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Sulfation of some chemically-modified heparins. Formation of a 3-sulfate analog of heparin

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ABSTRACT

A modified form of heparin containing residues of nonsulfated α-L-idopyranosyluronic acid (**7**) in place of the normal 2-sulfate (**1**) was sulfated with sulfur trioxide–trimethylamine in dimethylformamide at 0 and 25°. Examination of the reaction products by n.m.r. spectroscopy showed that sulfation occurred selectively at C-3 of residue **7**, to give a new polymer that may be described as a 3-sulfate analog of heparin. A slower substitution reaction led subsequently to sulfation at C-3 of 2-deoxy-2-sulfamino-α-D-glucopyranosyl 6-sulfate residues (**2**), although this was accompanied by partial *N*-desulfation of **2**. An analogous pattern of *O*-sulfation–*N*-desulfation was observed for the residues of **2** in two other modified heparins, one containing residues of 2,3-anhydro-α-L-gulopyranosyluronic acid and the other residues of α-L-galactopyranosyluronic acid, in place of residues of **1**. The *galacto* diastereomer exhibited relatively low regioselectivity, as it was found to be sulfated at C-2 or C-2,3, or both. Selective resulfation of free amino groups gave the products that were examined for anticoagulant activity and susceptibility to enzymolysis by heparinase. Antithrombin-binding affinity measurements were also carried out. Although none of the materials had significant anti-Xa activity, nor were they affected by heparinase, their patterns of binding to antithrombin-agarose were not dissimilar to that of heparin.

INTRODUCTION

The anticoagulant activity of heparin is attributable to its intricate pattern of sulfate and carboxyl substituents, which impart this polymer with an unusually high charge density. Representative are the four anionic groups of the major disaccharide repeating unit, consisting[1] of α-L-idopyranosyluronic acid 2-sulfate (**1**) and 2-deoxy-2-sulfamino-α-D-glucopyranosyl 6-sulfate (**2**) residues. An additional type of sulfate group is found[2] at C-3 of the trisulfated aminodeoxyhexose residue **3** that occurs in the antithrombin-binding pentasaccharide sequence of heparin, and a 6-sulfate group is located[3] on some 2-acetamido-2-deoxy-α-D-glucopyranosyl residues (**4**).

Studies with modified heparins and various oligosaccharides indicated that each of these negative charges is significant in ensuring a full expression of the anticoagulant activity of heparin. For example, little or no activity is exhibited[4] following selective hydrolysis of the sulfamino group of **2**, or in the absence of either the 3-sulfate group of **3** (ref. 5) or, in some instances, the 6-sulfate group of **2** (refs. 6 and 7). Consistent with these characteristics is complementary evidence that an increase in anionic character may enhance anticoagulant activity. This is illustrated by many examples[3] in which neutral polysaccharides have been converted through sulfation into anticoagulants

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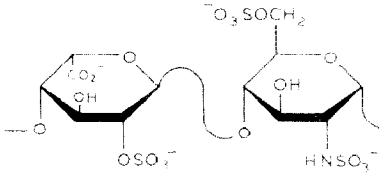
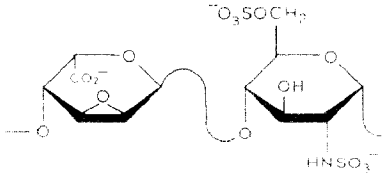
(often of relatively low activity). Similarly, such glycosaminoglycans as dermatan sulfate and heparan sulfate, which have lower charge densities and anticoagulant potencies than heparin, exhibit[8] enhanced U.S.P. activity following the introduction of additional sulfate groups.

In this context, it appeared worthwhile to undertake the sulfation of some modified forms[9,10] of heparin that retain little anticoagulant activity. In the formation of these novel polymers, uronic acid **1** of the heparin undergoes base-catalysed displacement of its 2-sulfate group and is converted into a 2,3-anhydro-α-L-gulopyranosyluronic acid residue (**5**, polymer A), and then into α-L-galactopyranosyluronic acid (**6**, polymer B) or α-L-idopyranosyluronic acid (**7**, polymer C) residues. For the polymer



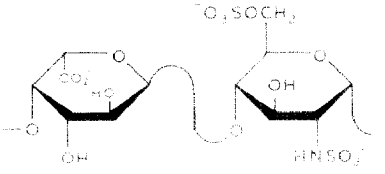
1 2 3 4

(Heparin)



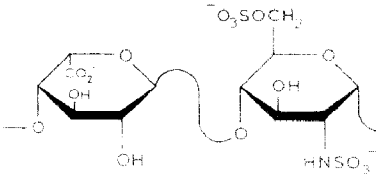
5 2

(Polymer A)



6 2

(Polymer B)

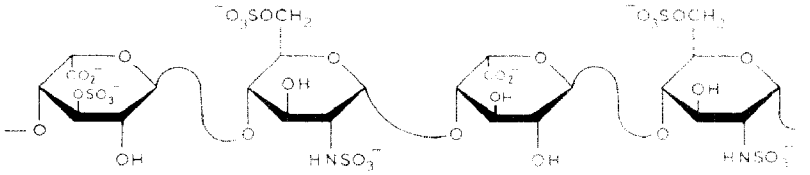


7 2

(Polymer C)

9 R = SO_3^-, R′ = H

10 R = R′ = SO_3^-



8 2 7 2

(Polymer D)

containing **7**, for example, selective substitution at OH-2 seemed to be a good possibility, according to a recent study[11] on the sulfation of a fully-desulfated heparin. This would amount to the reconstitution of an active heparin molecule and allow, *e.g.*, for site-specific labelling with ^{35}S for tracer applications, or ^{33}S for n.m.r. studies. As well, the introduction of sulfate at C-3 of residue **2** in any of these polymers, by increasing the content of trisulfated **3**, might generate antithrombin-mediated anti-Xa activity corresponding to that displayed[7] by a synthetic pentasaccharide containing *two* residues of **3**.

The present article describes the sulfation of the three modified heparins (polymers A, B, and C) under various experimental conditions, and the characterization of their sulfation products by n.m.r. spectroscopy, as well as by tests of their anticoagulant properties and susceptibility towards heparinase.

RESULTS AND DISCUSSION

Sulfation of polymer C. — Prior to sulfation, each of the polymers was converted into the pyridinium salt, to enhance its solubility in *N,N*-dimethylformamide used as the reaction medium. The sulfating reagent was sulfur trioxide–trimethylamine, and reactions were carried out at either 0 or 25°.

The product obtained by sulfation of polymer C at 0° for 6 h afforded a complex ^1H-n.m.r. spectrum which, at pD ~9.5, contained a prominent upfield signal (δ 2.8) attributable to a proton in position 1,2 to an amino group. It indicated that, unexpectedly, about 30% *N*-desulfation of residue **2** had occurred. To minimize structural heterogeneity in this product, selective *N*-sulfation was effected with the sulfur trioxide–trimethylamine reagent in aqueous sodium carbonate. The ^1H-n.m.r. spectrum of the neutral product isolated (Fig. 1B; at pD 9.5, the spectrum now contained no H-2 resonance due to free amine) differed in several respects from that of polymer C itself (Fig. 1A). As there are at least two I-1 (δ 5.0–5.1) and I-5 (δ 4.8–4.9) signals in Fig. 1B, sulfation of L-idopyranosyluronic acid residue **7** had taken place either incompletely or nonselectively (or both). The ^1H–^1H correlation (COSY) spectrum included in Fig. 1B is consistent with partial sulfation at only one position, based on the identification of cross-peaks for two I-2 and two I-3 signals. Of the latter, one is located far downfield (δ 4.7) of the second, which is attributable to the I-3 resonance of **7** (δ 4.1). One of the I-2 signals is also coincident with the I-2 signal of **7**, whereas the second (at δ 4.0) is displaced ~0.2 p.p.m. downfield.

In the tetrasaccharide sequence proposed, accordingly, to represent the product (polymer D) of sulfation of polymer C, some residues (**7**) remain unaffected, whereas others (**8**) bear a 3-sulfate group. The latter group accounts for the 0.6 p.p.m. deshielding difference between the two I-3 nuclei, and the moderate deshielding (by 0.2 p.p.m.) of proton I-2 of **8** by the neighboring 3-sulfate group. According to the relative intensity of signal I-3 at δ 4.7, the ratio of **7**:**8** in the polymeric product is ~2:3. Also noteworthy is the apparent absence of an I-2 cross-peak which would be indicative of a 2-sulfate group on an L-idopyranosyluronic acid residue (I-2 of residue **1** resonates at δ 4.4), nor is there evidence of appreciable sulfation elsewhere in polymer D.

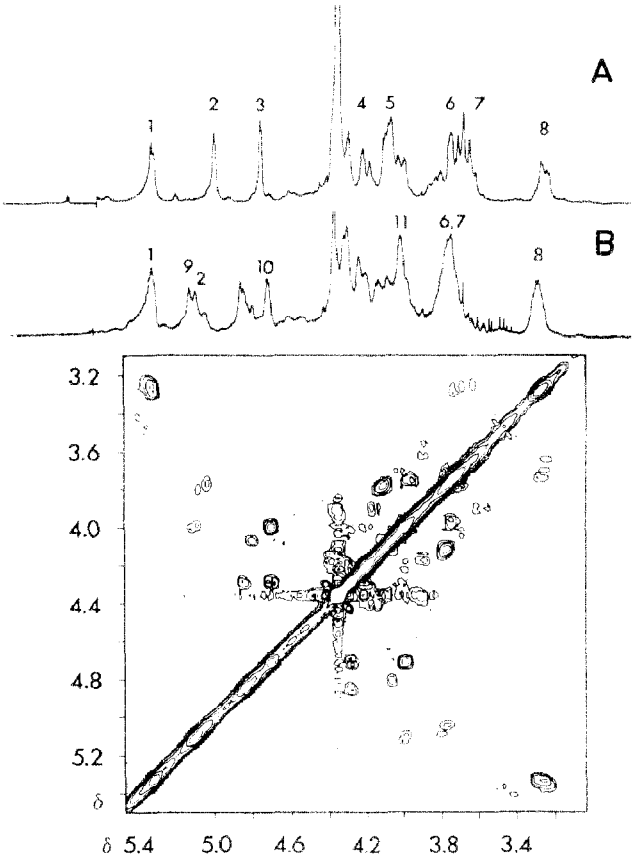


Fig. 1. ^{1}H-N.m.r. spectra (300 MHz), for solutions in D_2O at 65°, of: (A) Polymer C and (B) the product of sulfation of polymer C at 0° for 6 h, including (below) the corresponding COSY spectrum. Signal designations: 1, A-1; 2, I-1; 3, I-5; 4, A-6; 5, I-3; 6, I-2; 7, A-3,4; 8, A-2; 9, I'-1; 10, I'-3; and 11, I'-2. Structures: A, **2**; I, **7**; and I', **8**.

Under the conditions employed, therefore, the sulfation of modified heparin C led to selective substitution at OH-3 of the L-idopyranosyluronic acid residue (**7**), and hence the product represents a new, 3-sulfate analog (partially sulfated) of heparin, represented by the polymer D sequence.

When sulfation of polymer C was conducted at 25° for 2.5 h and 18 h, respectively, substitution at OH-3 of the aminodeoxyhexosyl residue **2** was also observed. Some hydrolysis of the sulfamino group occurred as well during these reactions and, again, was compensated for by selective *N*-sulfation. The ^{1}H-n.m.r. spectra of the two products (Figs. 2A and 2B) showed increasing complexity as the degree of substitution and level of structural inhomogeneity increased, and they must represent a large array of di-, tetra, etc. sequences. Evidence for the formation of 3-sulfate residues (**3**) was provided by signals at δ 4.55 and 3.50, attributable to resonances A-3 and A-2, respectively (by reference to the COSY spectra). Whereas neither of these signals was detected in Fig. 1B, both are relatively more prominent in Fig. 2A than 2B, as expected for a series of increasingly vigorous sulfation reactions.

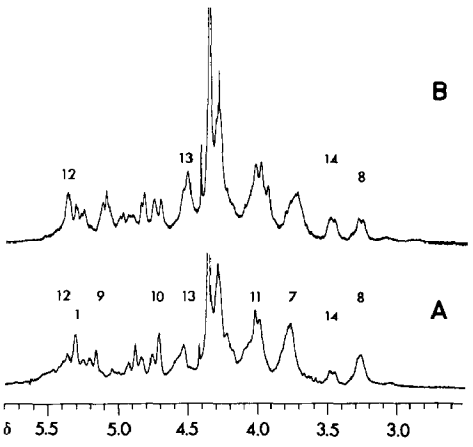


Fig. 2. ^{1}H-N.m.r. spectra (300 MHz), for solutions in D_2O at 65°, of: (A) The product of sulfation of polymer C at 25° for 2.5 h, and (B) the product obtained after 18 h at 25°. Signal designations: 1, A-1; 7, A-3,4; 8, A-2; 9, I'1; 10, I'-3; 11, I'-2; 12, A'1; 13, A'-3; and 14, A'-2. Structures: A, **2**; I', **8**; and A', **3**.

At least one prominent signal at δ 4.7–4.8 in Figs. 2A and 2B was determined (from the COSY spectra) to be that of I-3 showing, as anticipated, that residues of α-L-idopyranosyluronic acid 3-sulfate (**8**) are present in these polymers. Also found in these COSY spectra (but not that in Fig. 1B) was a cross-peak at δ 4.2 that must be attributed to an I-2 signal. This indicated, therefore, that at higher levels of sulfation, substitution also takes place at OH-2 of some (undetermined number of) L-idopyranosyluronic acid residues, either in addition to I-3 to give a 2,3-disulfate, or with the regeneration of residues of L-idopyranosyluronic acid 2-sulfate (**1**), as in the original heparin.

Our results with polymer C differ from those[11] reporting that the L-idopyranosyluronic acid residues of an extensively desulfated heparin, when subjected to resulfation, were more readily substituted at C-2 than C-3. As our reaction conditions were slightly different from those used by the earlier workers, we applied theirs to polymer C. Accordingly, the tributylammonium (rather than pyridinium) salt was prepared, and treated with sulfur trioxide–pyridine (rather than sulfur trioxide–trimethylamine) at room temperature for 2.5 h. The product of this reaction was found, as in our previous experiments, to have undergone partial *N*-desulfation and, after selective *N*-resulfation, to give ^{1}H- and ^{13}C-n.m.r. spectra closely resembling those obtained previously. Consequently, the two sets of reaction conditions appear, in fact, to give analogous results, *i.e.*, selective formation of the 3-sulfate derivative of L-idopyranosyluronic acid residues.

Another difference between the two studies was the presence of a 6-sulfate group on **2** in polymer C, in contrast to a polymer[11] with no sulfate at C-6. However, as the latter was shown[11] to be fully sulfated prior to substitution elsewhere, subsequent sulfation would have involved, essentially, polymer C, and rendered the two sets of experiments closely comparable.

^{13}C-N.m.r. spectra of the products of sulfation of polymer C. — The ^{13}C-n.m.r. spectra of these products are also worthy of comment, especially with respect to the formation of residues of trisulfate **3**. Owing to the great complexity of the spectra, few signal assignments were obtained for the secondary carbon atoms [despite attempts at correlation (HETCOR) with the ^{1}H-n.m.r. data available]. Nevertheless, reference to the distinctive downfield signals for the anomeric carbon atoms and those upfield due to ^{13}C-2 (A-2) in position 1,2 to the sulfamino group provided strong support for the conclusions based on the ^{1}H-n.m.r. data.

With the selective formation at 0° of residues of L-idopyranosyluronic acid 3-sulfate (**8**), the I-1 and A-1 signals in the partial spectrum in Fig. 3B both reflect nonequivalence, as compared with those for polymer C (Fig. 3A), although the A-2 resonance remains unchanged. Then, as a result of sulfation of the aminodeoxyhexosyl residue **2**, a new A-2 signal appeared slightly upfield at δ 59 (Figs. 3C and 3D), characteristic[12] of the A-2 resonance for residue **3** in heparin and related oligosaccharides. As with the corresponding ^{1}H-n.m.r. signals, its relative intensity increased as the degree of substitution would be expected to increase. (The minor peak at δ 57 in Fig. 3D reflects the presence, in this particular specimen, of a small proportion of the amine obtained prior to the selective *N*-sulfation mentioned earlier.)

Commensurate with the complexity observed for the (undisplayed) δ 65–85 region of these ^{13}C-n.m.r. spectra, is the multiplicity of peaks found in Figs. 3C and 3D, for the anomeric carbon atoms. Unexpected, however, was the appearance of the cluster at δ 101 due, presumably, to A-1. This suggested that the introduction of the 3-sulfate group to produce **3** induced a 2.5-p.p.m. deshielding change in the anomeric ^{13}C-n.m.r. signal, a large effect for which there appears to be no precedent.

Sulfation of polymer A. — In this instance, only OH-3 of the aminodeoxyhexose residue **2**, linked to the 2,3-anhydrido residue **5**, was available for substitution. When

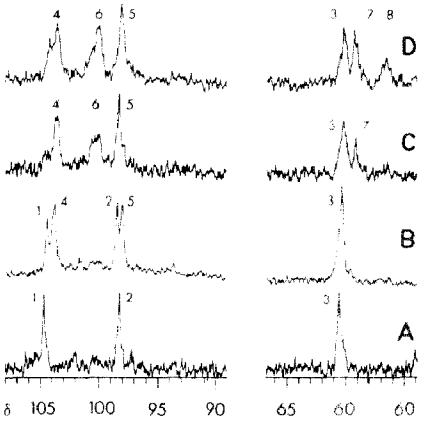


Fig. 3. Partial ^{13}C-n.m.r. spectra (75.4 MHz), for solutions in D_2O at 25°, of: (A) Polymer C; (B), (C), and (D), products of the sulfation of polymer C at 0° for 6 h, 25° for 2.5 h, and 25° for 18 h, respectively. Signal designations: I, I-1; 2, A-1; 3, A-2; 4, I'1; 5, A'-1; 6, A''-1; 7, A''-2; and 8, A'''-2. Structures: I, 7; A, 2; A', **2** linked to **8**; A'', **3**; and A''', free amine.

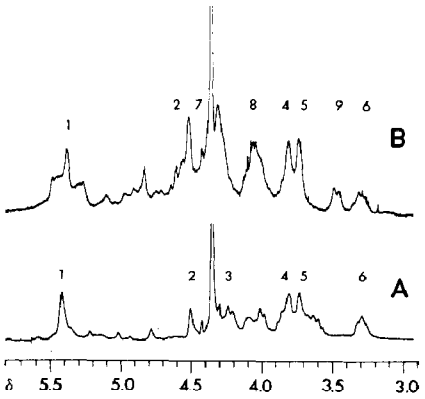


Fig. 4. ^{1}H-N.m.r. spectra (300 MHz), for solutions in D_2O at 65°, of: (A) Polymer A, and (B) the product of sulfation of polymer A at 25° for 20 h. Signal designations: 1, U-1 and A-1; 2, U-4; 3, A-6; 4, U-3; 5, U-2; 6, A-2; 7, A′-3; 8, A′-4; and 9, A′-2. Structures: U, **5**; A, **2**; and A′, **3**.

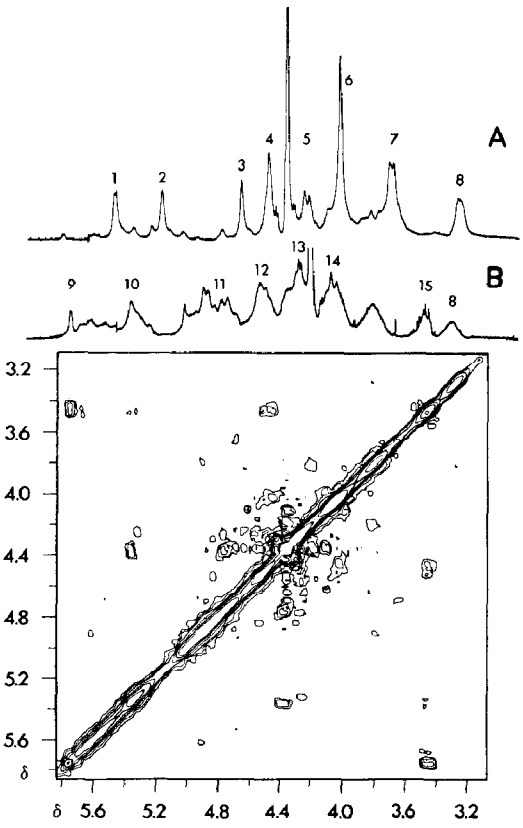


Fig. 5. ^{1}H-N.m.r. spectra (300 MHz), for solutions in D_2O at 65°, of: (A) Polymer B, and (B) the product of sulfation of polymer B at 25° for 5 h, including (below) the corresponding COSY spectrum. Signal designations: 1, A-1; 2, G-1; 3, G-5; 4, G-4; 5, A-6; 6, G-2,3; 7, A-3,4; 8, A-2; 9, A′-1; 10, G′-1; 11, G′3; 12, A′-3; 13, G′-2; 14, A′-4; and 15, A′-2. Structures: A, **2**; G, **6**; A′, **3**; and G′, **10**.

the polymer was subjected to sulfation at 25° for 2.5 h and 20 h, respectively, the results corresponded closely to those found for residue **2** of polymer C. According to the ^{1}H-n.m.r. spectrum in Fig. 4B (which may be compared with that in Fig. 4A of polymer A), about 0.5 sulfate group per residue had been introduced during 20 h, inasmuch as the two A-2 signals (δ 3.50 and 3.35, respectively) were of about equal intensity. This was confirmed by the presence in the ^{13}C-n.m.r. spectrum of the two corresponding A-2 signals (δ 60 and 59), analogous to those shown in Fig. 3D. Consequently, about one-half of this sulfation product (and a lesser proportion in the 2.5-h reaction) consisted of a disaccharide sequence of residues **3** and **5**.

As observed in the sulfation of polymer C, some *N*-desulfation also occurred in these reactions. It is noteworthy, therefore, that although the reaction medium was sufficiently acidic to cleave the sulfamino group of **2**, it had no apparent effect on the 2,3-anhydro ring, because the oxirane ^{1}H-n.m.r. signals (at δ 3.7–3.8, Figs. 4A and 4B) and ^{13}C-n.m.r. signals (δ 53–54) remained intact. Consequently, these experiments showed not only that residues **2** of polymers A and C are sulfated with equal facility, but they also suggested that the sulfation conditions are not likely to have involved appreciable degradation of the polymer, despite the *N*-desulfation observed.

Sulfation of polymer B. — The product of sulfation of this polymer for 5 h at 25° showed a markedly different ^{1}H-n.m.r. spectrum (Fig. 5B) from that of the starting material (Fig. 5A). (Once again, the partially *N*-desulfated initial product was *N*-resulfated). That substantial substitution of OH-3 of the aminodeoxyhexosyl residue **2** had taken place was evident from the prominent A-2 signal at δ 3.45 in Fig. 5B, in addition to that of **2**, as well as the complementary pair of A-2 resonances in the ^{13}C-n.m.r. spectrum of the product.

Despite the complexity of the spectrum in Fig. 5B, the presence of the required A-3 signal was confirmed (at δ 4.55) by its off-diagonal resonance in the COSY spectrum shown. Particularly noteworthy in the latter are two cross-peaks (at δ 4.4 and 4.3) that correlate glycosyluronic acid H-1 signals (at δ 5.40 and 5.35, respectively) with signals attributable to H-2 of that residue. This indicated that OH-2 of the L-galactopyranosyluronic acid residue (**6**) had been substituted, giving **9**, thus accounting for the downfield displacement of the signal of H-2 from a value of δ 4.0 (Fig. 5A) to δ 4.3 (Fig. 5B). In addition, the presence of a slightly more deshielded H-2 nucleus (resonance at δ 4.4) suggested that the product also contains residues of L-galactopyranosyluronic 2,3-disulfate (**10**); the resonance for H-3 in position 1,2 to a sulfate group appears to be at δ ~5.0.

Therefore, in contrast to the selectivity for substitution of OH-3 in an α-L-idopyranosyluronic acid residue, both OH-2 and OH-3 in residues of α-L-galactopyranosyluronic acid are converted into sulfate groups. This difference is consistent, to some extent, with recent observations[13] on the substitution of OH-2 and OH-3 of aldohexopyranose derivatives by phenyl chlorosulfate. Thus, methyl 4,6-*O*-benzylidene-α-D-galactopyranoside is susceptible to substitution at both OH-2 and OH-3, as found here for the 4-substituted α-L-*galacto* residue in polymer B. However, comparative data for the α-*ido* diastereomer are not available.

TABLE I

Fractions (%) of heparin and modified heparins eluted from an antithrombin-agarose column by sodium chloride

Polymer[a]	*Fractions*[b]				*Anti-Xa activity (Units/mg)*
	1	*2*	*3*	*4*	
Heparin (mucosal)	42.4	47.2	4.3	6.1	150
Heparin (high affinity)	0	66.2	14.0	19.8	210[c]
Heparin (1:1 high:low affinity)	41.5	39.4	2.3	16.8	[d]
Modified heparin C	59.4	38.5	2.1	0	9
Sulfated C	45.8	43.3	5.4	6.5	16
Modified heparin A	40.2	44.6	8.7	2.4	26
Sulfated A	31.7	52.0	13.2	1.6	26

[a] Five mg. [b] Eluted at NaCl concentration of 0.01, 0.05, 0.10, and 2.0M, respectively. [c] USP activity (units/mg). [d] USP activity of the low-affinity fraction, 40 units/mg.

Anticoagulant properties of the products of sulfation of the modified heparins. — As already noted, polymers A, B, and C were virtually devoid of anticoagulant activity, measured by anti Xa and U.S.P. assays. In view of the considerations offered in the Introduction section, it was noteworthy to now find (Table I) that anti Xa assays carried out with several of the sulfation products showed little or no increase in activity. Among samples included as controls, which gave assays of the order expected (Table I), were heparin fractions known to have a high- and a low-affinity, respectively, for antithrombin III. Consequently, the results suggested that by increasing the content of trisulfated aminohexosyl residue **3** in a heparin-type polymer, without also ensuring that residues of α-L-idopyranosyluronic acid 2-sulfate are present and appropriately located, an enhanced interaction with antithrombin will not occur.

Supplementary to these observations were measurements on the antithrombin III-binding characteristics of the sulfation products, by means of affinity chromatography. A column containing antithrombin III linked to a matrix of agarose was used to compare the fractionation behavior of the modified heparins with respect to that after they were sulfated. By analogy with procedures reported by others, the elution of material from the column was effected at varying concentrations of sodium chloride (Table I). Although the commercially-supplied antithrombin-agarose was found to have a weaker binding capacity than the immobilized antithrombins described in the earlier studies, its elution profiles (Table I) generally paralleled the results given in those studies for heparin and fractions of heparin. This is shown by the differences among a standard mucosal heparin, a fraction having a high affinity for antithrombin, and a mixture of the latter with a low-affinity fraction, in the percent distribution of their

fractions eluted from the column. Under comparable conditions, sulfated polymer C exhibited an elution profile similar to that of heparin, despite its having virtually no anti-Factor Xa activity. Also evident is the fact that although the sulfation of polymer C altered its elution profile towards the higher affinity end, there was little effect on the binding of polymer A upon sulfation (nor on its anti-Xa activity). With these modified heparin polymers, therefore, there appears to be no relationship between their interactions with antithrombin as measured, respectively, by affinity-binding and anti-Xa activity.

Measurements of activated, partial thromboplastin time (aPTT) for two of the products of sulfation of polymer C (represented by Figs. 1B and 2B) gave clotting times of 108 and 125 s, respectively, as compared with a value of > 500 s for beef lung heparin (all at level of 10 μg/mL). These values for sulfated C correspond to appreciable levels of potency relative to, *e.g.*, a weakly anticoagulant[8] specimen of dermatan sulfate, for which clot formation was detected in only 31 s.

The finding that some *N*-desulfation occurred during the sulfation of the polymers suggested that, despite the precautions taken, our reaction conditions were not sufficiently anhydrous to prevent hydrolysis of the acid-labile[4] sulfamino group. Without subsequent *N*-resulfation, a reduction in anticoagulant potency might be expected[4]. Consequently, these observations may be related to reports[9,14] of reduced activity of heparin and heparan sulfate that had been subjected to sulfation under reaction conditions (that appear to have been) comparable to ours.

Tests for activity by heparinase. — It was noted earlier[10] that modified heparin polymers A, B, and C are not substrates for the heparinase of *Flavobacterium heparinum*, presumably because they lack residues of α-L-idopyranosyluronic acid 2-sulfate (**1**). Replacement of the latter, in sulfated polymer C, by residues of α-L-idopyranosyluronic acid 3-sulfate (**8**), proved to be ineffective in stimulating enzymolysis by the heparinase. The products of sulfation of polymers A and B were also unaffected by the enzyme.

EXPERIMENTAL

Materials and general methods. — The preparation of the modified heparin polymers A, B, and C, was described earlier[9]. The 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 ^1H-probe and a 5-mm broad-band probe, respectively, and are referenced to the signal of internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (δ 0.0). The 2D (^1H ^1H) COSY and (^{13}C ^1H) HETCOR experiments were performed with the Varian pulse sequences, and utilized for verifying most of the spectral assignments given. All samples (as the sodium salts) were treated with D_2O by repeated addition and evaporation of their solutions prior to n.m.r. analysis, and these ^1H-exchanged products were dissolved in D_2O to give solutions containing 4% (w/w) of polysaccharide for ^1H-n.m.r., and 10–20% (w/w) for ^{13}C-n.m.r. spectroscopy.

Sulfation of the modified heparins. — The experiments were carried out at either 0

or 25° for varying periods of time. A representative sulfation reaction[15] was the following:

Polymer C (100 mg) in water (10 mL) was converted into the acid form by ion exchange, and then into the pyridinium salt by neutralization of the solution with pyridine, followed by lyophilization. The solid residue was dispersed in dry *N,N*-dimethylformamide (20 mL), SO_3–trimethylamine complex (1.2 g) was introduced, and the mixture was stirred for 2.5 at 25°. Water (60 mL) was added, and the solution was dialyzed against 0.3% $NaHCO_3$ solution for 2 h, then against distilled water for 48 h, passed through a column of Chelex ion-exchange resin (Na^+), and lyophilized. Further purification of the product was effected by dissolving it in water (2 mL), introducing ethanol (5 vols.), and recovering the precipitated solid by lyophilization, and finally by centrifugation; yield 86 mg.

As the ^{1}H-n.m.r. spectrum of the product in D_2O at pD 9.5 showed that a free amino group had been formed (signal at δ 2.8, characteristic of H-2 in position 1,2 to an amino group), *N*-sulfation was carried out: The product was dissolved in water (8 mL), Na_2CO_3 (60 mg) and SO_3–trimethylamine complex (60 mg) were added, and the solution was heated at 50–55° for 20 h. Acetic acid was introduced, and the neutral solution was dialyzed against running water for 20 h, treated with Chelex ion-exchange resin (Na^+), and lyophilized. The solid product (46 mg), which no longer produced the signal for free amine at δ 2.8 in D_2O at pD 9.5, gave the ^{1}H-n.m.r. spectrum shown in Fig. 2A.

Antithrombin-binding measurements. — The procedure employed was similar to that described by Cerskus *et al.*[16]. Antithrombin III-agarose (Sigma; 1.5 g) was equilibrated with starting buffer (0.02M Tris; pH 7.4) for 72 h at 4°, and used as an affinity column (15 × 1.0 cm) thermostatically-controlled at 4°. A solution of the sample (~5 mg) in starting buffer was applied to the column and eluted stepwise with 60-mL portions of the buffer containing NaCl at concentrations of 0.01, 0.05, 0.1, and 2.0M. Fractions eluted from the column were detected and estimated by the carbazole method[17], absorption measurements being made at λ 530 nm and referenced to a calibration curve based on unfractionated heparin.

Measurements of heparinase activity. — The sample (10 mg) in 100:1 (0.25M) sodium acetate–calcium acetate buffer (pH 7.0; 2.0 mL), was treated with *Flavobacterium heparinum* heparinase (10 units; Sigma) at 30°. Activity was monitored by measurements at λ 230 nm. A second portion of the heparinase was introduced after 3 h, and the reaction mixture was kept at 30° for an additional 20 h. Following heat inactivation of the enzyme, the solution was evaporated and the residue was subjected to D_2O exchange and examined in that solvent by ^{1}H-n.m.r. spectroscopy. As a control, unfractionated heparin, treated in the same fashion, was degraded almost fully into the Δ^4-unsaturated disaccharide[1].

Measurement of anticoagulant potency. — Tests for anti-Xa activity were performed by a chromogenic assay using an Actichrome Heparin Kit (Ortho Diagnostics Inc., Don Mills, ON).

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