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Regioselectivity in the sulfation of some chemically-modified heparins, and observations on their cation-binding characteristics *

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ABSTRACT

Two modified forms of heparin, polymers A and B, have been prepared, one containing residues of nonsulfated α-L-idopyranosyluronic acid (**3**) and the other residues of α-L-galactopyranosyluronic acid (**7**), in place of the normal α-L-idopyranosyluronic acid 2-sulfate (**1**). In addition, both A and B contained 2-acetamido-2-deoxy-α-D-glucopyranosyl 6-sulfate residues (**6**) in place of the corresponding *N*-sulfated residues (**2**) of the original heparin. These polymers were subjected to sulfation under various conditions. Examination of the products by NMR spectroscopy showed that polymer A was sulfated initially at position-3 of residue **3**, and that slower substitution occurred at position-3 of **6**. By contrast, polymer B exhibited low regioselectivity, as sulfation occurred with about equal facility at positions-2 and -3 of **7**, and -3 of **6**. The sulfation products had no significant anti Xa activity. Based on the paramagnetic effects of Cu^{2+} and chemical shift displacements induced by Ca^{2+}, NMR spectroscopy was used to compare cation-binding properties of A and B with those of heparin. In contrast to heparin, which forms a complex with Cu^{2+} detectable at a level of $< 10^{-3}$ mol per dimeric unit of the polymer, neither A nor B exhibited an interaction with the cation. However, polymer A was found to bind Ca^{2+}, in this respect being distinct from the related modification, **1** → **6**, which contains a 2-sulfate group in **1**, as well as from polymer B.

INTRODUCTION

The sulfate group of the α-L-idopyranosyluronic acid 2-sulfate residue (**1**) of heparin undergoes facile, base-catalyzed, displacement with retention of configuration at C-2, whereby the major disaccharide repeating unit (**1** → **2**) is converted into sequence **3** → **2**[1,2]. Sulfation of this modified heparin with sulfur trioxide–trimethylamine results[3] in regioselective substitution of OH-3 of **3**, accompanied by a slower reaction at OH-3 of **2**, to give another modified form of heparin, **4** → **5**.

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* Dedicated to Professor Jean Montreuil.

Although the 4-linked residue of α-L-idopyranosyluronic acid in dermatan sulfate appears to be structurally equivalent to uronic acid component **3** in the heparin series, it exhibits no such regioselectivity, because it forms both the 2- and 3-sulfates with equal ease[4]. We have suggested[4] that this striking difference may be caused by a screening of OH-2 of **3** by the neighboring amino sugar residue (**2**), and that the orientation of the corresponding amino sugar residue in dermatan sulfate is such as to leave both OH-2 and -3 relatively unhindered.

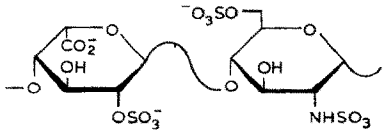
Some atypical ^{1}H and ^{13}C chemical shifts observed[5] for the *N*-acetyl analog of heparin (**1** → **6**) raised the possibility that the molecular conformation of this polymer differs from that of heparin itself. Whether a 2-acetamido group would, in turn, contribute to a change in the regioselectivity of sulfation of a polymer comprised of residues of **3** and **6** (polymer A) became a question worth pursuing. An additional incentive for undertaking this study was the finding[6] that *N*-acetyl analog **1** → **6** is unique among modified forms of heparin, and among other glycosaminoglycans, in binding cupric ion (Cu^{2+}) as strongly as heparin. Among species that do not bind this cation under comparable conditions[6] is modification **3** → **2**, which suggests a requirement for the 2-sulfate group of the uronic acid residue in heparin. Would this be true as well for modification **3** → **6**?

Also described here is a complementary study on sulfation and cation-binding characteristics of another related macromolecule, **7** → **6** (polymer B), in which the uronic acid component has the α-L-*galacto* (**7**), rather than the α-L-*ido*, configuration.

RESULTS AND DISCUSSION

Preparation and sulfation of polymer A (**3** → **6**).—Heparin from beef lung, although less readily-available than hog mucosal heparin, was used for the preparation of polymer A because of its more homogeneous structure[7]. First, the *N*-sulfate group of **2** was selectively hydrolysed[8] in 19:1 dimethyl sulfoxide–water, and the liberated amino group was *N*-acetylated to form residue **6**. Then the 2-sulfate group of uronic acid residue **1** was displaced, as described[1,2] for heparin itself, by lyophilization at pH 12.5, so that, overall, repeating sequences of **1** → **2** were converted into sequences of **3** → **6** (polymer A). NMR spectra of the product indicated that this interconversion had occurred to at least 80% judging, for example, from the group of major ^{1}H resonances in Fig. 1A, the identities of which were confirmed by a 2D COSY spectrum. As shown by the comparative data listed in Table I, the ^{1}H chemical shifts for residue **3** are generally close to those for the corresponding residue in modified heparin **3** → **2**, and also those of residue **6** are close to the chemical shift values for **6** in heparin analog **1** → **6**. This suggests that the conformational characteristics of the individual residues were not materially altered by the interchanging of neighboring constituents.

The sulfation of polymer A was carried out as described previously for modification **3** → **2**, by reaction of its pyridinium salt with sulfur trioxide–trimethylamine in



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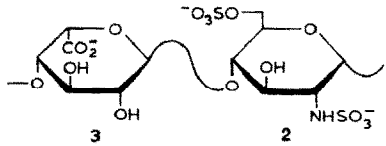
Heparin



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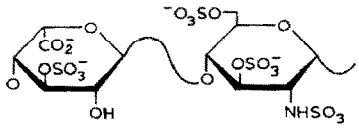

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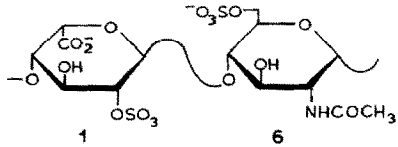

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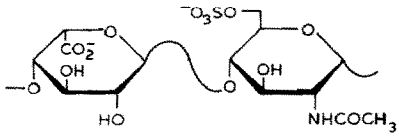

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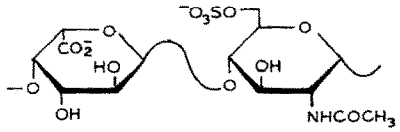
Polymer A



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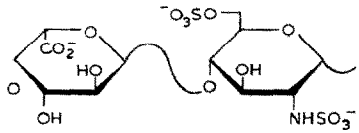
Polymer B



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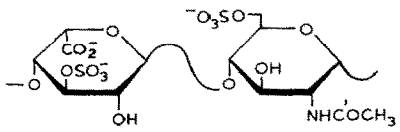

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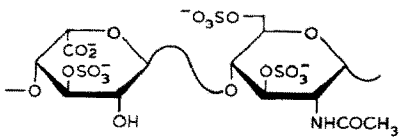

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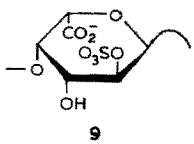

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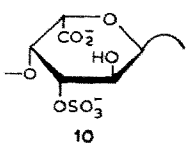

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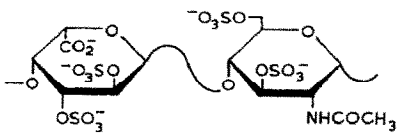

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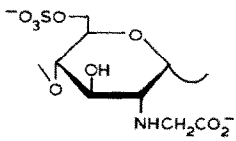

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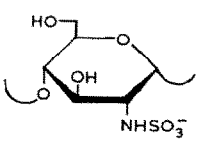

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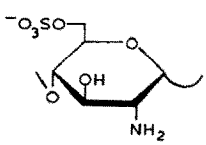

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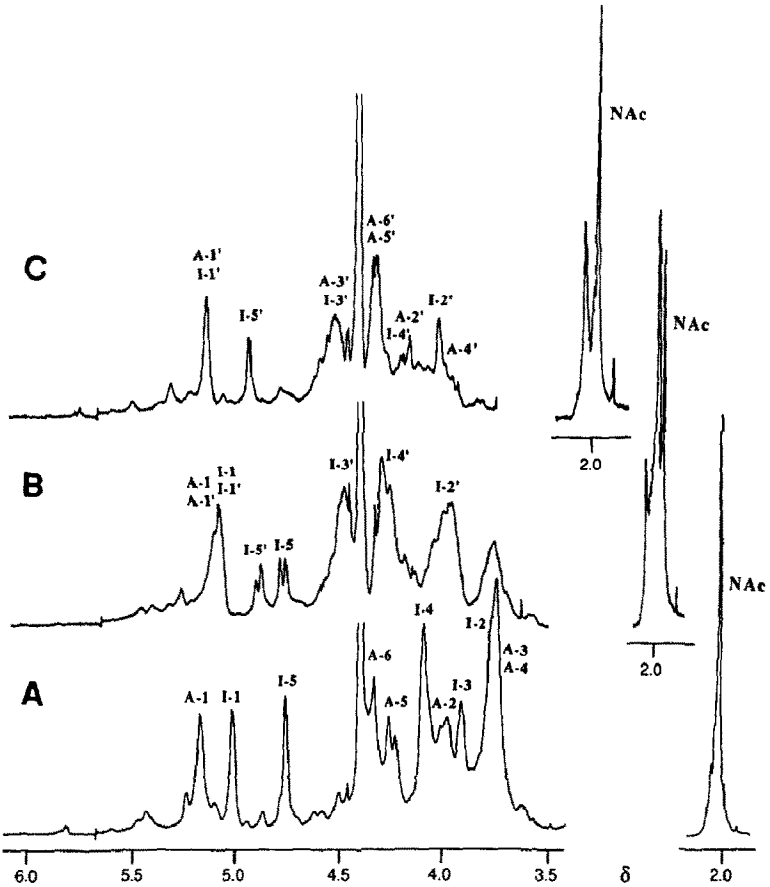

Fig. 1. ^{1}H NMR spectra (300 MHz), for solutions in D_2O at 65°, of: (A) polymer A; (B) the product of sulfation of polymer A at 15° for 5 h; and (C) the product of sulfation at 25° for 24 h. Signal designations: A, aminodeoxyhexose; I, uronic acid; primed signals (A-1′, etc.) are associated with the formation of sulfate substituents; NAc, acetamidomethyl (its relative intensity has been reduced by about one-half).

N,*N*-dimethylformamide, although it proved to be less reactive. Following treatment with the reagent at 0° for 6 h, conditions under which **3** → **2** had undergone partial substitution, polymer A was recovered intact. However, substantial sulfation occurred when the reaction temperature was raised to 15°. This is evident from the ^{1}H NMR spectrum of the product (Fig. 1B), which shows several marked departures from the chemical shift pattern in Fig. 1A, particularly for resonances of residue **3**.

With the aid of a COSY spectrum, it was found that a portion of the H-3 signal (I-3) of **3** had undergone a major downfield displacement of 0.6 ppm (Table II), from an original value of δ 3.88 to 4.48 (signal I-3′, Fig. 1B). This is commensurate only with the presence of a sulfate group at C-3. An accompanying change was a less pronounced deshielding of the I-2 and -4 resonances, by 0.2 ppm, as expected[3,4]

TABLE I

Comparison of ^{1}H chemical shifts (δ) [a] for individual residues of polymers A (**3** → **6**) and B (**7** → **6**) with those of other modified heparins

Residues compared	Polymer	Resonances				
		U-1	U-2	U-3	U-4	U-5
3	**3** → **2** [b]	5.0	3.8	4.1	4.1	4.8
	3 → **6**	5.0	3.7	3.9	4.1	4.7
7	**7** → **2**	5.2	3.9	4.0	4.4	4.6
	7 → **6**	5.1	3.9	4.0	4.4	4.6
		A-1	A-2	A-3	A-4	A-6
6	**1** → **6**	5.1	4.0	3.8	3.8	
	3 → **6**	5.1	4.0	3.7	3.7	4.3
6	**3** → **6**	5.1	4.0	3.7	3.7	4.3
	7 → **6**	5.3	3.8	3.7	3.7	4.2

[a] Of the uronic acid residues (U = I, L-*ido*; G = L-*galacto*), or the aminodeoxyhexose residues (A). [b] Data from ref. 1.

for substitution at position-3, but *not* -2. Consequently, this spectrum is evidence for the regioselective formation of residues of a 3-sulfate, as in **4** → **6**.

Particularly striking in this spectrum is the complexity displayed by the I-5 resonance, which is represented by 3–4 components over a 0.15 ppm range. Those centered at δ 4.75 are attributed to as yet-unsulfated residues of **3** and, accordingly, those at δ 4.88 to I-5′ of an approximately equal number of residues (**4**) that have undergone substitution. Probably, this intermediate level of sulfation is also the origin of the "doublet" structure of the *N*-acetyl signal (δ ~2.0). The

TABLE II

^{1}H chemical shift displacements ($\Delta\delta$) due to *O*-sulfate substituents introduced during the sulfation of polymers A and B [a]

Resonance [b]	Polymer A(δ) (**3** → **6**)	Product(δ) (**4** → **8**)	$\Delta\delta$	Polymer B (**7** → **6**)	Product (**11** → **8**)	$\Delta\delta$
U-1	4.97	5.10	+0.13	5.11	5.56	+0.45
U-2	3.72	3.99	+0.27	3.92	4.84	+0.92
U-3	3.88	4.48	+0.60	4.00	5.01	+1.01
U-4	4.06	4.25	+0.19	4.42	4.84	+0.142
U-5	4.71	4.90	+0.19	4.56	4.90	+0.34
A-1	5.13	5.10	−0.03	5.34	5.33	−0.01
A-2	3.95	4.13	+0.18	3.82	4.16	+0.34
A-3	3.73	4.48	+0.75	3.70	4.60	+0.90
A-4	3.73	4.09	+0.36	3.70	4.06	+0.36
A-6	4.30	4.30	0	4.18	4.30	+0.12

[a] The underlined, appropriately large, downfield shifts correspond to the positions of the sulfate groups formed. [b] Of the uronic acid residue 9U = **I**, L-*ido*; G = L-*galacto*), or the aminodeoxyhexose residue (A).

additional splittings in the I-5 signals are taken to be a reflection of long-range effects associated with mixed populations of sequences **3** → **6** and **4** → **6**.

The anomeric proton signals in Fig. 1B are slightly shifted in opposite directions, relative to their positions in Fig. 1A, so that they now overlap. For the I-1 signal, deshielding is consistent with the presence of the 3-sulfate group, although why two components of this signal are not observable, as for the I-5 signal, is unclear. Also unexpected is the *upfield* displacement of the A-1 signal, because if sulfation at C-3 of **6** had occurred, deshielding would be anticipated. In fact, evidence of ~25% substitution at OH-3 was provided by the ^{13}C NMR spectrum of the product, from the presence of an A-2 signal (δ 55.2) upfield of the main A-2 signal (δ 56.5). This information was not perceptible from Fig. 1B, nor its COSY counterpart. Similarly, the ^{13}C, but not the ^{1}H spectrum, suggested that ~10% sulfation had occurred at C-2 of **3**, because it contained a minor I-1 signal (δ 101.1) that could have arisen by an increase in the shielding of C-1 of **3** if a sulfate group had been created on the adjacent carbon atom.

Overall, then, the reaction between polymer A and sulfur trioxide–trimethylamine at 15° for 6 h had resulted in substitution mainly of OH-3 of **3**, to a level of ~0.5 sulfate groups per residue, and of OH-3 of **6** to a level of ~0.25 sulfate groups, whereas OH-2 of **3** appeared to have been far less affected than either of these.

Substitution at OH-3 of both residues was almost complete when the reaction was conducted at 25° for 24 h. According to Fig. 1C and its COSY counterpart, the product of this reaction was more homogeneous in constitution than that represented by Fig. 1B. Strong signals due to protons A-3′ and I-3′ now overlap downfield at δ ~4.5, which represents a deshielding of each nucleus by ~0.5 ppm (Table I). By contrast, a prominent upfield signal, identified as that of I-2 of **3**, showed directly that OH-2 had undergone relatively little substitution, and consistent with this also was ^{13}C spectral evidence based on the I-1 signal, such as cited above, indicating that few of the L-iduronic acid residues contained a 2-sulfate substituent. Consequently, the product obtained in this experiment was constituted mainly of residues of **4** and **8**.

These findings establish that OH-3 of the α-L-idopyranosyluronic acid residue (**3**) of polymer A is far more highly susceptible than OH-2 to sulfation with the sulfur trioxide–trimethylamine reagent. They are closely analogous, therefore, to the observations made[3] with modified heparin **3** → **2**, although the degree of selectivity is slightly higher in the present instance. Hence, if the replacement of the *N*-sulfate of residue **2** by an *N*-acetyl substituent has an effect on molecular conformation, as surmised at the outset, it is not in the direction of an increased exposure of OH-2 towards sulfation. The observations that OH-2 and -3 of the α-L-idopyranosyluronic acid residue in dermatan sulfate are substituted with equal facility indicates that regioselectivity in the heparin series is not attributable to a greater chemical reactivity of OH-3 than of OH-2. Indeed, the converse has sometimes been observed (e.g., see ref. 9) in base-catalyzed sulfation reactions of

carbohydrate hydroxyl groups. More likely, it appears that the 2-acetamido-D-glucopyranosyl residue (**6**), as well as its 2-deoxy-2-sulfoamino counterpart (**2**), are oriented with respect to OH-2 of **3**, so as to strongly retard the rate of reaction of the latter with sulfur trioxide–trimethylamine. Also in each instance, OH-3 of the amino sugar residues (**2** or **6**) undergoes a substantially slower reaction than OH-3 of **3**, perhaps because of greater crowding within the *e,e,e* orientation of the 2,3,4-ring substituents of these residues.

Preparation and sulfation of polymer B (**7** → **6**).—Also readily accessible from heparin is a modified form (**7** → **2**)[1,10] in which the main uronic acid unit is the α-L-*galacto* diastereomer (**7**). As both OH-2 and OH-3 of **7** in this polymer are sulfated with almost equal facility[3], it appeared worthwhile to prepare the *N*-acetyl analog in this series as well, i.e., **7** → **6** (polymer B), and examine its substitution pattern. If, as found with polymer A, the replacement of an *N*-sulfate by an *N*-acetyl group has relatively little effect on OH reactivity within the neighboring uronic acid units, then a low degree of regioselectivity was to be expected. This proved to be correct.

For the preparation of polymer B, *N*-desulfation of **7** → **2** was carried out with aqueous dimethyl sulfoxide, and the product was selectively *N*-acetylated. The polymer obtained had a *galacto*-to-*ido* ratio of ~ 5:1, judging from the relative intensities of the H-1 (G-1, I-1) and I-5 (G-5, I-5) signals in the spectrum reproduced in Fig. 2A. These, and the other, signal assignments were obtained with the aid of a ^1H–^1H correlation (COSY) spectrum.

NMR spectra of the products of reactions between polymer B (pyridinium salt) and sulfur trioxide–trimethylamine established that all of the free hydroxyl groups were substituted with relative ease. As seen in Fig. 2B (analyzed with the aid of its COSY counterpart), the product of the reaction at 15° for 6 h, gave partial signals that represent deshielding of many protons, notably G-1, -2, -3, and -4, and also A-3 and -4. Overall, the pattern of deshielding indicates that OH-2 and -3 of residue **7** and OH-3 of residue **6** had undergone sulfation (see Table II). For example, the chemical shift of the minor anomeric proton (G-1′) signal, at δ 5.55, represents a 0.4 ppm downfield displacement, which can best be accounted for by the formation of a sulfate group at C-2 (as in **9**). Similarly, two strongly deshielded G-3 component signals (G-3′) are found at δ 5.01 and 4.70, respectively, as compared with a value of δ 4.00 for G-3 of **7** (see Fig. 2A and Table II), which is probably indicative of the presence of 2,3-(**11**) and 3-(**10**) substituted forms of **7** in the reaction product. That a 3-sulfate derivative of residue **6** (i.e., **8**) had been produced, is evidenced by the signal (A-3′) at δ 4.60, which accounts for a major proportion of the A-3 protons in the polymer. Although this spectrum did not afford satisfactory integral values, visual examination of the relative intensities of the appropriate signals suggests that the order of reactivity of the three hydroxyl groups of polymer B was OH-3 (of **6**) > OH-3 (of **7**) > OH-2 (of **7**).

When polymer B was subjected to a reaction temperature of 25°, for 24 h, it was almost fully sulfated, according to the ^1H and ^{13}C NMR spectra of the product.

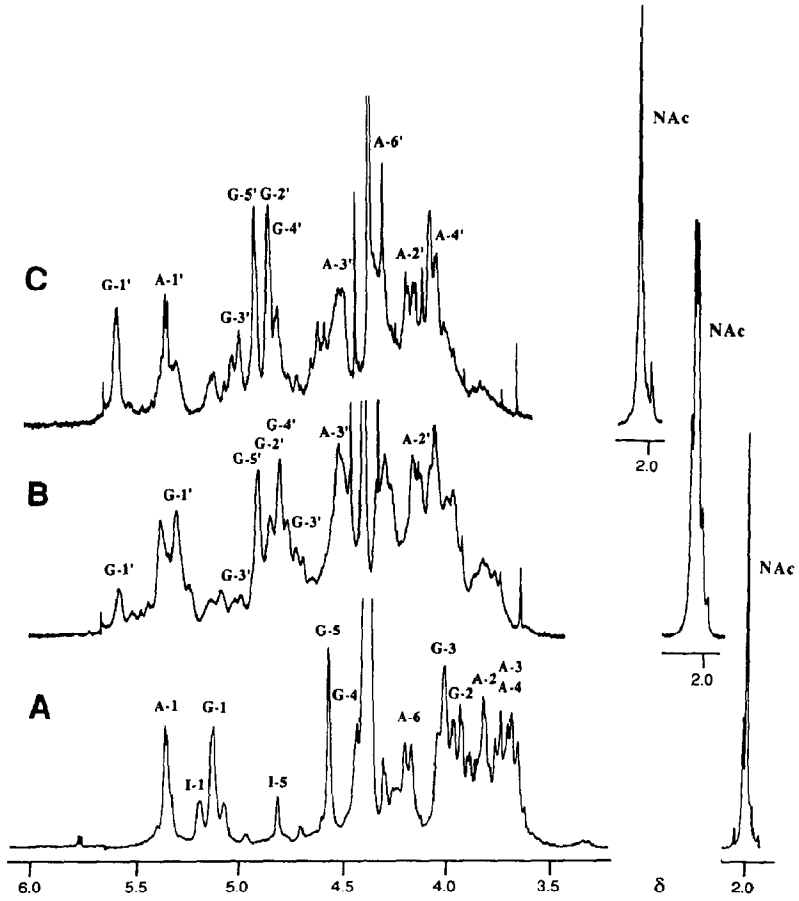

Fig. 2. ^{1}H NMR spectra (300 MHz), for solutions in D_2O at 65°, of: (A) polymer B; (B) the product of sulfation of polymer B at 15° for 5 h; and (C) the product of sulfation at 25° for 24 h. Signal designations: A, aminodeoxyhexose; I, uronic acid; primed signals (A-1′, etc.) are associated with the formation of sulfate substituents; NAc, acetamidomethyl (its relative intensity has been reduced by about one-half).

The latter spectrum provided good evidence of this, because it consisted essentially of a group of signals that individually accounted for 12 of the 14 ^{13}C nuclei required by a major repeating sequence, such as **11** → **8**. In the ^{1}H NMR spectrum (Fig. 2C), the anomeric proton signal (G′-1, δ 5.56) is relatively far stronger than in Fig. 2B, evidence of a large increase in the level of substitution at C-2. Similarly, signal G′-3, attributable to the 2,3-disulfate (**11**), is now more prominantly displayed at δ 5.00. An examination of the chemical shift data for all of the major, assigned signals (Table III), which records particularly large downfield displacements for H-2 and -3 of the uronic acid residue (now residue **11**), and for H-3 of the amino sugar residue (now residue **8**) confirmed that all three positions were affected in the sulfation process.

TABLE III

Comparative selectivity in the binding of cations by heparin and modified heparins.

Polymer	Binding affinity [a] for		Anionic charges/oligosaccharide unit
	Cu^{2+}	Ca^{2+}	
1 → **2** (Heparin)	+	+	4
3 → **2**	−	+	3
7 → **2**	−	+	3
7 → **2**	−	+	3
1 → **6**	+	−	3
3 → **6** (Polymer A)	−	+	2
7 → **6** (Polymer B)	−	−	2
1 → **12**	−	+	4
3 → **12**	−	+	3
3 → **13**	−	+	2
3 → **14**	−	−	3
11 → **8**	−	+	5

[a] Monitored by ^1H NMR spectroscopy at 10^{-3} mol of Cu^{2+}, or 2 mol of Ca^{2+}/repeating unit.

Clearly then, the sulfation of polymer B involved a much lower degree of regioselectivity than observed for polymer A, which is analogous to the results obtained[3] with the related polymer, **7** → **2**. It is also worth noting that the most highly sulfated forms of A and B exhibited virtually no anticoagulant (anti Xa) activity.

Observations on cation-binding.—An interaction between Cu^{2+} and heparin at a concentration of $\sim 10^{-3}$ mol per disaccharide unit (**1** → **2**), is readily detectable[5] as a marked broadening of several heparin ^1H and ^{13}C signals (notably I-1 and I-5) due to the paramagnetic effect of the cation on the nuclear relaxation times. Of an array of other molecules tested[5,6] by ^1H NMR spectroscopy, only the *N*-acetyl analog of heparin (**1** → **6**) responds in the same fashion. Although this indicates that the *N*-sulfate group of **2** is not essential for binding, the 2-sulfate group of residue **1** of heparin appears to be, because polymer **3** → **2** exhibited[5] no interaction with Cu^{2+}. In polymer A, residue **3** had been combined with *N*-acetylated residue **6** as an additional variation in structure, and the fact that its NMR spectrum (Fig. 2A) also proved to be unaffected by Cu^{2+}, again suggested that the 2-sulfated uronic acid residue (**1**) is required for binding. Nevertheless, the presence of this residue in a heparin analog need not ensure binding, because another modification, **1** → **12**, in which the *N*-substituent was carboxymethyl, exhibited no interaction with Cu^{2+}. In this overall context, not surprisingly, polymer B was found to be inactive as well.

Table III contains a summary of the various modified anionic polymers prepared from heparin, and their response towards Cu^{2+}. Included in Table III are corresponding data for the binding of calcium ion (Ca^{2+}), which induces ^1H chemical shift displacements[11]. The most prominent of the latter involve deshielding of the H-1 and -5 protons of the uronic acid units. Accordingly, in the presence

of 2 mol of Ca^{2+} per disaccharide unit, the I-1 and I-5 signals of polymer A were shifted downfield by 24 and 18 Hz, respectively; (for comparison, the corresponding values for heparin are[6] 29 and 25 Hz). This finding contrasts notably with evidence[12] that the *N*-acetyl analog (**1** → **6**) of heparin does not bind Ca^{2+}, despite its having an extra sulfate substituent. That is, the higher total anionic charge density of **1** → **6** than for polymer A would be expected[13,14] to be more favorable for binding of Ca^{2+}. As Table III shows, however, binding does occur over a wide range of anionic charge, perhaps because such other factors as intercharge distances are also critical[5,14].

Polymer B provides a second example among the group of a molecule that does *not* bind Ca^{2+}. Although it contains only two anionic charges, both polymer A and a modification[15] comprised of residues **3** and **13** are equally deficient in this respect, and yet they *do* interact with Ca^{2+}. Nevertheless, by converting polymer B into a more highly sulfated species, i.e., **11** → **8**, an ability to bind Ca^{2+} was acquired: this was determined from the observation that the G′-1 and G′-5 signals of **11** (Fig. 2C) were displaced downfield by 18 and 16 Hz, respectively, upon the introduction of Ca^{2+}. A possible factor in this instance is the greater anionic charge density (a total of five) of this polymer.

As already noted, polymer B was found not to bind Cu^{2+}, based on the absence of any change induced in its ^1H NMR spectrum (Fig. 2A) by the cation. It is noteworthy, however, that this negative response towards Cu^{2+} included the minor signals present that are attributable to chemically unmodified residues of **1**. That

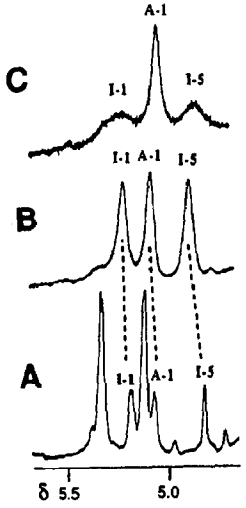

Fig. 3. Partial ^1H NMR spectra (300 MHz) for solutions in D_2O at 65° of: (A) polymer B, emphasizing the presence of minor signals due to unreacted polymer **1** → **6**; (B) intact polymer **1** → **6**, showing the signals for I-1, A-1, and I-5; and (C) sample as in spectrum (B), following the introduction of 2×10^{-3} mol of Cu^{2+}/disaccharide unit. N.B. The addition of Cu^{2+} to the solution represented by (A) induced no change.

is, although these residues represent the survival of a few intact sequences of **1** → **6**, their I-1 and I-5 signals were unaffected by the Cu^{2+}. As seen in Figs. 3A and 3B, respectively, there is a close correspondence in chemical shift between these signals (and also the minor A-1 signal associated with them) and those of the parent polymer, which emphasizes their structural relationship. Their contrasting behavior in terms of Cu^{2+} binding, however, is striking; whereas the signals in Fig. 3A remained unchanged despite the presence of Cu^{2+} in the sample, the I-1 and I-5 peaks in Fig. 3B were severely broadened by the cations presence (Fig. 3C). One possibility suggested by these findings is that, for the binding of Cu^{2+} to occur, the anionic groups involved must not only have appropriate interchange distances, but also cover a substantial segment of the macromolecular surface (we assume that the sequences of **1** → **6** are broadly dispersed, rather than that intact, unreacted molecules of **1** → **6** remain).

EXPERIMENTAL

Materials and general methods.—Beef lung heparin was furnished by the Upjohn Company of Canada. The NMR spectra were recorded with a Varian XL300 spectrometer operating at 300 MHz for ^1H and 75.4 MHz for ^{13}C. The 2D (^1H–^1H) COSY experiments were performed with the Varian instrument pulse sequences and the data were utilized for verifying most of the assignments listed. Chemical shifts are referenced to the signal of internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (δ 0.0). All samples (as the sodium salts) were treated with D_2O by repeated addition and evaporation of their solutions prior to NMR analysis, and these ^1H-exchanged products were dissolved in D_2O to give solutions containing 4% (w/w) of polysaccharide for ^1H NMR, and 10–20% (w/w) for ^{13}C NMR spectroscopy. In the cation-binding experiments, the appropriate quantity (generally 2–10 μL) of either $CuSO_4 \cdot 5H_2O$ or $CaCl_2 \cdot 2H_2O$ of the required molarity in D_2O, was introduced into the NMR tube.

Preparation of polymer A.—Beef lung heparin (150 mg) in water (10 mL) was converted into the acid form by ion exchange, and then into the pyridinium salt by neutralization with pyridine, followed by lyophilization. For selective hydrolysis[8] of the 2-*N*-sulfate group, the residue was dissolved in Me_2SO (10 mL) containing 5% of water, the solution was stirred at 55° for 1.5 h, diluted with an equal volume of water, and its pH adjusted to 9.5 with 0.1 M NaOH. Following dialyzis against distilled water for 24 h, the product was recovered by lyophilization (yield, 103 mg). *N*-Acetylation was then carried out, as recently described[5], to give polymer **1** → **6**, (yield, 93 mg). A solution of this product (40 mg) in water (10 mL) was adjusted to pH 12.5 with 0.1 M NaOH, and then lyophilized to effect[1,2] displacement of the 2-sulfate group of **1**, affording polymer A as a pale yellow powder, which was characterized by ^1H and ^{13}C NMR spectroscopy (for ^1H data, see Fig. 1A and Table I); ^{13}C NMR: δ 178.4 ($NCOCH_3$), 177.7 (CO_2), 104.9 (I-1), 97.5 (A-1), 80.4 (A-4), 77.1 (I-4), 72.8-71.6 (I-2, -3, -5, A-3), 68.8 (A-6), 56.5 (A-2), and 24.8 (CH_3).

Sulfation of polymer A.—The experiments were carried out under conditions closely analogous to those described[3] for the sulfation of polymer **3** → **2**, at 0° for 6 h, 15° for 6 h, and 25° for 24 h. A representative sulfation reaction is the following. Polymer A (100 mg) was converted into the pyridinium salt, which was dispersed in dry *N*,*N*-dimethylformamide (20 mL). Sulfur trioxide–trimethylamine complex (1.2 g) was introduced, and the mixture was stirred at 15° for 6 h. Water (60 mL) was added, the solution was dialyzed against 0.3% $NaHCO_3$ for 2 h, and then against distilled water for 48 h, passed through a column of Chelex ion-exchange resin (Na^+), and lyophilized (yield, 80 mg); for ^1H NMR, see Fig. 1B.

Preparation of polymer B.—A solution of beef lung heparin (0.33 g) and Na_2CO_3 (0.15 g) in water (16 mL) was heated under reflux for 24 h, for conversion of residue **1** into **7**, as described elsewhere[10]. Employing the same conditions as used in the preparation of polymer A, the pyridinium salt (200 mg) of the product was subjected to selective hydrolysis for removal of the 2-sulfate group, followed by selective *N*-acetylation, to give polymer B (yield, 180 mg); for ^1H NMR, see Fig. 2A.

Sulfation of polymer B.—These experiments were carried out under the same conditions as described for polymer A. The products recovered from the sulfation reactions at 15° for 6 h, and at 25° for 24 h, are represented by the ^1H NMR spectra shown in Figs. 2B and 2C.

Measurement of anticoagulant potency.—Tests for anti Xa activity were performed by a chromogenic assay using a "Spectrolyse Heparin" kit (Interhematol Inc., Hamilton, ON).

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