systems have remarkably similar surface compositions and topographies.

This study has, for the first time, shown that data from XPS($\theta$) measurements can provide information bearing on the question of phase separation behavior in multicomponent polymer systems. Our results on the triblock copolymers clearly suggest that the components in the copolymers are partially miscible in the surface region and that this miscibility is a result of electronic interactions between the PEO and PS blocks in the copolymers. Additional studies to explore these interactions in related systems are warranted and are in progress.

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# Studies on the Conformation of Heparin by $^1H$ and $^{13}C$ NMR Spectroscopy

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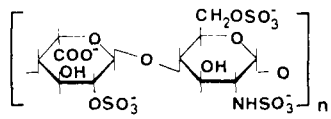
ABSTRACT: A convolution-difference 270-MHz $^1H$ NMR spectrum of heparin has afforded a complete set of interproton coupling data for the major constituent residues of heparin, i.e., $\alpha$-L-idopyranosyluronic acid 2-sulfate and 2-deoxy-2-sulfamino-$\alpha$-D-glucopyranose 6-sulfate. According to these data, the conformation of the iduronic acid residue is $^1C_4$(L) or a slightly distorted form thereof, whereas that of the aminodeoxyhexose residue is $^4C_1$(D); the exocyclic $CH_2OSO_3^-$ group of the latter residue favors the gauche,gauche rotamer. The 5-proton of each residue exhibits selective line broadening and a large chemical shift displacement in the $pK_a$ region of the uronide carboxyl group, which indicates that these particular protons are in close proximity within the molecule. This observation helps to define a solution conformation for the polyelectrolyte chain. Other information on stereochemical aspects, and on pH and temperature effects, is provided by $^{13}C$ NMR spectra. The latter are utilized also to highlight differences between beef lung (B type) and hog mucosal (A type) heparins, associated with the presence of minor constituent sugar residues, particularly through the acquisition of a difference $^{13}C$ spectra. It is also shown that signal dispersion in $^{13}C$ spectra of heparin is satisfactory at relatively low field (22.6 MHz) as well as at 67.9 MHz, provided that conditions of pH and ionic strength are optimized.

Heparin is a carbohydrate polymer that is widely distributed in animal tissues and is best known for its use in therapy as a blood anticoagulant. It is classed[2,3] as a mucopolysaccharide, or glycosaminoglycan, although it also may be aptly described as a glycosaminoglycuronan since it is formally a copolymer of a hexosamine and a uronic acid.




1 n = 10-30

Most of the heparin molecule is accounted for[4-6] by repeating disaccharide unit 1 that consists of a residue of $\alpha$-L-idopyranosyluronic acid 2-sulfate and of 2-deoxy-2-sulfamino-$\alpha$-D-glucopyranose 6-sulfate, each of which is glycosidically linked through position 4. This repeating sequence represents[7-9] at least 85% of heparins from beef

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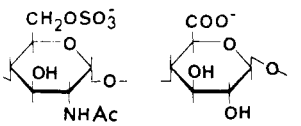
lung (B type[7]) and about 75% of those from intestinal mucosa (A type[7]). The balance of the molecule is constituted largely of residues of 2-acetamido-2-deoxy-α-D-glucose (2) and β-D-glucuronic acid (3), although their

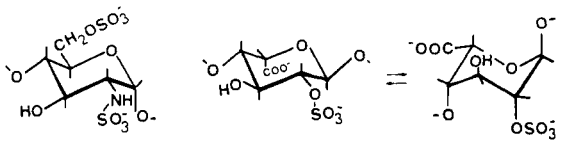


2 3

modes of bonding and distribution within the polymer, as well as their degrees of sulfation, have yet to be established unequivocally. In addition, a "linkage region" comprising two residues of D-galactose and one of D-xylose located at the reducing end serves to bind the polysaccharide to a peptide matrix.[6]

The anticoagulant property of heparin is associated[11] with its binding to the plasma protein antithrombin-III, although it appears[12] that this interaction does not solely determine the degree of activity observed. Moreover, it is well recognized[6,13] that heparin functions not only in preventing the clotting of blood but also, for example, as a potent antilipemic agent, by releasing lipoprotein lipase. In this latter role, the activity is fully maintained by heparins almost completely devoid of anticoagulant activity.

An understanding of how heparin functions at the molecular level requires detailed information about the geometry of the polymer chain. A necessary adjunct to this is a firm assessment of the conformations of the monomeric residues. According to potential energy calculations on neutral isomeric monosaccharides,[14,15] the aminodeoxy-α-D-glucose residues should exist in the $^4C_1$(D) conformation 4. This is consistent with the evidence



4 5a 5b

available and is fully confirmed by data to be presented below. By contrast, the calculations also infer that the residues of α-L-iduronic acid should favor $^4C_1$(L) (**5a**) and $^1C_4$(L) (**5b**) conformations almost equally. NMR studies,[16] in which methyl idopyranuronsides were used as models, suggest that the $^1C_4$ conformation is the more stable (the equilibrium is to the right (**5b**)). However, this need not be true for the polymer, since the iduronic acid therein is largely O-sulfated and is flanked by other anionic residues of the chain. In attempting to define the overall shape of the heparin molecule, therefore, we begin by focusing on the nature of the iduronic acid moiety.

Earlier findings,[7] based on a partial analysis of $^1$H NMR spectra of heparins, rule out a $^4C_1$(L) stereochemistry (**5a**) because the associated trans diaxial orientation of H-1 and H-2 would require a $J_{1,2}$ value of ~9 Hz, whereas, in fact, the observed coupling is only ~3 Hz. The latter value is much more consistent with *e,e* orientation of these protons in structure **5b** or one having a closely related geometry. Additional support for this conclusion is available from $^{13}$C chemical shift,[17] coupling,[18] and relaxation[19] data. Moreover, the currently accepted molecular model of heparin in the solid state,[10] compatible with X-ray fibre data and stereochemical criteria, is based on conformation **5b**, as well as **4** for the aminosugar residue. Hence, the two axial glycosidic bonds in **5b** account for a larger helical pattern than would be generated if, as in **5a**, these bonds were equatorial.

Until now, a complete analysis of the $^1$H NMR spectrum of heparin has not been feasible because several signals overlap heavily, even at 270 MHz. Another adverse factor has been signal broadening due to the effect of moderately high viscosities at the solution concentrations employed and, probably, of paramagnetic ions present as trace impurities. Here, however, we report full analyses of 270 MHz spectra obtained through the use of a resolution-enhancement ("convolution-difference") method,[20] already applied successfully to proteins. Assignment of all of the $^1$H signals has been effected through spin–decoupling, and the interproton coupling constants were then obtained by computer simulation of the spectra. In turn, the use of heteronuclear spin–decoupling has permitted the assignment of all signals in the corresponding 67.9-MHz $^{13}$C spectrum. These combined data, and others based on pH-induced changes in the spectra, are discussed in relation to the conformations of individual residues as well as to the overall shape of the polymer chain.

## Experimental Section

Beef lung heparin (B type, sodium salt) (155 USP units/mg) was obtained from Upjohn, Kalamazoo, Mich.; it has been characterized in earlier studies.[7,8,17] Hog mucosal heparin (A type, sodium salt) (150 USP units/mg) was a reference standard furnished by J. A. Cifonelli and M. B. Mathews, University of Chicago. Disaccharide **6**, i.e., 4-*O*-(2-sulfo-α-L-idopyranuronosyl)-2,5-anhydro-D-mannose, was prepared by R. Helleur, McGill University, through deaminative degradation of heparin followed by gel chromatography.[21] Deuterium oxide (99.9%, Merck) was used as the solvent, following several exchange treatments with 99.7% $D_2O$. Prior to H–D exchange, heparin solutions were treated with a chelating resin (Chelex-100, Bio-Rad Laboratories) which promotes narrowing of $^{13}$C signals, particularly that of a carboxyl group.

NMR spectra were measured with a Bruker HX-270 spectrometer operating at 270 MHz (for $^1$H) or 67.9 MHz (for $^{13}$C) and also with a Bruker WH-90 spectrometer operating at 22.6 MHz (for $^{13}$C). Convolution-difference spectra were obtained by the procedure described by Campbell et al.,[20] and the LAOCOON system was used for computer simulation of spectra. Chemical shifts were measured with reference to internal TSP for $^1$H spectra and to internal methanol for $^{13}$C spectra. Data for the latter were normalized with respect to $Me_4Si$ ($\delta(CH_3OH) - \delta(Me_4Si_{ext}) = 50.1$). For titrations, reference was made to internal acetone ($\delta$ 2.24).

For the measurements of changes in chemical shift with pH, samples were prepared in the following manner: a small portion of the sodium heparin solution (above) was drawn from the NMR tube into a long-tipped Pasteur pipet containing Amberlite IR-120 ($D^+$) resin (~1 g, moist, prewashed with $D_2O$), then it was returned to the tube, the pD of the whole solution was measured (pD = pH + 0.41) with an elongated glass microelectrode (Ingold, Model 6975), and the spectrum was recorded. This procedure was repeated as the pD was lowered incrementally over the range from 6.5 to 2.0.

## Results

**(a) Spectral Analyses. $^1$H and $^{13}$C Chemical Shifts and Coupling Constants.** The resolution obtained for the spectrum of beef lung heparin at 270 MHz (Figure 1a) is slightly better than that found earlier at 220 MHz, as would be expected; several weak signals that are seen more clearly here than in the latter spectrum are attributed to minor constituents of heparin (see below). A much more dramatic improvement is evident (Figure 1b), following convolution-difference processing of the spectrum. By computer simulation using the parameters listed in Table I, a calculated spectrum is obtained (Figure 1c) that closely matches spectrum 1b. Table I contains data obtained in this manner at both 35 and 90 °C.

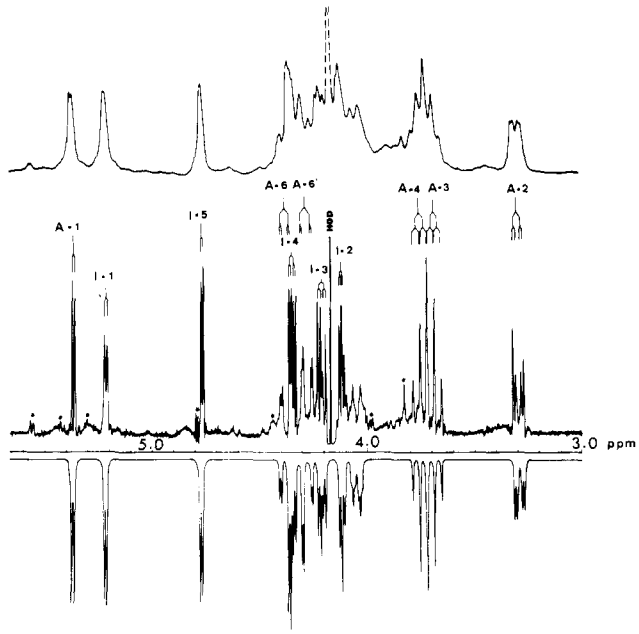



**Figure 1.** (a, top) $^1H$ NMR spectrum (270 MHz) of beef lung heparin (B type) in $D_2O$ (40%, w/v) at 90°; (b, middle) after convolution-difference processing; (c, bottom) computer simulation of spectrum b (A, aminodeoxyglucose; I, iduronic acid).

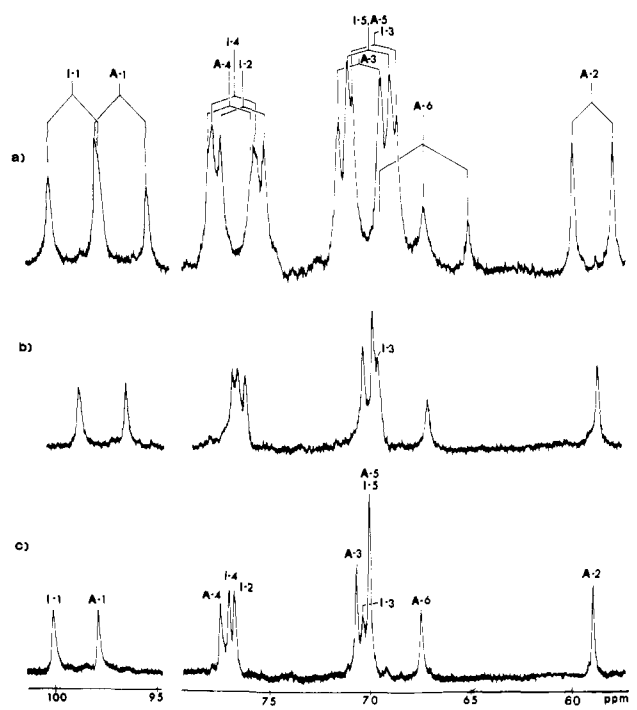



**Figure 2.** $^{13}C$ NMR spectra (69.7 MHz) of beef lung heparin (B type) in $D_2O$ (20%, w/v): (a) $^1H$ coupled (40 °C); (b) $^1H$ decoupled (40 °C); (c) $^1H$ decoupled (70 °C). Carboxyl region is not shown (A, aminodeoxyglucose; I, iduronic acid).

Figure 2 illustrates 67.9-MHz $^{13}C$ spectra of beef lung heparin. The $^1H$ decoupled spectrum (Figure 2b) consists essentially of 12 signals including that (not shown in the figure) produced far downfield by the uronosyl carboxyl carbon (I-6). In the $^1H$ coupled spectrum, the latter signal again appears as a singlet (again, not shown), whereas the triplet at 67 ppm (Figure 2a) can be attributed only to the primary carbon (A-6) of the aminosugar moiety. Hence, both in terms of their typical chemical shift values and multiplicities, resonance signals I-6 and A-6 are readily identifiable. The assignments given in Figures 2a and 2b for the other nuclei are based on selective single-frequency

Table I
$^1H$ Spectral Parameters[a] for Heparin (Sequence 1)

| | aminodeoxyglucose residue | | iduronic acid residue | |
|---|---|---|---|---|
| | 35 °C | 90 °C | 35 °C | 90 °C |
| $\delta_1$ | 5.40 | 5.37 | 5.22 | 5.22 |
| $\delta_2$ | 3.28 | 3.30 | 4.35 | 4.35 |
| $\delta_3$ | 3.67 | 3.69 | 4.20 | 4.21 |
| $\delta_4$ | 3.77 | 3.75 | 4.11 | 4.12 |
| $\delta_5$ | 4.03 | 4.05 | 4.82 | 4.77 |
| $\delta_6$ | 4.41 | 4.38 | | |
| $\delta_{6'}$ | 4.28 | 4.28 | | |
| $J_{12}$ | 3.66 | 3.57 | 2.64 | 3.29 |
| $J_{23}$ | 9.98 | 9.88 | 5.90 | 6.10 |
| $J_{34}$ | 9.09 | 8.91 | 3.44 | 3.60 |
| $J_{45}$ | 9.23 | 9.23 | 3.09 | 3.14 |
| $J_{56}$ | 2.92 | 2.92 | | |
| $J_{56'}$ | 2.15 | 2.15 | | |
| $J_{66'}$ | −11.23 | −11.23 | | |

[a] Chemical shifts in ppm from internal TSP; coupling constants in Hz. Solvent, deuterium oxide.

Table II
$^{13}C$ Spectral Parameters[a] for Heparin (1)

| | aminodeoxyglucose residue | | iduronic acid residue | |
|---|---|---|---|---|
| | $\delta$ | $^1J_{CH}$ | $\delta$ | $^1J_{CH}$ |
| C-1 | 97.88 | 170[b] | 100.12 | 172[b] |
| C-2 | 58.95 | 138 | 76.64 | 150 |
| C-3 | 70.72 | 144 | 70.05 | 150 |
| C-4 | 77.37 | 150 | 77.10 | 150 |
| C-5 | 70.15 | 144 | 70.15 | 144 |
| C-6 | 67.45 | 150 | 175.51 | |

[a] Chemical shifts in ppm from external MeSi; coupling constants in Hz. Solvent, deuterium oxide; temperature, 40 °C. [b] Due to signal overlap at 69.7 MHz, these values are taken from the 22.6-MHz spectra.

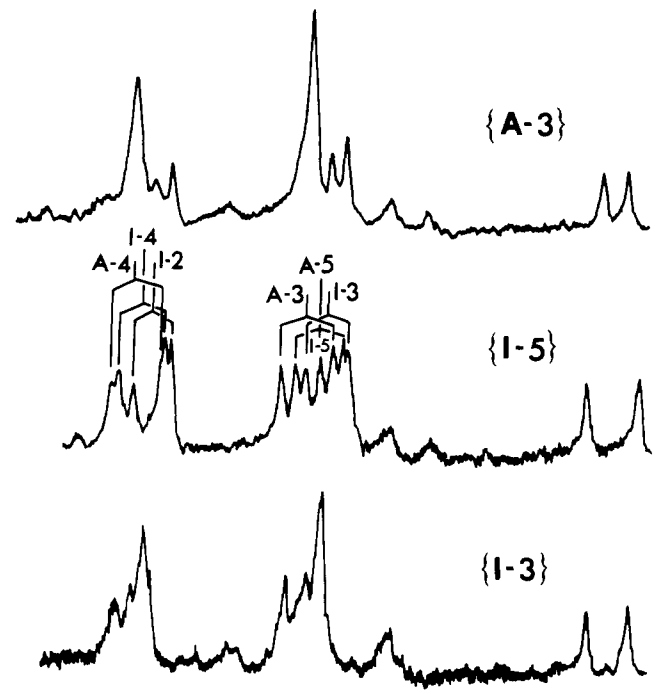



**Figure 3.** Partial $^{13}C$ NMR spectra corresponding to Figures 2a and 2b, showing effects of single-frequency proton decoupling: (a, top) irradiation of AH-3; (b, middle) irradiation of IH-5; (c, bottom) irradiation of IH-3 (A, aminodeoxyglucose; I, iduronic acid).

$^1H$ decoupling, as illustrated in Figure 3 for the cluster of signals in the region 69–71 ppm. Chemical shifts and direct

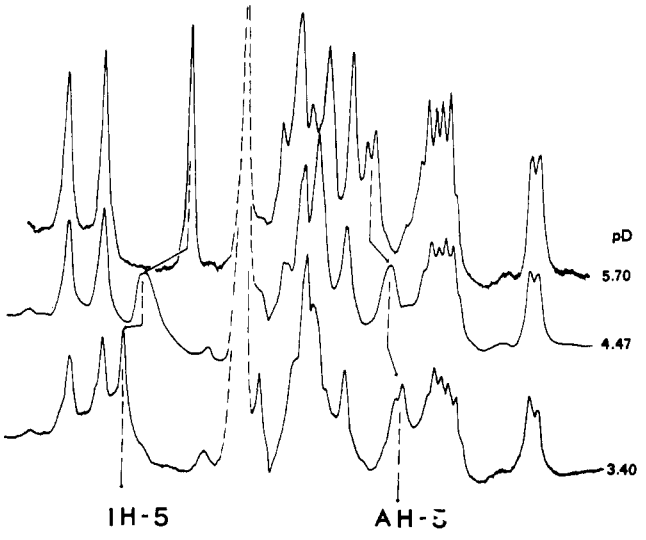



**Figure 4.** $^1H$ NMR spectra (270 MHz) of beef lung heparin at different values of pD (10% (w/v) in $D_2O$; 40 °C), showing substantial shift and selective broadening of IH-5 and AH-5 signals (A, aminodeoxyglucose; I, iduronic acid).

$^{13}C$–$^1H$ coupling constants ($^1J$) measured from these spectra are recorded in Table II.

**(b) Temperature and Concentration Dependence.** $^1H$ chemical shifts are affected only slightly by temperature (Table I). By contrast, some $^{13}C$ nuclei are relatively sensitive to a change in temperature (Figure 2c), most notably carbon-3 (I-3) of the iduronosyl residue, which becomes less shielded as the temperature is raised. An accompanying response of the iduronsyl residue is some increase (Table I) in $^1H$ coupling constants. Changes in concentration of neutral solutions of heparin over a range of 1–40% (w/v) for $^1H$ spectra and of 5–40% for $^{13}C$ spectra had only minor influences on chemical shifts or interproton coupling constants.

**(c) Effects of pH.** Marked changes in $^1H$ and $^{13}C$ spectra of heparin are induced by varying the pH. As is illustrated in Figure 4, signals IH-5 and AH-5 are especially affected both in chemical shift and line width; they become selectively broadened at pD ~4.5 and return to a "normal" band width at lower pD. Although these spectra have not been completely analyzed, it appears that most of the interproton coupling constants remain the same over the pD range of 8.0 to 1.5. According to the observed variations in $\delta$ with changes in pD, which give the "titration curves" plotted in Figure 5, protons respond in either of two ways toward an increase in acidity: downfield shifts are found in the order I-5 > I-3 > I-4 > A-2, A-1 > I-1 and upfield shifts are found in the order A-5 > A-4 > I-2. Of the $^{13}C$ signals, A-1 (and also probably I-5) exhibits a downfield shift, whereas upfield displacements are evident for I-6 > I-3 > I-2.

Table III
Apparent $pK_a$ of the Carboxyl Group of L-Iduronic Acid Residues of Heparin as a Function of Concentration

| concn, % (w/v) | $pK_a$ | method |
|---|---|---|
| 20 | 3.93 | $^{13}C$ NMR |
| 15 | 4.06 | IR[23] |
| 10 | 4.60 | $^1H$ NMR |
| 1 | 5.03 | $^1H$ NMR |
| ~0.1 | 5.10 | CD[24] |

From the titration curves, apparent dissociation constants have been derived for the carboxyl group of the L-iduronic acid residues, using the method of Bradbury and Brown.[22] As is shown in Table III, which also contains data obtained by IR spectrophotometry[23] and circular dichroism,[24] the $pK_a$ increases with decreasing concentration over the range 0.1–20% from a value of 3.9 to 5.1.

**(d) Influence of Structural Heterogeneity. A- and B-Type Heparins.** Most heparin preparations are obtained from hog mucosa (A type) although, formerly, beef lung heparin (B type) was more common. All of the main peaks in $^1H$ and $^{13}C$ spectra of an A-type heparin correspond to those found in Figures 1 and 2 for the B-type molecule, and interproton coupling data obtained from its convolution-difference $^1H$ spectrum are essentially the same as those listed in Table I for the B type. However, the two heparins show a distinct difference in that minor signals are much more prominent in the mucosal spectra. Although this is evident from a comparison of $^1H$ spectra, the difference is emphasized by the large number of relatively weak signals observable in $^{13}C$ spectra of acidified solutions (Figure 6). Particularly noticeable are peaks in Figure 6a which denote the presence of an extra anomeric carbon (at 105 ppm, likely due to 3) and an acetamido $CH_3$ (at 25.5 ppm, due to 2[4,25]) in the A-type preparation. The

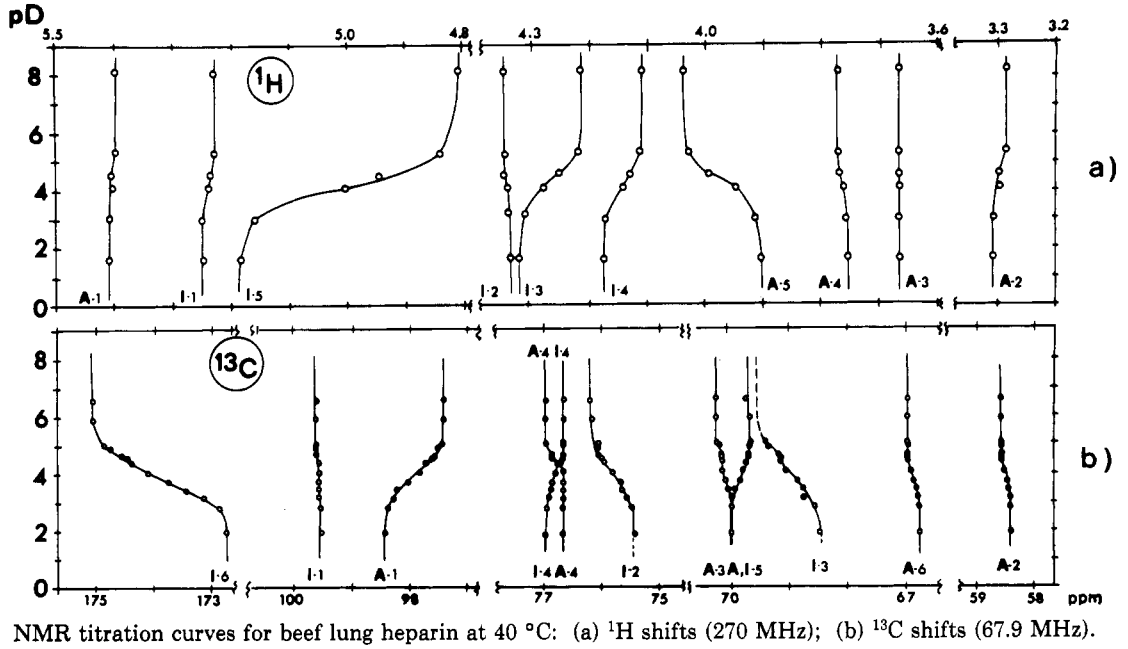



**Figure 5.** NMR titration curves for beef lung heparin at 40 °C: (a) $^1H$ shifts (270 MHz); (b) $^{13}C$ shifts (67.9 MHz).

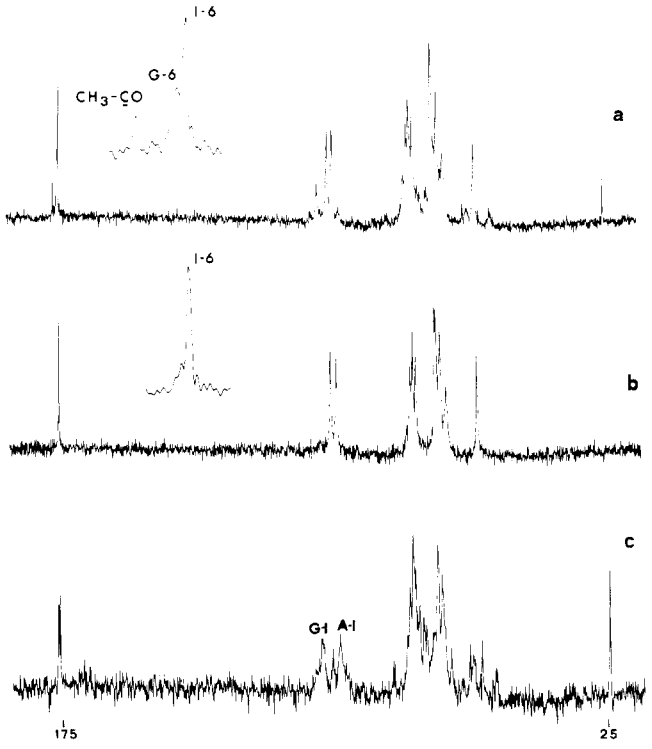



**Figure 6.** $^{13}C$ NMR spectra (22.6 MHz) of heparin in $D_2O$ (20%, w/v), at pD 1.5 and 40 °C, in the presence of 0.8 M NaCl. (a) Hog mucosal heparin (A type); inset, expansion of the spectrum in the region of 175 ppm. Upfield shifts ($\Delta\delta$) observed in going from pD 5.2 to 1.5 are 1.8 for I-6, 1.5 for G-6, and 0.1 for C=O. (b) Beef lung heparin (A type); inset, expansion of the spectrum in the region of 175 ppm. (c) Difference between spectra of A- and B-type heparins, both as sodium salts in $D_2O$ (20%, w/v) at 40 °C.

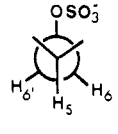
latter also exhibits three signals in the carbonyl region (inset, Figure 6a), although these are found to be virtually coincident (at ~175 ppm) at neutral pH. Since acidification causes an upfield displacement of I-6, as noted above, the major carbonyl signal observed here must be that of the iduronosyl residue in **1**, whereas the most deshielded of the signals is attributable to an acetamido C=O (residue **2**) and the intermediate peak to the carboxyl (C-6) of glucuronic acid[25] (residue **3**). For B-type heparin, by contrast, the carbonyl region consists essentially of only one signal (inset, Figure 6b) attributable to I-6 of **1**. Therefore, these spectra are fully consistent with the earlier evidence cited above to the effect that beef lung heparin is represented almost wholly by sequence **1**, whereas heparin from hog mucosa may contain 25–30% of residues **2** and **3**.

Another way in which $^{13}C$ spectra of these two heparins may be compared is by use of a computer to subtract a B-type spectrum from that of an A type. The difference spectrum obtained in this way (Figure 6c) provides a display of signals arising spectifically from the minor constituents. Accordingly, one observes resonances attributable to **2** and **3** in the form of two carbonyl peaks (COOH) (C=O) at low field and two prominent carbon peaks in the region of 100 ppm. Of the latter, one signal (G-1) corresponds in chemical shift to carbon-1 of a *β-gluco* moiety, and A-1 corresponds to the anomeric carbon of a 2-acetamido-2-deoxy-*α-gluco* moiety, as required by formulas **2** and **3**, respectively. Since there are several signals in the regions C-2 and C-6 (~60–65 ppm),[17] it appears that the minor structural sequences in the polymer contain various kinds of aminodeoxysugar residues: some are O-sulfated at position-6, whereas others are not, and some are N-sulfated, whereas others are N-acetylated. It is noteworthy that a similar variety of structural features is found[26] in heparan and, in fact, that Figure 6c closely resembles the $^{13}C$ spectrum of a major fraction of heparan sulfate.[27]

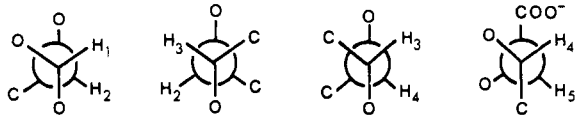
The 22.6-MHz spectra shown in Figure 6a merit comment in relation to $^{13}C$ spectra of heparin reported initially[4,17] as well as those above, at 67.9 MHz. All of the signals in the latter are at least partly resolved and, in combination with the 270-MHz $^1H$ spectral data, have now been assigned. However, it should be noted that this information applied expressly for the sodium salt of heparin. The earlier $^{13}C$ spectra also dealt with the sodium salt although, because of the low field employed, severe overlap of many signals was observed.[4,17,28] The spectra in Figures 6a and 6b differ in that the solutions were at low pH and contained added sodium chloride; this increase in ionic strength improves the signal dispersion even at neutral pH,[29] and further enhancement ensues upon acidification. Hence, under such conditions, $^{13}C$ spectra of heparin at relatively low field can be more serviceable.

## Discussion

Several conformational features of the major portion of the heparin molecule (**1**) are clearly evident in the complete set of data furnished by the present study. As expected for the aminodeoxyhexose moiety, the large $^3J$ values of ~9 Hz (Table I) show[30] that C–H bonds 2–5 must all be axial and hence that the conformation is $^4C_1$(D), as in formula **4**. Similarly, the value of $J_{1,2}$ is in keeping with the (*a,e*) α-D configuration assigned for this residue. Other information concerns the rotational conformation of the $CH_2OSO_3^-$ group. Since $J_{5,6}$ and $J_{5,6'}$ are substantially equivalent and small, the gauche,gauche conformation **7**




**7**




is favored over the other two fully staggered rotamers about the C-5,C-6 bond. This stereochemistry differs from those found for nonsulfated models, α,β-D-glucose[31] and 2-acetamido-2-deoxy-α,β-D-glucose,[32] and suggests that the bulky 6-sulfate substituent is projected as far as possible from the pyranose ring.

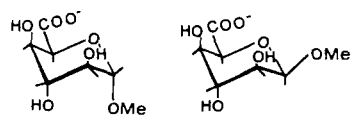
Although interproton coupling constants give only an approximate measure of dihedral angle, it is obvious that the L-iduronic portion of **1** cannot possess the $^4C_1$(L) conformation **5a**, because all of its $^3J$ parameters are far too small (2.6–5.9 Hz) for trans-diaxial orientations. Therefore, the vicinal protons must be gauche, as illustrated in Newman projections a–d, or quasigauche. Due to the influence of electronegative substituents, $^1H$–$^1H$ coupling associated with gauche orientations may range from 0.8–5.5 Hz.[33,34] A low value (0.8–2 Hz) is to be expected when, as in d, two oxygen atoms are antiperiplanar with respect to the protons, one of intermediate size (2–3.5 Hz) when there is only one antiperiplanar oxygen (as in a), and largest (3.5–5 Hz) when neither of the antiperi-

Table IV
Comparative $^3J_{HH}$ (Hz) for Idopyranosyluronic Acid Moieties

| | temp, °C | $J_{12}$ | $J_{23}$ | $J_{34}$ | $J_{45}$ | $J_{24}$ |
|---|---|---|---|---|---|---|
| 9[16] | 40 | 1.9 | 3.5 | 3.5 | 2.3 | 1.2 |
| 8[16] | 40 | 4.0 | 6.0 | 6.0 | 3.5 | |
| 1[a] | 35 | 2.6 | 5.9 | 3.4 | 3.1 | |
| 6 | 35 | 3.1 | *b* | *b* | 2.8 | |

[a] Data from Table I. [b] Obscured by signal overlap.

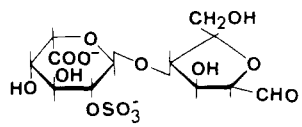
planar atoms is oxygen (as in b and c).[34] Such characteristics are observable (Table IV) in the spectra of methyl $\alpha$- and $\beta$-D-idopyranosiduronic acids[16] (8 and 9, respectively) as well as in data obtained from the convolution difference 270-MHz $^1$H spectrum of disaccharide 6. For



8



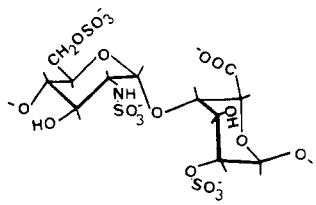
9



6

the $\beta$-glycoside 9, long-range coupling between H-2 and H-4 established unequivocally that these protons are equatorially oriented (forming part of a "W" arrangement[35]). Hence, the segment of this molecule corresponding to projection b or c is characterized (Table IV) by a coupling of ~3.5 Hz and that to d by a value of ~2.3 Hz.

Since the corresponding values of $J_{3,4}$ and $J_{4,5}$ for heparin are closely similar, O-4 and C-5 in the polymer must be axial and equatorial, respectively, as required by the $^1C_4$(L) conformation **5b**. Furthermore, $J_{1,2}$ = 2.6 Hz is consistent with the orientation of substituents depicted in projection a for the $\alpha$-L anomer in this conformation. The value of $J_{2,3}$ is slightly large, a characteristic also found recently[36] for the $\alpha$-L-iduronic acid residues in dermatan sulfate. Since the latter residues have no 2-sulfate group, the presence of this substituent in heparin is not likely to account for an increase in $J_{2,3}$. A more plausible source of the increase could be a minor distortion of the pyranose ring in the vicinity of C-2 and C-3. By comparison, however, the $\alpha$-glycoside 8 exhibits several large $^3J$ values (Table IV). These are much more likely to be representative of a significant skewing of the ring or of a substantial contribution from the alternate chair **5a**. Therefore, we conclude that the iduronic acid residues of heparin strongly favor the $^1C_4$(L) conformation as in sequence **10**. A slight skewing of the chair appears to take place and, judging from the increase observed in $J_{1,2}$ (Table I), to become more pronounced as the temperature is raised.



10

Conformational formula **10** has the C-1,O-1 bond of each residue in an axial orientation. Additional support for this geometry comes from direct $^{13}$C–$^1$H coupling constants ($^1J$). As is shown in Table II, coupling at the anomeric position is ~170 Hz in both instances, a value characteristic[18,37,38] of an axial C-1,O-1 bond. The other C–H couplings are in the range 138–150 Hz, the smallest value being associated with the relatively low electronegativity of the amino substituent; no apparent relationship with conformational features seems to be extractable at the present from these $^1J$ values.

Small changes observed in $^1$H and $^{13}$C chemical shifts with variations in temperature and concentration may be attributable to minor conformational readjustments, such as in the angles of rotation about the interresidue glycosidic bonds and/or in the local conformation of substituent groups. Thus, the notable temperature-induced deshielding of the iduronosyl C-3 (Figure 2) could be associated with a rearrangement of the polymer chain, such that the shielding influence of adjacent residues is altered, and/or with a change in the relative proximity of the 2-sulfate group. The latter is a less likely factor, however, because there are no comparable effects on the chemical shifts of nearby C-2 or H-2. It is noteworthy that this C-3 signal (I-3) is also the most strongly affected by interaction of heparin with $Ca^{2+}$ ions.[39]

The pH-induced changes (Figures 4 and 5) undoubtedly reflect the influence of the state of ionization of the carboxyl group on the magnetic shielding of nuclei close to the ionization site as well as the conformation of the polyelectrolyte chain. Since there is little impact on interproton coupling, the conformations of individual residues are virtually unaffected. Hence, the observed displacements in chemical shift probably result from contraction or extension of the polyelectrolyte chain as negative charges are removed or generated. Accompanying changes in the orientation of glycosidic linkages may then be accounted for by the chemical shift variations exhibited by nuclei of the anomeric centers.

As is shown in Table III, p$K_a$ values obtained from the NMR titration curves (Figure 5) increase with decreasing concentration, an effect common for polyelectrolytes as a result of chain–chain interactions.[40] When normalized for the different concentrations at which they were derived, the NMR values are in line with those afforded by IR[23] and circular dichroism[24] measurements.

The strong upfield shift of the AH-5 signal (Figure 5) is especially striking because, at first sight, the proton is remote from any region in which ionization changes occur. However, a molecular model, proposed for crystalline heparin by Atkins and Nieduszynsky,[10] places AH-5 in relatively close proximity to the carboxyl group of an adjacent iduronosyl residue. Possibly, then, this spatial arrangement is also to be found in solution (Figure 7), in which case AH-5 is positioned so as to sense changes in the polarization of the $COO^-$/COOH groups. Also, a decrease in the distance between the latter and AH-5 as the polyelectrolyte chain contracts should promote an increase in the magnetic shielding of this proton (and vice versa). Strong support for such a possibility is furnished by the pronounced, selective broadening of *both* the IH-5 and AH-5 signals in the region close to the p$K_a$ (Figure 4). This effect, attributable to a slow exchange of $H^+$ between the COOH and $COO^-$ groups when they are approximately equally represented in the heparin molecule, points to a

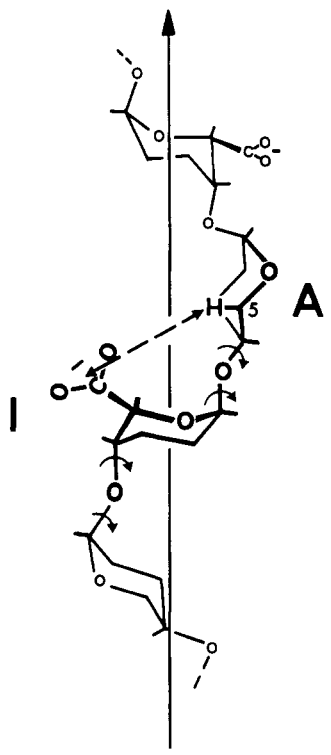



**Figure 7.** Representation of heparin as repeating sequences of **10**, showing the proximity of AH-4 to the carboxyl group of a neighboring L-iduronosyl residue.

close spatial relationship between the IH-5 and AH-5 sites along the chain as in Figure 7.

The anticoagulant activities of beef lung and hog mucosal heparin are not equivalent, according to different methods of assay.[12] This focuses attention on the manner in which the two heparins differ chemically: the much higher proportion of minor constituents, such as **2** and **3**, in mucosal heparin is demonstrated with particular clarity by a comparison of its $^{13}C$ spectrum (Figure 6b) with that of beef lung (Figure 6a). Since the $^{13}C$ and $^{1}H$ chemical shift and coupling parameters for the major residues of both A and B heparins are closely similar, it appears that the conformation of that portion of each molecule represented by Figure 7 is not materially altered by the presence of the minor constituents. However, the conformation of a given segment of a molecular chain should be affected materially on replacement of a residue of $\alpha$-L-iduronic acid by one of $\beta$-D-glucuronic acid, because their ring conformations would differ. Therefore, it is possible that variations in biological activity are related to such factors as the distribution pattern of minor types of residues within the main framework of repeating units of biose **10**; about this, relatively little is yet known.[41,42] Differences in fine structure also may be related to the recent finding[42,43] that a given heparin can be fractionated into material of high and low anticoagulant activity and that the binding sites to antithrombin-III are confined in heterogeneous segments of heparin chains.[44,45]

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