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NUCLEAR MAGNETIC RESONANCE SPECTRA OF HEPARIN IN ADMIXTURE WITH DERMATAN SULFATE AND OTHER GLYCOSAMINOGLYCANS. 2-D SPECTRA OF THE CHONDROITIN SULFATES

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ABSTRACT

Characteristics of the ^{1}H-n.m.r. spectra of heparin admixed with other glycosaminoglycans are described with respect to the identification of the latter as possible contaminants of pharmaceutical heparins. Chemical shift differences are sufficiently large, particularly with the aid of resolution enhancement, to allow for the detection of dermatan sulfate, chondroitin 4- or 6-sulfate, hyaluronic acid, or heparan sulfate as a minor constituent in the presence of heparin. The acetamidomethyl resonance region (δ 1.95–2.15) is especially useful in this context, both for identification and quantitative estimation. Whereas dermatan sulfate is a common contaminant of pharmaceutical heparin preparations, in some instances comprising 10–15 percent of the polymer mixture, the other glycosaminoglycans, by contrast, were not detected in such preparations. Two-dimensional heterocorrelation and homocorrelation n.m.r. experiments have provided ^{1}H- and ^{13}C-chemical shift data that complete or verify (or both) previous information available for heparin, dermatan sulfate, and chondroitin 4- and 6-sulfates (chondroitins A and C).

INTRODUCTION

In the isolation of heparin, various procedures have been developed[1,2] to remove other glycosaminoglycans, protein, etc. that are co-extracted from the bovine or porcine tissue. As is well known[2,3], different preparations of heparin may vary appreciably in chemical composition and physical properties, as well as biological activity, although the origins of such differences often remain elusive. Nuclear magnetic resonance spectroscopy is effective for detecting[4] variations in chemical composition among heparins, as well as for differentiating[5] between a heparin and other glycosaminoglycans. This latter type of application is examined here in relation to our recent finding[6] that some pharmaceutical heparins contain unexpectedly high levels of dermatan sulfate, and the possibility that other glycosaminoglycans or additional kinds of contaminants might be present as well. A number of features of the polysaccharide spectra are also described, as a supple-

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ment to earlier n.m.r. data on glycosaminoglycans, in taking advantage of the current availability of 2-dimensional n.m.r. techniques.

RESULTS AND DISCUSSION

The ^{1}H-n.m.r. spectra in Figs. 1A and 1B are representative, respectively, of those of beef lung and hog mucosal heparins described[4,5] in earlier publications. In both spectra, the same group of major signals are produced by the main constituent sugars, *i.e.*, 2-deoxy-2-sulfamino-α-D-glucopyranose 6-sulfate (**1**) and α-L-ido-pyranosuronic acid 2-sulfate (**3**). Clearly, however, Fig. 1B also shows a group of relatively strong minor signals, as compared to those in Fig. 1A, consistent with the observation that the hog mucosal heparin contains substantially higher proportions

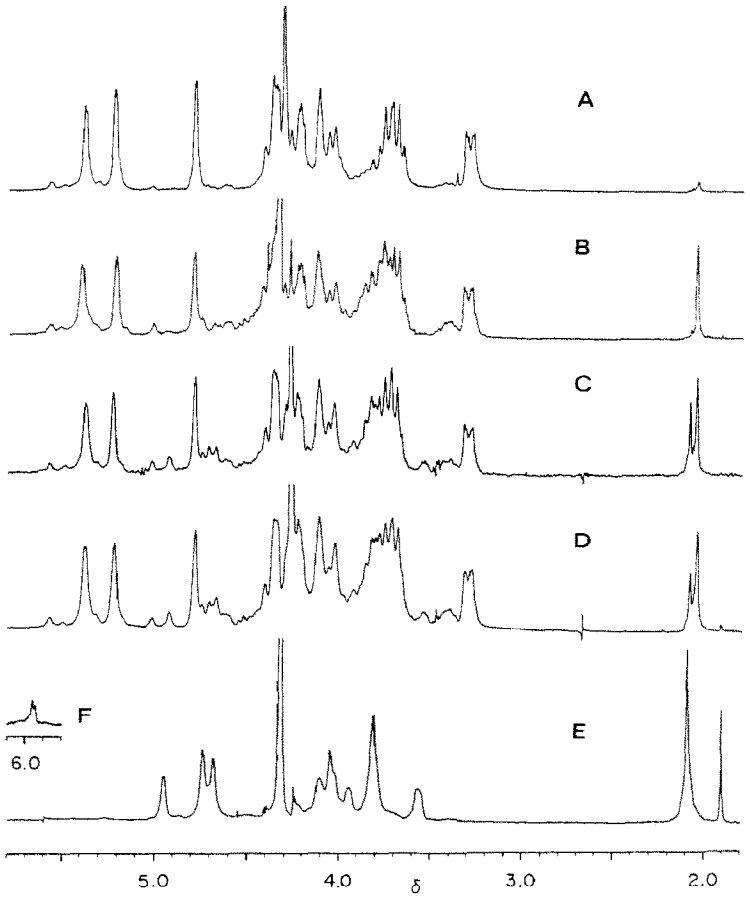
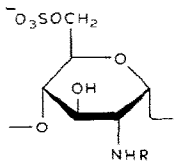


Fig. 1. ^{1}H-N.m.r. spectra (300 MHz) recorded at 72.5°, showing the δ 1.8–5.8 regions of: (A) beef lung heparin, (B) hog mucosal heparin, (C) a commercial heparin containing dermatan sulfate, (D) a 9:1 mixture of heparin and dermatan sulfate, (E) dermatan sulfate, and (F) the δ 5.8–6.1 region of dermatan sulfate after chondroitinase ABC treatment.



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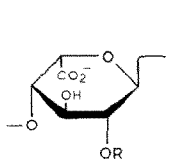
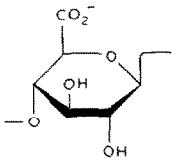
2 R = Ac



3 R = SO_3^-
4 R = H



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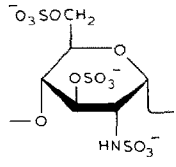

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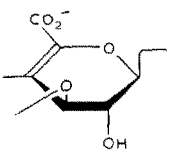

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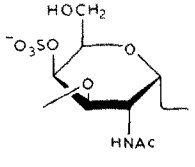

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of residues of 2-acetamido-2-deoxy-α-D-glucopyranose (**2**) (δ 2.0, NCOMe), β-D-glucopyranosuronic acid (**5**) (δ 4.6, H-1), and α-L-idopyranosuronic acid (**4**) (δ 5.0, H-1), than does the bovine heparin. These latter residues are more preponderant as well[7], in the molecules of relatively low molecular-weight which, in fact, give spectra[8] more closely akin to that of heparan sulfate, than to the spectra of hog mucosal heparin fractions constituted mainly of **1** and **3**, or to the beef lung spectrum in Fig. 1A.

Among unidentified minor resonances in Figs. 1A and 1B is one at δ 5.55 which, with resolution enhancement, appears to be a doublet ($J \sim 3$ Hz). It probably is due to H-1 of a residue structurally-related to **1**, inasmuch as it is decoupled by selective irradiation at the resonance frequency (δ 3.28) of H-2 of **1**. However, it is unlikely to come from the 3,6-disulfate (**6**) contained[9] in the antithrombin-binding sequence because, as part of a pentasaccharide fragment, **6** produces[10] its H-1 signal farther upfield.

The spectrum reproduced in Fig. 1C is that of a pharmaceutical preparation of hog mucosal origin marketed for clinical use. It closely resembles Fig. 1B, as expected, although it contains several other signals — at δ 4.93, 4.71, 4.67, 3.55, and 2.08 — all of which are attributable[5,6] to the related glycosaminoglycan, dermatan sulfate (chondroitin sulfate B, Fig. 1E). This fact is emphasized by the close correspondence between Fig. 1C and the spectrum (Fig. 1D) of a 9:1 mixture of the mucosal heparin (Fig. 1B) and a specimen of dermatan sulfate. A related observation is the selective effect on spectrum 1C caused by the introduction of chondroitinase ABC enzyme: most of the dermatan sulfate signals are displaced, there is the expected[11] appearance (at δ 5.95) of the olefinic proton signal (see Fig. 1F) of the α,β-unsaturated uronic acid elimination fragments (**7**), and a greater separation of the methyl signals produced by the 2-acetamido-2-deoxyhexose residues in dermatan sulfate *(galacto,)* and heparin *(gluco,)*, **8** and **2**, respectively.

The pharmaceutical heparin represented by Fig. 1C is one of a dozen prep-

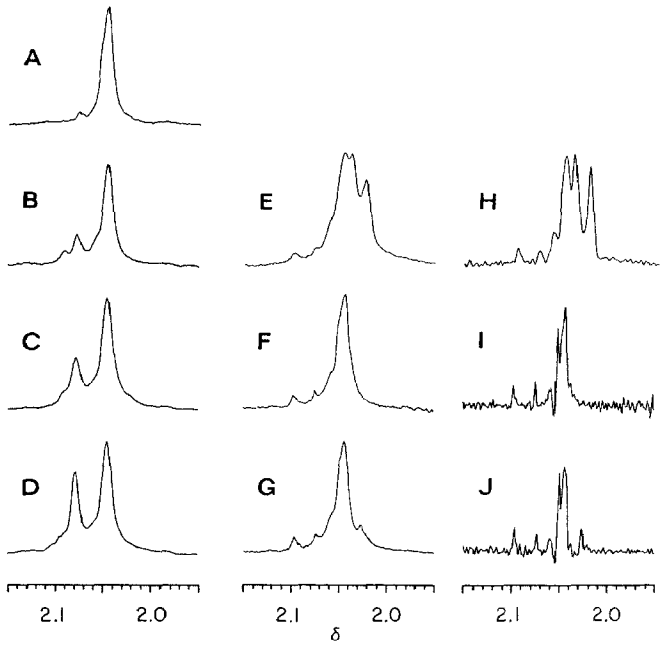


Fig. 2. The δ 1.95–2.15 region of the ^{1}H-n.m.r. spectra showing the acetamidomethyl signals produced by: (A) hog mucosal heparin, (B,C,D) commercial hog mucosal heparin samples, (E) a mixture of heparin and chondroitin 4- and 6-sulfates, (F) a mixture of heparin and heparan sulfate, (G) a mixture of heparin and hyaluronic acid, and (H,I,J) resolution-enhanced versions of E, F and G, respectively.

arations reported[6] to contain from 2 to 12 percent of dermatan sulfate, and ten additional specimens examined more recently also fall into this category. Although the heparins were marketed for clinical use by many different suppliers, the extent of contamination in different batches from *one* selected supplier has been found to vary over approximately the same range. We are unaware of other reports of heparins that contain an appreciable percentage of dermatan sulfate. The heparins examined in early n.m.r. studies of 15–20 years ago[4,5] showed no evidence of its presence, whereas the oldest of the contaminated preparations described here was packaged in 1984.

Resonances of acetamidomethyl groups. — In the 300-MHz spectrum of a porcine mucosal heparin that, according to chemical analysis, contains no dermatan sulfate, the methyl signal due to the 2-acetamido-2-deoxy-D-glucose residue (**3**) is accompanied in the region of δ ~2.0 by two, very minor, resonances (Fig. 2A). Some other heparins, aside from those obviously contaminated with dermatan sulfate (see Fig. 2D), produce three to four minor signals in this region (Figs. 2B,C). Although one of those lesser signals might be due to the presence of a small proportion of dermatan sulfate, the others are not attributable to chondroitin 4- or 6-sulfate, which also contain acetamido substituents. That is, the methyl resonances of these latter polymers are found distinctly upfield (Fig. 2E). It is worth noting that, although chondroitin 4- and 6-sulfates have occasionally been found[12] in

TABLE I

N.M.R. PARAMETERS FOR CHONDROITIN 4- AND 6-SULFATES, DERMATAN SULFATE AND HEPARIN[a]

Resonance	*Chondroitin 4-sulfate*		*Chondroitin 6-sulfate*		*Dermatan sulfate*		*Heparin*	
	$\delta(^1H)$	$\delta(^{13}C)$	$\delta(^1H)$	$\delta(^{13}C)$	$\delta(^1H)$	$\delta(^{13}C)$	$\delta(^1H)$	$\delta(^{13}C)$
U-1	4.48 (8.0)	106.29	4.51 (8.0)	106.81	4.97 (3.0)	105.66	5.24	101.36
U-2	3.405	75.25	3.40	75.50	3.585 (6.0)	71.99	4.37	78.33
U-3	3.590	76.59	3.585	76.82	3.975 (4.0)	73.59	4.425	71.44
U-4	3.75–3.89	83.32	3.78	83.87	4.12	82.50	4.14	78.19
U-5	3.690	79.64	3.72	79.41	4.755	72.11	4.805	71.71
U-6		176.90		176.78		176.58		176.22
A-1	4.615 (6.0)	103.63	4.585 (6.0)	104.01	4.67–4.89 (6.0)	104.70	5.40	98.96
A-2	3.94–4.09	54.47	4.035 (6.0)	53.78	4.01–4.09	54.90	3.32	60.01
A-3	3.94–4.09	78.38	3.88	82.78	4.01–4.09	78.19	3.705	71.90
A-4	4.755 (1)	79.35	4.14–4.28	70.30	4.705 (1)	78.87	3.785	78.64
A-5	3.75–3.89	77.46	3.965	75.54	3.75–3.90	77.42	4.065	71.26
A-6	3.75–3.89	63.84	4.14–4.28	70.30	3.75–3.90	63.81	4.26–4.45	68.61
NAc (CH_3)	2.04	25.41	2.037	25.41	2.11	25.47		24.91
(C=O)		177.64		177.64		177.75		

[a]Values in parentheses are observed spacings, in Hz.

preparations of beef lung heparin, they obviously are not present in the sample represented by Fig. 1A. The acetamido substituents of two other glycosaminoglycans, heparan sulfate (Fig. 2F) and hyaluronic acid (Fig. 2G), produce methyl signals close to, although not coincident with, that of the major methyl peak in the heparin spectrum. Resolution enhancement was useful for distinguishing between methyl signals in the latter mixtures (Figs. 2H–J).

Overall, then, it appears possible to determine the presence of more than 1–2 percent of any of these various polymers in a heparin preparation, by reference to the resonance region of the methyl protons. Although less sensitive, the methyl ^{13}C nuclei provide[13] another means for detecting glycosaminoglycans admixed with heparin. This is seen from the ^{13}C-chemical shift data of Table I, which show that the acetamidomethyl signal produced by heparin is clearly separated from that of the other polymers.

Based on these observations overall, a re-examination of n.m.r. spectra of many heparin samples recorded over an extended period in this laboratory, led to the conclusion that the methods used in the manufacture of heparin effectively remove all other glycosaminoglycans, aside from dermatan sulfate.

Other heparins. — As already noted, heparin from beef lung contains far fewer residues of 2-acetamido-2-deoxy-D-glucose (**2**) than does that from hog

mucosa, as shown (Fig. 1A) by the presence of only a minor methyl resonance at δ 2.05 in comparison with that in Fig. 1B. Accordingly, the resonance at δ 3.4, attributed to H-2 of **2**, is correspondingly weak [H-2 of β-D-glucosyluronic acid residues (**5**) also absorbs in this region]. Another marked distinction, however, is the fact that none of the several beef lung preparations as yet examined, such as that represented by Fig. 1A and spectra in Refs. 4 and 5, has been found to contain dermatan sulfate. We are unaware of a basis for this difference relative to the hog mucosal samples just described.

One specimen of a heparin extracted from beef mucosal tissue has been examined. In accord with its composition as determined[14] by enzymolysis, the ^{1}H-n.m.r. spectrum of this material is more closely analogous to that of hog mucosal, than of beef lung heparin. This is evident from a relatively strong acetamidomethyl signal that it produces, as well as other characteristics, including those of the downfield portion of the spectrum (not shown). It is also worth noting that the beef mucosal sample appeared to be free of dermatan sulfate.

Quantitative measurements. — An estimate of the dermatan sulfate content of the heparin samples was obtained from the integrated areas of appropriate resonance signals. Thus, the acetamidomethyl signals of dermatan sulfate and hog mucosal heparin are readily distinguished from each other, as also are the anomeric signals of the L-iduronic acid residues in the two polymers. Nevertheless, integrals of these analogous peaks cannot be compared directly, because the heparin resonances are not integer values. Its acetamidomethyl signal may represent only 15–20% of the total hexosamine, whereas the remainder is represented[4] by the H-2 signal of residues having the 2-sulfamino substituent. Similarly, the H-1 signals of the 2-sulfate and nonsulfated forms of L-iduronic acid together correspond to only 75–80% of the uronic acid content, and hence to a noninteger measure of the latter. Based on these considerations, we tested three different integral ratios ($R1$, $R2$, and $R3$) for the analysis of known mixtures of heparin and dermatan sulfate, as follows: (*a*) *Eq. 1* where *Acd* is the area of the acetamidomethyl (3 H) signal of dermatan sulfate, *Ach* is the area of the acetamidomethyl (3 H) signal of heparin, and *AH-2* is the area of the H-2 signal of the 2-sulfamino heparin residues; (*b*) *Eq. 2* where *Acd* and *Ach* are as under $R1$; and (*c*) *Eq. 3* where $I - Id$ is the area of

$$R1 = 1/3\ Acd/(1/3\ AcH + AH\text{-}2) \qquad (1)$$

H-1 of iduronic acid residues in dermatan sulfate, $I - I$ is the area of H-1 of

$$R2 = 20\ (Acd/Ach) \qquad (2)$$

iduronic residues in heparin, and $I - Is$ is the area of H-1 of iduronic acid 2-sulfate residues in heparin.

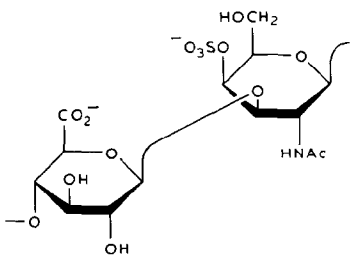
$$R3 = 80\ [I - \mathrm{1d}/(I - Is + I - I)] \qquad (3)$$

TABLE II

COMPOSITION OF HEPARIN–DERMATAN SULFATE MIXTURES, BASED ON PROTON INTEGRAL[a] RATIOS (R)

Dermatan sulfate (%)				
Mixture	*Added*[b]	*R1*	*R2*	*R3*
1	2.5	3.0	3.0	2.5
2	5.0	3.9	4.2	5.0
3	7.5	6.7	7.2	7.0
4	10.0	8.5	10.4	9.5
5	12.5	11.9	12.0	15.5

[a]Without resolution enhancement. [b]Percent (w/w) of dermatan sulfate added to hog mucosal heparin.



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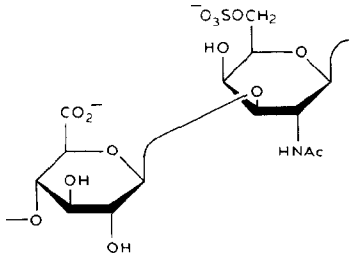

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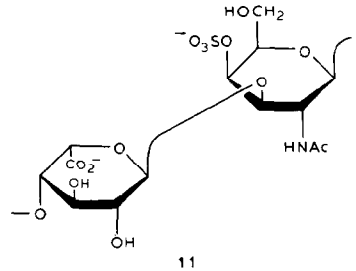

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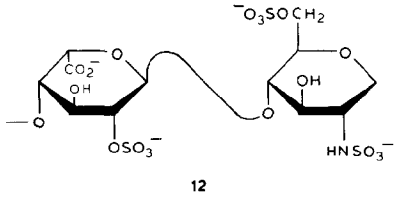

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The results obtained, expressed in Table II as percent dermatan sulfate, are in good agreement with the theoretical values, which cover the range at which dermatan contamination has been found[6] to occur. $R1$ values are the most useful, because they do not require a knowledge of the percent composition of the heparin, whereas this must be assumed in the calculation of $R2$ or $R3$. It has also been assumed that signals chosen to represent dermatan sulfate correspond closely to one ($I - 1d$) and three (Acd) proton integral values. This appears to be justified by the characteristics of the n.m.r. spectra of dermatan sulfate, which are considered further in the next section.

Measurement of the products formed[15] from dermatan sulfate by chondroitinase ABC, by use of absorption spectroscopy, may provide an alternative means for the analysis of such mixtures.

N.m.r. spectra of chondroitin 4- and 6-sulfates and dermatan sulfate. — Chondroitin 4- and 6-sulfates, and dermatan sulfate (chondroitin sulfates A, C, and B) have structures represented by the disaccharide repeating units **9**, **10**, and **11**, respectively. Both ^{1}H- and ^{13}C-n.m.r. spectroscopy have been used[5,13,16] in the structural characterization of these glycosaminoglycans. Although the ^{1}H-n.m.r. spectra suffer from line-broadening and signal overlap, more recent studies[17,18] at high field have provided considerable improvement in signal dispersion. Resolution enhancement of the 270-MHz spectra of the chondroitin sulfates permitted coupling data to be obtained and used in an analysis of the conformational characteristics of the respective uronic acid residues. ^{13}C-N.m.r. spectroscopy offers the advantage of greater signal dispersion and relatively narrower linewidths. However, a major limitation is that signal assignments for the ^{13}C-spectra have to be made on the basis of chemical shift comparisons and the use of model compounds.

In evaluating the n.m.r. data available for the chondroitin sulfates, it was anticipated that improvements in ^{1}H-signal dispersion at 300 MHz, particularly for chondroitin 6-sulfate, could be used to verify earlier ^{13}C-n.m.r. assignments with the aid of 2-D heteronuclear and homonuclear-correlated spectroscopy (HETCOR and COSY, respectively) experiments. In the following discussion, which describes the results obtained from 2D-n.m.r. experiments on the three chondroitin sulfates, some reassignments are reported, and others confirmed, by this procedure.

Analysis of the 300-MHz spectrum of chondroitin 6-sulfate (Fig. 3), with the aid of ^{13}C assignments already available[13], afforded the data listed in Table I. The three upfield signals bear a striking similarity to the upfield resonances of chondroitin 4-sulfate, which have been assigned to UH-2, UH-3, and UH-5, respectively. This is not unexpected, as both chondroitin 4- and 6-sulfates contain a 4-*O*-substituted β-D-glucopyranosyluronic acid residue. Correlations between two of the upfield ^{1}H signals (δ 3.40 and 3.72) and resonances assigned as UC-2 and UC-5 confirmed the assignments of UH-2 and UH-5 (Table I). The resonance expected to correlate with UC-3, however, did not. This indicated, perhaps, that the assignment of UC-3 should be interchanged with AC-5, the latter being correlated with

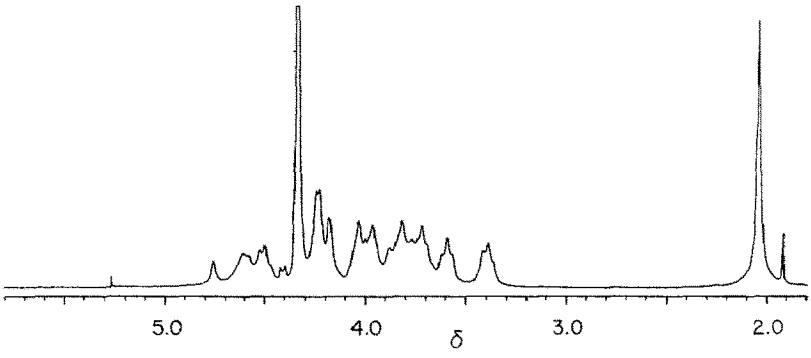

Fig. 3. The ^{1}H-n.m.r. spectrum (300 MHz) of a commercial preparation of chondroitin 6-sulfate recorded at 72.5°.

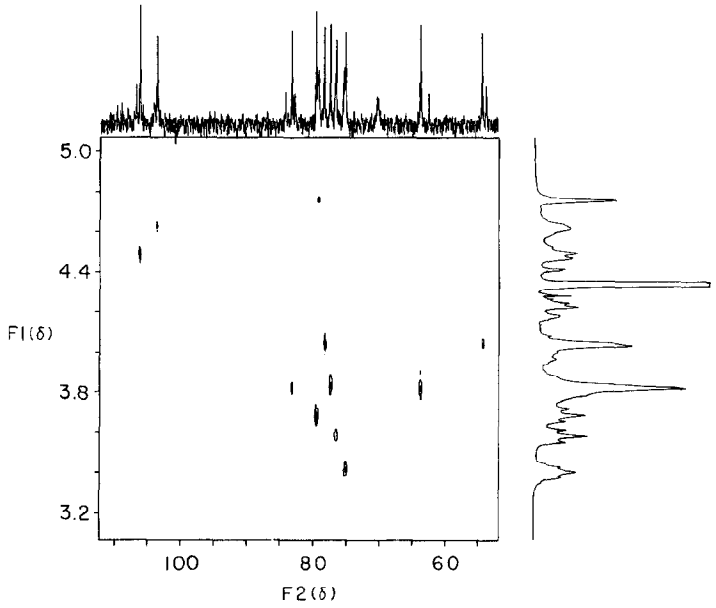

Fig. 4. ^{1}H–^{13}C-Heterocorrelation plot obtained for chondroitin 4-sulfate at 72.5°.

a resonance at δ 3.97, as might be anticipated for a proton adjacent to the sulfated C-6 position. The remaining glycosyluronic acid assignments were consistent with the ^{1}H- and ^{13}C-chemical shift data already reported[13,17,18].

Providing further support for the proposed reassignment of UC-3 and AC-5 of chondroitin 6-sulfate was a heterocorrelation experiment performed on chondroitin 4-sulfate (Fig. 4). Since the glycosyluronic acid residues in both are analogous, their correlation patterns could be directly compared. It was found that, although the upfield UH-2 and UH-3 resonances correlated with those that had been assigned as UC-2 and UC-3, the UH-5 signal showed connectivity to a ^{13}C signal at δ 79.64, and not to that previously assigned as UC-5. Despite the fact that the chemical shift of UH-5 proved to be that expected for chondroitin 4-sulfate, the signal itself (Fig. 4) had the appearance of a triplet, rather than a doublet, structure. This anomaly, however, was caused by the overlapping UH-3 resonance of chondroitin 6-sulfate, which was present at a level of about 25–30% in the commercial chondroitin 4-sulfate preparation used. These observations are consistent with the assignment of UC-5 (δ 79.41) and the reassignment of UC-3 for chondroitin 6-sulfate, and also necessitate changes for UC-4, AC-3, and AC-4. The UC-4 signal cannot be unambiguously assigned from the heterocorrelation data, although it can be limited to either the signal at δ 83.32 or δ 77.46, both of which differ from previous data. The UC-4 assignment given in Table I was facilitated by the data obtained for dermatan sulfate, and the results of a 2D homonuclear-correlated spectrum of chondroitin 4-sulfate (see later). The downfield AH-1 and AH-4 signals allowed for a facile assignment of AC-1 and AC-4 of chondroitin 4-sulfate, and

confirm the reassignment of AC-4 to a resonance slightly downfield (δ 79.35). Like UC-4, AC-3 is not unambiguously assignable from heterocorrelation of the ^{1}H and ^{13}C spectra. However, the upfield position of AC-3 of dermatan sulfate supported the designation of AC-3 to the signal at δ 78.38, and of UC-4 to δ 83.32. The upfield positions of AC-2 and AC-6 allowed them to be distinguished, and correlated to AH-2 (δ 3.94–4.09) and AH-6,6′ (δ 3.75–3.89), whereas AH-5 and the corresponding nucleus, AC-5, have the chemical shifts expected. In the δ 3.94–4.09 region, the AH-3 signal overlaps that of AH-2 and provided support for the assignment of AC-3 at δ 78.38.

A homocorrelated ^{1}H-spectrum of chondroitin 4-sulfate supported ^{1}H assignments for UH-1, 2, 3, 4, and 5, through cross-peak connectivities. As well, connectivity is established between AH-1 and AH-2, and AH-4 and AH-3. It was difficult to attribute this latter correlation to AH-3, as both the $J_{3,4}$ and $J_{4,5}$ ^{1}H-coupling constants are relatively small, and may not result in a cross peak. However, $J_{3,4}$ (3.2 Hz) is larger than $J_{4,5}$ (0.8 Hz) for the 2-acetamido-2-deoxy-β-D-galactopyranosyl residues, thus making a correlation of AH-4 with AH-3 more likely than with AH-5. Based on this assignment of AH-3 to an overlapping multiplet at δ 3.94–4.09, the heterocorrelation showed AC-3 at δ 78.38, and supported the UC-4 assignment at δ 83.32.

Dermatan sulfate contains a 2-acetamido-2-deoxy-β-D-galactopyranosyl 4-sulfate residue (**8**) in common with chondroitin 4-sulfate. Its idosyluronic acid protons have been assigned previously, as have the ^{13}C-signals of both residues. The heteronuclear-correlated spectra for dermatan sulfate confirmed chemical shift assignments for AH-1,2,6,6′, and UH-1, as the corresponding ^{13}C signals are easily distinguishable. The downfield UH-5 resonance correlated with a ^{13}C peak at δ 72.11 which, therefore, was assigned to UC-5, in agreement with previous data. Known assignments for UH-2, 3, and 4 allowed the respective carbon resonances to be located. UC-2 and UC-3 are situated at the expected chemical shifts positions, whereas UC-4 was assigned to the resonance slightly downfield at δ 82.50. The position of AC-4 is also relocated slightly downfield, and has the broad linewidth characteristics seen for AC-4 in chondroitin 4-sulfate. Although attempts were made to improve the signal linewidth by ion-exchange (Chelex) treatment, for the removal of possible trace metal-ion impurities, no significant change in the AC-4 signal resulted. However, the intensity of UC-5 was enhanced, as might be expected. Finally, the AC-3 signal was assigned to an upfield position rather than, as previously, to the downfield resonance now attributed to UC-4. It is this observation which provides further support for the AC-3 reassignment (δ 78.38) for chondroitin 4-sulfate to an upfield location, whereas UC-4 is assigned to the downfield carbohydrate region.

It is noteworthy that the ^{1}H- and ^{13}C-chemical shift assignments for the uronic acid residue of chondroitin 4-sulfate are similar to those for chondroitin 6-sulfate, whereas the 2-acetamido-2-deoxy-β-D-galactose signals of chondroitin 4-sulfate agree with those of dermatan sulfate. This reflects the structural analogies between

these polymers. Nevertheless, some assignments find poor agreement with calculated chemical-shift values. The most notable is AC-3 which, for chondroitin 6-sulfate, appears in the downfield region (δ 82.78) owing to its involvement in the glycosidic linkage. The introduction of a 4-sulfate group (as in chondroitin 4-sulfate and dermatan sulfate), however, causes an upfield shift of ~4.5 p.p.m., substantially greater than expected[13], based on a β-substituent effect.

2-D N.m.r. spectrum of beef lung heparin. — As a complement to the one-dimensional n.m.r. data available for the relatively homogeneous type of heparin from beef lung, this latter polymer was subjected to 2D n.m.r. analysis using ^1H–^{13}C heterocorrelation and ^1H–^1H homocorrelation experiments. The chemical shift assignments obtained are summarized in Table I. Despite considerable signal overlap in the ^1H spectrum, and a lack of dispersion in regions of the ^{13}C spectrum, the heterocorrelation procedure gave connectivities showing good dispersion in the ^1H dimension, which allowed for facile assignments to be made. The ^{13}C assignments were based on the previously[16] analyzed ^1H spectrum of beef lung heparin, and agreed with published ^{13}C data, except for the relatively minor reversal in the order of the IC-3 and IC-5 resonances. Furthermore, the proton assignments were fully supported by the ^1H–^1H homocorrelation experiment.

In conclusion, carbon–proton heterocorrelation spectroscopy has been useful for assigning some previously-unidentified proton signals, as well as for confirming or reassigning ^{13}C signals. Although the ^1H–^{13}C correlations are not always unambiguous in themselves, they can be incorporated into the more usual rationalizations based on chemical environment and model compounds, to provide a powerful assignment strategy for such complex carbohydrate polymers. It has also been demonstrated that where reasonable signal dispersion is obtained, the ^1H-homocorrelation experiment can be used in conjunction with the heterocorrelation data, in order to help resolve ambiguous structural assignments.

EXPERIMENTAL

N.m.r. spectra were recorded with a Varian XL300 spectrometer operating at 300 MHz for ^1H and 75.4 MHz for ^{13}C, equipped with a 5-mm proton probe and a 5-mm broad-band probe, respectively. All polysaccharide samples were treated by repeated addition and evaporation of their solutions with deuterium oxide, prior to ^1H-n.m.r. analysis, and the residues were then dissolved in deuterium oxide to give solutions containing 1–2% (w/w) of polysaccharide. The ^1H-spectra were acquired at 72.5°, and were referenced to the signal of internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (δ 0.0). ^{13}C-N.m.r. spectra were also recorded at 72.5° for solutions containing 10–20% (w/w) of polysaccharide in deuterium oxide with internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate as reference (δ 0.0).

The 2-D heterocorrelation experiment was performed with the Varian-supplied pulse sequence[19]. Generally, a 3.0-p.p.m. region was chosen in the ^1H dimension (δ 2.8–5.8), and a 50-p.p.m. region (δ 55–105) on the ^{13}C axis, with 1024

× 128 data points. The 2-D homocorrelation experiment was also executed with the Varian-supplied software[20]. A sweep width corresponding to ~3.0 p.p.m. (δ 2.8–5.8) was generally chosen, and collected in 512 × 256 data-point blocks.

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