decided.

One further point merits comments. It is highly probable that a small proportion of the uronosyl residues in chondroitin B possess the D-*gluco* configuration (21–23). As found with heparin, the current spectral data are difficult to reconcile with a substantial content of a third type of residue. However, there is a relatively weak, broad, signal at about 4.5 p.p.m., which has not been commented on above, the chemical shift and spacing of which are close to those of H-1 signals in Fig. 4C (especially signal *c*) associated with the β-D-gluco configuration. Tentatively, then, this minor signal is attributed to H-1 of β-D-glucosyluronic acid residues in dermatan, its relative intensity showing that such residues could comprise perhaps 10% of the polymer.



11
Chondroitin A

Chondroitin Sulfate A

Chondroitin sulfate A (**11**) differs from dermatan in possessing a β-D-glucuronosyl rather than α-L-iduronosyl residue (24, 25). Comparatively then, the presence of only three low field signals rather than four (Fig. 4C) is not unexpected in view of the relatively high field resonance for H-5 of D-glucosiduronic acids (Fig. 2). The chemical shifts and broad spacings of signals *b* and *c* appear to be consistent with the assigned β-D-anomeric configurations of the two different residues in **11**, and the much narrower signal (*a*) may reasonably be ascribed to H-4 of the sulfated hexosaminyl residue. As with dermatan, the total intensities of these low field signals relative to the intensity of the *N*-acetyl proton signal (3:2.7) corresponds to just under a 50% content of 2-acetamido-2-deoxy-hexose. Again also, the overall relative intensity of the other signals is slightly high, amounting to about ten, rather than nine, protons. Although the upfield

[9]Signals produced by *N*-acetyl groups have been observed, particularly in experiments at 100 MHz, to be relatively highly sensitive to saturation effects.

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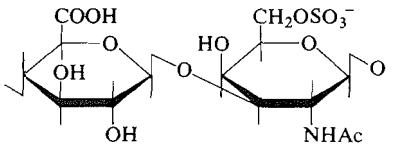
signals, *d* and *e*, have chemical shifts close to that of AH-2 of Fig. 1, H-2 of the 2-acetamido-2-deoxy-D-galactosaminyl residues would be expected to resonate further downfield because of deshielding by the axial C_4—O bond. In any event, attempts to relate these signals to the *b* and *c* doublets by spin-decoupling were unsuccessful. More probably, *d* and *e* are due to the 6-protons of the hexosaminyl residue; the tilt of signal *e* suggests that the shift of the adjacent H-5 is slightly downfield. Analogously, the upfield signal *e* of Fig. 4B also is probably produced by an H-6 of the hexosaminyl residue, but in this instance shifts for the second H-6 and for H-5 are likely to be almost coincident. Hence, the perturbed pattern observed for *e* could result from averaging of the negative geminal coupling and positive vicinal coupling. Although this type of hexosaminyl residue is common to **10** and **11** its environment is different in the two polymers, and therefore some spectral differences are to be expected.



12
Chondroitin C

Chondroitin Sulfate C

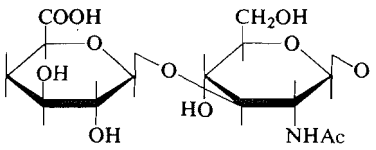
Because the spectrum of chondroitin sulfate C (Fig. 5A) is not as well resolved as that of chondroitin A, a spectral comparison of these closely related polymers (compare **12** and **11**) is difficult. However, the fact that an *O*-sulfate group is located at C-6 of the hexosaminyl residue of the former mucopolysaccharide (26, 27) rather than at C-4, is reflected in the absence of a signal corresponding to *a* in Fig. 4C. Similarly, there are proportionately weaker signals at about 3.8 p.p.m. and stronger ones at 4.2–4.3 p.p.m. (signal *a*) in contrast with the situation in Fig. 3C, which corresponds to the expected downfield displacement of H-6 signals for protons α- to the 6-*O*-sulfate group (compare also with signal AH-6 of Fig. 1B).

Hyaluronic Acid and Keratan Sulfate

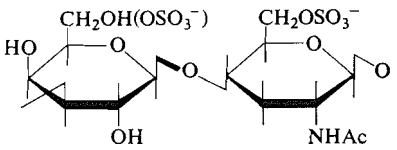
Hyaluronic acid, which forms highly viscous solutions, furnishes a poorly resolved spectrum (Fig. 5B). The anomeric proton signals (*a*) are almost obscured by their broadness and by the excessively broad HOD signals. However, in accord with the fact that this polymer contains no *O*-sulfate or other such strongly deshielding groups (**13**) (28, 29) the overall spectrum is produced at relatively high field. On the same basis, the spectrum of keratan sulfate (Fig. 5C) appears to detect the presence of several strongly deshielded protons (*a* and *b*) in addition to those (most probably signal *c*) that are located α- to the 6-*O*-sulfate group, as in **14** (30, 31).

In summary, the p.m.r. spectra of mucopolysaccharides at 220 MHz are sufficiently well resolved to permit more satisfactory assignments for many individual resonance signals than was possible with spectra obtained at lower frequency. The improved separation of signals also enhances quantitative evaluation of the spectra. This is of particular advantage because of difficulties inherent in the analysis of mucopolysaccharide composition. For heparin the current p.m.r. data, together with chemical and enzymic evidence, lead to the proposition that two types of residues (**7** and **15**), approximately equimolar, constitute most of the molecule. Accordingly also, they do not support the widely-accepted structure for heparin in which D-glucosyluronic acid (**2**) is depicted as the major uronosyl component. Differences between the two types of heparins are more clearly evident at 220 MHz, and the possibility is raised that these types may differ in having, or not having, heparitin as a contaminant.

Spectra of chondroitins A, B, and C appear to



13
Hyaluronic acid



14
Keratan sulfate

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be closely in accord with formulae (**11**, **10**, and **12**, respectively) that have been proposed for these mucopolysaccharides, and the samples examined give no indication that contaminating polymers are present in significant proportion. In terms of the ratio of constituent residues, heparitin probably has a more heterogeneous type of structure than do these other mucopolysaccharides, and it may have a substantial content of L-idosyluronic acid. The spectrum of keratan sulfate is not easily reconciled with formula **14**; the fact that this material has as yet received only relatively limited attention chemically allows for the possibility that there may yet be some revision of the formula.

In general, chemical and enzymic studies of the mucopolysaccharides have led to the view that these polymers are constituted basically of alternating residues of hexosamine and uronic acid. The current p.m.r. spectra serve to reinforce this view.

Experimental

The p.m.r. spectra were obtained with a Varian HR-220 MHz or HA-100 MHz spectrometer. Samples were given a preliminary deuterium-exchange by repeated treatments with deuterium oxide. Most spectra were recorded at about 70 °C because the higher temperature favored improved resolution, although lower temperatures were used also in minimizing the obscuring-effect of the DOH signal. Hyaluronic acid was recorded at a maximum of 50 °C, because it was precipitated from solution above this temperature. The 100 MHz spectra were obtained with the aid of an internal capillary containing tetramethylsilane which, however, caused a downward displacement of the signals by about 0.5 p.p.m.; accordingly, the actual spectra are corrected by −0.5 p.p.m. The 220 MHz spectra are not shift calibrated, although of precise width (i.e., 1000 Hz), and hence the chemical shifts in p.p.m. are given relative to the 100 MHz spectra.

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