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N.M.R. SPECTROSCOPIC OBSERVATIONS RELATED TO THE FUNCTION OF SULFATE GROUPS IN HEPARIN. CALCIUM BINDING *VS.* BIOLOGICAL ACTIVITY

LYNN AYOTTE AND ARTHUR S. PERLIN
Department of Chemistry, McGill University, Montreal, Quebec H3A 2A7 (Canada)
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ABSTRACT

Chemically-modified heparins containing different combinations of N- and O-sulfate groups were prepared. Characterized by high field ^{1}H- and ^{13}C-n.m.r. spectroscopy, the polymers exhibited chemical shift variations in general accord with shielding differences expected on removal of sulfate substituents, and additional variations that probably arose from conformational changes in the polymers. Whereas the anticoagulant activity of heparins, as measured by USP, anti-Xa, and thrombin-time assays, was invariably reduced by the chemical transformations effected, the ability of heparin to bind calcium ions was found to be dependent on retention of the 2-sulfamino group, whether or not O-sulfate groups were present. The results suggest that the 2-sulfamino group is essential for maintaining a molecular conformation consistent with the ability for the L-iduronic acid residues to complex with calcium ions. Also, they show that although the anticoagulant and calcium-binding properties of heparin may be interdependent, they are not determined by the same structural entities in the polymer.

INTRODUCTION

Owing to its polyelectrolyte nature[1], heparin forms complexes with such proteins as antithrombin-III, which is associated with anticoagulant effects, or with lipoprotein lipase, in promoting antilipemic action. The binding of calcium ion to heparin is another interaction of interest, because it is regarded as important for anticoagulant activity, as well as for stabilizing heparin-lipoprotein-lipase complexes.

In a number of studies, it has been shown[2–8] that calcium ion binds preferentially to the carboxyl groups of heparin. Nevertheless, the 2-sulfamino group plays an essential role[8] in the formation of the chelate, for, on selective hydrolysis of this group, the specific binding of calcium is no longer observed. Although this effect parallels the almost total loss of anticoagulant activity on removal of the N-sulfate group, it is not known whether these two observations are related to, or independent of, each other. Evidence bearing on this question is presented here, together

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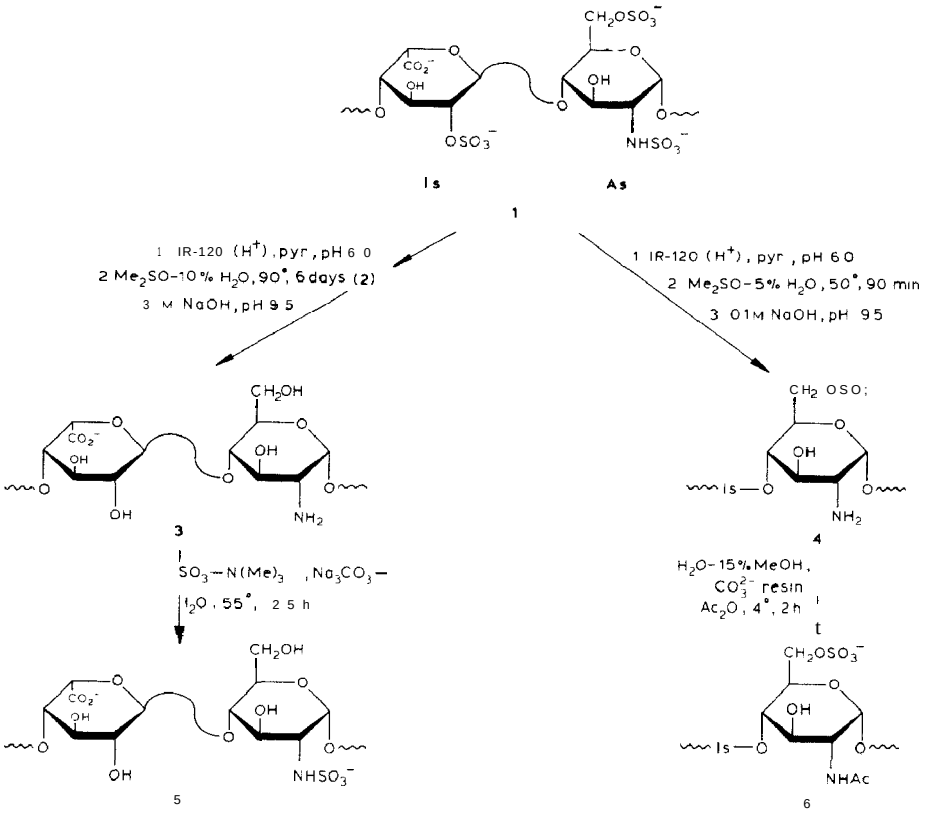

Scheme 1. Chemical modification of the major constituent residues of heparin. Abbreviations: Is, α-L-idopyranosyluronic acid 2-sulfate residue, As, 2-deoxy-2-sulfamido-α-D-glucopyranosyl 6-sulfate residue.

with calcium-binding and biological properties of other chemically-modified heparins, notably, some deficient in O-sulfate groups. N.m.r. spectroscopy has been employed throughout this study for characterization of the modified heparins as **well** as for the measurement of their binding with calcium ion.

RESULTS AND DISCUSSION

Chemical modification of heparin. — Hog mucosal heparin was subjected to various treatments, as illustrated in Scheme 1, to alter its content of N- or O-sulfate (or both) groups. The reaction conditions used are described briefly, following which the n.m.r. spectra of the products are presented in support of the structures proposed.

Selective removal of the relatively labile N-sulfate group of heparin (1) was effected[9] by heating a solution of the pyridinium salt of the polymer in 19:1 dimethyl sulfoxide-water for 1.5 h at 50°, affording compound 4. In 9:1 dimethyl sulfoxide-water at 90°, O-sulfate groups also were subject to hydrolysis: in 20 h the

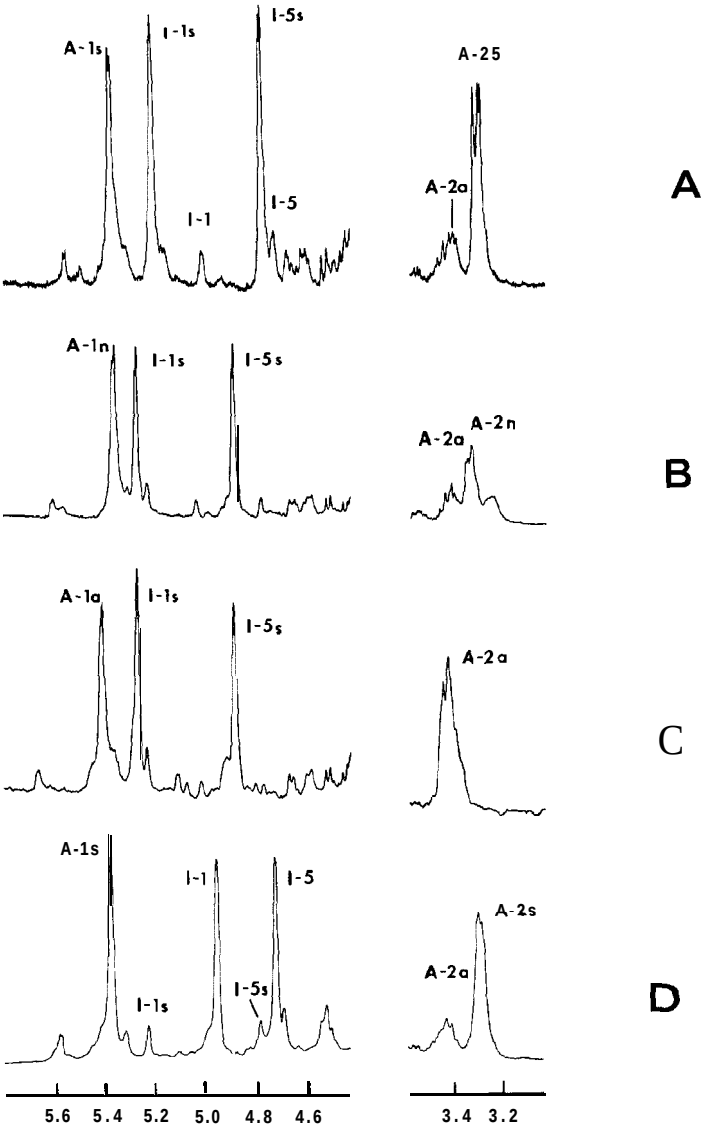

Fig. 1. Partial 400-MHz, ^{1}H-n.m.r. spectra of chemically-modified heparins, showing regions of H-1 and H-15 resonances (δ 5.6-4.6) and H-2 resonances (6 3.5-3.2): (A) Unmodified hog mucosal heparin; (B) compound 4; (C) compound 6; and (D) compound 5. Abbreviations: s, sulfated residue; a. acetamido; and n, amino.

6-sulfate group of aminodeoxyhexose residues was almost totally removed, together with 37% of the 2-sulfate group of L-iduronic acid residues (compound 2) whereas the latter figure was increased to 89% (compound 3) during reaction for 6 days. Polymer 4 was selectively *N*-acetylated[10] with acetic anhydride and a basic

TABLE I

^{1}H-CHEMICAL SHIFTS (δ) FOR HOG MUCOSAL HEPARIN (1) AND CHEMICALLY-MODIFIED FORMS **2, 4, 5**, AND **6**[a]

Proton[b]	***Compound***				
	1	2	4	5	6
A-1		5.48.5.42	5.39 (5.11)[c]		
A-1a					5 42
A-IS	***5.36***			***5.39***	
A-2			3.25 (2.78)		
A-2a	3.40		3 42		3.43
A-2s	3.30			3 29	
I-1	4.99	***4.94***		4.97	
I-1s	5.19	***5.26***	***5.29 (5.24)***	5 24	5.28
I-5	4 71	***4.73***		3.74	
I-5s	4.75	***4.91***	***4 94 (4.92)***	4.80	4 89
G-1	4.51		***4.53***		4.52
G-2	3 44		***3.42***		
Ac	2 04		***2.05***	2.06	2.03

"Measured at 400 MHz for a solution in D_2O at 80°, relative to Internal TSP. [b]Abbreviations: A, 2-ammo-2-deoxy-u-D-glucopyranosyl residue, **I**, α-L-idopyranosyluronic acid residue; G, β-D-glucopyranosyluronic acid residue: a, N-acetylated; **s**, *N*- or O-sulfate at C-2; and Ac, acetamido 'Measured at 200 MHz, pD 9.0, 70°.

ion-exchange resin in aqueous methanol, yielding 6. In addition, by selective *N*-sulfation[11] with sulfur trioxide-trimethylamine in aqueous sodium carbonate, 3 was converted into 5, i.e., a modified heparin containing only about 5% of the O-sulfate groups present initially, but with the same proportion of sulfamino groups as present in the original heparin. For further study, all of the products were converted into sodium salts.

N.m.r. ***spectroscopic analysis of*** *chemically-modified* ***heparins.*** — Among signals readily assignable in the ^{1}H-n.m.r. spectrum of N-desulfated heparin (4) (Fig. 1B and Table I) are A-l and A-2 due to the aminodeoxyhexose residue. Although they are in virtually the same positions (Fig. 1A) as for unmodified heparin[12], the signals were displaced upfield[13] at pD 9.0 (Table I)*. As this was not observed with heparin itself and as no residual signals were detected at δ 5.3 and -3.3, it is clear that 4 had retained no N-sulfate groups. This was confirmed by the ^{13}C-n.m.r. spectrum (Table II), which showed that although there were no signals at δ 99.5 and 60.5, corresponding[14] to C-l and C-2, respectively, of the amino-deoxyhexose residue of heparin, new signals attributable to A-l and A-2 appeared at δ 93.9 and 56.8. A strong upfield shift (by -6.6 p.p.m.) upon N-desulfation also was observed for signal A-3 (Table XX). That no appreciable loss of O-sulfate had occurred was indicated by the lack of a change in the intensity of the C-6 signal

*Small differences between chemical shifts given in this paper and those reported earlier'?-" are attributed to such variables as operating temperature and standard of reference.

TABLE11

^{13}C-CHEMICAL SHIFTS (δ) FOR HOG MUCOSAL HEPARIN (**1**) AND CHEMICALLY-MODIFIED FORMS **4, 5**, AND **6**[a]

Carbon atom	*Compound*			
	1	4	5	6
A-1		93.9		
A-1a				93.6
A-1s	99.5		98.4	
A-2		56.8		
A-2a		56.3	56.6	56.8
A-2s	60.5		60.6	
A-3	72.0	65.4		65.5
A-6			***62.3***	
A-6s	69.0			68.8
I-1		103.8	104.3	104.0
I-1s	101.8	101.3		101.4
G-1	104.6	104.5	104 8	104.6
CO(I)	176.6	176.9	177.0	177.0
CO(Ac)				177.1
Ac	24.6	24.6	24.6	24.7

[a]Measured at 100 MHz for a solution in D_2O at 25". relative to internal TSP. [b]Abbreviations: A, 2-amino-2-deoxy-α-D-glucopyranosyl residue; **I**, α-L-idopyranosyluronic acid residue; G, β-D-glucopyranosyluronic acid residue; a. N-acetylated; **s**, *N*- or O-sulfate at C-2; and Ac, acetamido.

(A-6, δ 62.3) of aminodeoxyhexose residues devoid of a 6-sulfate group, relative to that of **A-6s** or in the intensity of signal I-1 (6 103.8) of nonsulfated L-iduronic acid residues, relative to that of **I-1s**.

It is noteworthy that proton signals **I-1s** and **I-5s** of 4 (Table I) experienced downfield displacements of 0.10 and 0.19 p.p.m., respectively, as compared with the spectrum of heparin. However, there appeared to be no associated change in the conformation of the L-iduronic acid ring of 4, as the spin-spin coupling patterns for these protons (data not shown) were unchanged.

N-Acetylation of 4, as seen in the ^{1}H-n.m.r. spectrum of modified heparin 6, caused small downfield shifts in signals A-1 and A-2, and slightly greater ones for **I-1s** (by 0.08 p.p.m.) and 1-5s signals (by 0.14 p.p.m.) (Fig. 1C and Table I). Changes in the ^{13}C-n.m.r. spectrum introduced by the N-acetylation step were, uniformly, minor (Table II). Consequently, the spectra of 6 were distinguished mainly by the presence of ^{1}H- and ^{13}C-resonances due to the *N*-acetyl group.

Among the most notable alterations observed in ^{1}H-n.m.r. spectra upon *O*-desulfation were those involving the nuclei listed in Table I for compound 2, in which about one-third of the L-iduronic acid residues are nonsulfated. (The spectrum of 3, the almost completely desulfated compound, was more poorly resolved, because of line broadening, as commonly observed with neutral polysaccharides.) The anomeric proton signal (A-1) was split into two components, that at δ 5.42 decreasing in intensity relative to the signal at δ 5.48. as the 2-sulfate

group of the L-iduronic acid residue was removed progressively (*i.e.*, **2→3**). This latter process also was characterized by relative increases in signals I-l and I-S (Table I) which, being well separated from other signals, were used for analysis of the 2-sulfate content of the samples isolated.

Compound 5, which contains a 2-sulfamino group and -90% of non-0 sulfated L-iduronic acid, exhibited chemical shifts for A-l, A-2. I-l, and I-5 that are close to those for the original heparin (Fig. 1D and Table I). That is, the deshielding effect experienced by these protons upon N-desulfation, although not counterbalanced by N-acetylation (as seen with **6**), is fully offset (Table I) by restoration of the sulfamino function, despite the low O-sulfate content of the material. Close similarities also are found in ^{13}C-chemical shifts for A-l, A-2, and I-1 of 5, in comparison with those of heparin (Table II). As signal **A-2s** (6 60.6) is 3.8 p.p.m. downfield of that for the amino-containing product (4. δ 56.8), and distinct from the weak signal (6 56.2) due to the minor acetamidodeoxyhexose residues. the formation of 5 had clearly involved complete *N*-resulfation. Similarly, the absence of a signal at δ -69 demonstrates that, in the preparation of 5 (as well as of precursor **3**), there had been complete 0-desulfation at O-6 of aminodeoxyhexose residues, whereas there now is an appropriate upfield signal (6 62.3) attributable to C-6 bearing a hydroxyl group.

It is worth noting that the spectra of the modified heparins contained no signals to suggest that depolymerization had accompanied the preparation of these materials.

The binding of calcium ion by chemically-modified heparins. — Removal of the N-sulfate group of heparin (1) produced a modified polymer[15] (4) that has a markedly reduced capacity to bind calcium ions. It appears possible that the loss of the N-sulfate group and the associated decrease in its chelating ability are also accompanied by a change in the molecular conformation because, as shown earlier (Table I), 'H-resonances of L-iduronic acid residues (**I-1s** and 1-5s) are affected. being notably downfield of those of 1. If the difference in size of the sulfamino and amino group were to be a contributing factor, then the introduction of an acetamido group (a functionality that occurs in native heparins) might be expected to have some effect on chemical shift. However, protons **I-1s** and **I-5s** of the *N*-acetyl derivative (6) have the same chemical shifts as for 4 (Table I). Furthermore. according to comparable ^{1}H-n.m.r. measurements, polymer 6 did not appreciably bind calcium ion.

Although the sulfamino group is necessary for calcium binding to be observed[8], the O-sulfate groups of heparin were found, by contrast, to play no significant role in the chelation process. Hence, the effects of calcium ion on the 'H-n. m.r. spectrum of compound 5 (Table III) were analogous to, though less pronounced than, those observed for heparin under comparable conditions. In this instance, the measurements were made at pD 8.5 with both the unmodified heparin and polymer 5, because the spectrum of 5 was much better resolved at the higher pD than in D_2O. The chemical-shift changes for heparin that arc induced by

TABLE III

DISPLACEMENTS OF ^{1}H-RESONANCES[a] INDUCED BY THE INTERACTION OF HEPARIN AND MODIFIED HEPARIN (5) WITH CALCIUM ION[b]

Protons'	*Heparin (pD6.0)*[d]	*Heparin (pD8.5)*[e]	*5 (pD 8.5)'*
A-l	-10	-12	- 4
I-l	32	38	24
I-5	20	20	16

[a]In Hz at 200 MHz. Minus denotes an upfield shift. [b]At 1.25 mol of Ca/heparin disaccharide repeating unit. [c]Other protons exhibited lesser, or no, displacement. [d]From ref. 8. [e]pD adjusted with Na_2CO_3.

TABLE IV

BIOASSAYS OF HEPARIN AND MODIFIED HEPARINS **4, 5, AND 6**

Specimen	*Assay*		
	USP (U/mg)	*Anti-Xa (U/mg)*	*TT*[a] *(s)*
Heparin	142	107	⩾200
4	⩽5	⩽5	13.3
5	⩽5	⩽5	13 3
6	⩽5	⩽5	13.1

[a]Normal plasma blank, 12.4 s.

TABLE V

O-DESULFATION OF HEPARIN

Reaction conditions			O-*Desulfated* ***iduronic acid*** *(%)*
Solvent	***Temp. (degrees)***	***Time (h)***	
Me_2SO–10% H_2O	80	5	negligible
Me_2SO–10% H_2O	90	20	37.5 (product 2)
Me_2SO–15% H_2O	90	48	48.3
Me_2SO–10% H_2O	87-92	140	89.3 (product 3)

calcium ion are constant over this pD range (Table III), which accords with pH-titration data for ionization states of the polymer[7,14]. Consequently, it is evident that calcium binding took place when a 2-sulfamino group was present, despite the removal of the other sulfate groups from the polymer and its overall lower charge density.

It also is noteworthy, in the light of comments presented earlier, that *N*-resulfation gives rise to a ^{1}H-n.m.r. spectrum for 5 in which the signals of L-iduronic acid (**I-1s** and **I-5s**, as well as I-l and **I-5**) are all virtually restorted to their positions in the spectrum of unmodified heparin (Table I). Consequently. these findings

suggest that the 2-sulfamino moiety is the principle determinant providing the appropriate environment for L-iduronic acid residues and the attendant overall molecular conformation, consistent with the ability of the polymer, ***i.e.,*** 1 or 5, to complex strongly with calcium ions".

Bio-assays of *chemically-modified* ***heparins.*** — The chemically modified heparins, when tested by the whole blood assay (USP), as well as for their specificity in inhibiting coagulation Factor Xa (anti-Xa), were found to be completely inactive (Table IV). Since the USP and anti-Xa assays reflect complex-formation between heparin and antithrombin, these results are consistent with the findings of others[1,16,17], because there can be few intact antithrombin-binding sequences in the modified polymers.

Measurements of thrombin time (TT) also were carried out to test for the inhibition of blood clotting through interference with the fibrinogen-fibrin interconversion, which is facilitated by calcium ion. However, in this test as well (Table IV), none of the modified heparins showed an appreciable effect. Consequently, although calcium ion is believed to play a role in the anticoagulant function of heparin, and the structural requirements that allow for the chelation of calcium ion are shared with heparin by polymer 5, the latter is unable to interfere with the fibrinogen-fibrin interconversion through its calcium-binding capacity alone.

EXPERIMENTAL

General. — ^{1}H-N.m.r. spectra were recorded with a Varian XL-200 or Bruker WH-400 spectrometer using samples, pre-exchanged with D_2O, at a concentration of -20 mg/mL of D_2O, at 70". Chemical shifts are reported with respect to internal sodium 4,4-dimethyl-4-sila-(2,3-$^2H_4$)pentanoate (TSP). ^{13}C-N.m.r. spectra were recorded with a Bruker WH-90 or Varian XL-200 spectrometer. at a sample concentration of -100 mg/mL of D_2O. Chemical shifts are reported with respect to internal methanol (δ 51.5 in D_2O with reference to internal TSP). Dialysis was performed against distilled water (5–6 exchanges)** for 48 h. Spectrapor membrane tubing was used: a molecular weight cutoff at 3500 and a dry cylinder diameter of 11 .5 mm was used for samples of $\leq$100 mg, and for larger samples, a molecular weight cutoff of 6000-8000 and 20.5 mm. Solutions were evaporated under reduced pressure at a bath temperature not exceeding 55–60°.

Calcium-binding measurements. — Binding between calcium ion and heparin and its derivatives was monitored by ^{1}H-n.m.r. spectroscopy at 200 MHz. Spectra

*Perhaps the chemical shift displacements observed represent a conformational change induced by a specific binding of calcium, and are not due simply to electrostatic changes when Ca^{2+} and CO_2^- interact

**For some preparations. where calcium-binding measurements were not involved. dialysis against tap water also was used. As noted by a referee. this procedure introduced the possibility that those preparations contained ions in addition to sodium Nevertheless, paramagnetic cations likely were absent, because such resonances as I-l and I-S should then have been unusually broad.

were recorded for solutions in D_2O at 70° with a Varian XL-200 spectrometer using TSP as internal standard. Solutions of the polymers were prepared at a concentration of 20 mg/0.5 mL. Ca^{2+} ions were introduced into the n.m.r. tube in the form of a 10% solution of $CaCl_2$ in D_2O, in appropriate amounts.

Biological assays. — Overall anticoagulant activity was measured by the USP assay method described by Foster, and anti-Xa activity by the procedure of Yin et *al.*[18], in the laboratory of Choay S.A., Paris. Thrombin-time determinations were performed by Mrs. S. Atkinson, Montreal General Hospital. Samples were prepared by dissolving 13.6 mg of material in 2.0 mL of water to produce a concentration of 1000 units/ml, based on the USP assay (147 USP units/mg) of unaltered hog mucosal heparin. For each sample, an aliquot quantity was first diluted to 1/40 in M NaCl and 0.01 mL of the latter solution was mixed with 0.5 mL of human pooled plasma, to give a working concentration of 0.5 unit of heparin (or derivative) in plasma. Thrombin times were then performed on these plasma samples by use of 0.1 mL of M NaCl, 0.2 mL of plasma, and 0.1 mL of human thrombin (5 units/ml). Thrombin times are expressed in terms of the time taken for each sample to clot.

Chemical modification of hog mucosal heparin (Upjohn). Pyridinium heparinate. — Pyridinium heparinate was prepared by the method described by Inoue and Nagasawa[9]. A solution of sodium heparinate (800 mg) in water (35 mL) was applied to a column of Amberlite IR-120 (H+) ion-exchange resin (BDH) (35 mL) previously washed with water, 10% HCl, and water. The resultant acidic solution and washings were combined, titrated with pyridine to pH 6.0, and lyophilized, to give pyridinium heparinate (840 mg) as a white powder.

N-Desulfuted heparin (4). — N-Desulfated heparin was prepared by the method described by Inoue and Nagasawa[9]. Pyridinium heparinate (500 mg) was dissolved in dimethyl sulfoxide (25 mL) containing 5% of water; the solution was stirred for 1.5 h at 50°, then diluted with water to a volume of 50 mL, and its pH adjusted to 9.5 with 0.1M NaOH. The solution was dialyzed against running tap water overnight and against 5-6 exchanges of distilled water over a period of 48 h, filtered, and lyophilized; yield, 360 mg of a white fluffy powder. The ^{13}C-n.m.r. spectrum at 22.6 MHz showed that the A-2 signal was completely shifted upfield, compared to that of the unaltered heparin, and overlapped the C-2 signal of *N*-acetylated residues, indicating that N-desulfation was complete. The n.m.r. data suggested also that virtually no hydrolysis of sulfate ester groups had occurred, as there was no evidence for an increase in the proportion of residues devoid of an O-sulfate group at C-6.

N-Desulfuted, N-reacetylated heparin (6). — N-Desulfated, N-reacetylated heparin was prepared by a method similar to that described by Danishefsky[10]. *N*-Desulfated heparin (200 mg) was dissolved in water (20 mL), methanol (3 mL) was introduced at 0°, and the solution was added to Dowex 1-X8 (CO;) anion-exchange resin (7.5 mL). At 0°, acetic anhydride (0.3 mL) was added dropwise with stirring, the mixture was stirred at 4" for an additional 2 h, the resin was removed by filtration, and washed with water. The clear filtrate (total volume, 40 mL) was dialyzed

against running tap water overnight, and then against distilled water for 40 h, and lyophilized; yield, 198 mg of white powder.

N,O-*Desulfated heparin (3).* — A solution of pyridinium heparinate (500 mg) in dimethyl sulfoxide (50 mL) containing 10% water was stirred for 6 days at 87–92°, and the slightly yellow solution was cooled and diluted with water to a final volume of 200 mL (necessary to dissolve material precipitating upon the addition of water). The solution was titrated with M NaOH to pH 9.5, concentrated to ~113, dialyzed against running tap water overnight and against distilled water for 48 h. and lyophilized to give a powdery product (229 mg, 3). According to ^{1}H-n.m.r. data (200 MHz), 89.3% of the iduronic acid residues were *O*-desulfated at O-2, and from ^{13}C-n.m.r. data (50.3 MHz), the hexosaminc residues were completely *O*-desulfated at O-6. In attempting to obtain completely desulfated heparin. chemically modified heparins with intermediate degrees of *O*-sulfation were obtained. Reaction conditions, and the proportion of 0-desulfated iduronic acid. as measured from the area of I-l (S) and I-5 (OH) signals in the ^{1}H-n.m.r. spectra at 200 MHz, were as given in Table V. The most highly 0-desulfated material was poorly soluble in water, although very soluble in dimethyl sulfoxide. At the concentration needed in D_2O for a ^{1}H-n.m.r. spectrum. a portion of it remained undissolved, and spectra of the supernatant solution and the precipitate were recorded individually. Both spectra indicated that the degree of 0-desulfation of iduronic acid (at O-3) was virtually the same. although the more soluble material showed the strongest CH, signal, which suggests that the rate of 0-desulfation in aqueous dimethyl sulfoxide is independent of the length of the polymer chain or its structural heterogeneity.

N,O-*Desulfated,* N-*resulfated heparin (5).* — N-Resulfation of the N. *O*-desulfated heparin was carried out under conditions similar to those described by Lloyd *et al.*[11]. *N,O*-Desulfated heparin containing 89.3% 0-desulfatcd iduronic acid (100 mg) was dissolved in water (16 mL), Na_2CO_3 (100 mg) was introduced, and the solution was added to trimethylamine-sulfur trioxide complex [SO, ·$N(CH_3)_3$, 100 mg; Aldrich]. The mixture was stircd for 24 h at 5.5". cooled, diluted to a total volume of ~35 mL in order to dissolve suspended material, and dialyzed against distilled water for 48 h. The dialyzate was applied to a column of Amberlite IR-120 (H^+) ion-exchange resin (7-8 mL) to remove ammonium ions. titrated to pH 9.5 with 0.1 M NaOH, dialyzed against distilled water for 48 h. and lyophihzed. *N,O*-Desulfated, N-resulfated heparin (5) was obtained as a white. huffy powder (yield, 113 mg). There was no indication from its ^{1}H-n.m.r. spectrum at 200 MHz that the polymer had suffered degradation during the sulfation reaction. Although the material was very soluble in water. the addition of Na_2CO_3 to the n.m.r. sample caused an improvement in signal resolution (possibly by increasing the ionic strength). N, 0-Desulfated heparin containing 37.5% of *O*-desulfated iduronic acid also was N-resulfated, as just described. A solution of partially *O*-desulfated material (100 mg) and Na_2CO_3 (140 mg) in water (2.5 mL) was added at 0° to SO, . $N(CH_3)_3$ complex (70 mg), the mixture was stirred for 25 h at 55°, then diluted to IO mL with water, dialyzed for 72 h with stirring against

several changes of distilled water until all of the material had dissolved, and the solution was lyophilized. The N-resulfated product obtained as a white powder (yield, 106 mg), exhibited a singlet at δ 2.92, suggesting that the material had been isolated as a mixed trimethylammonium and sodium salt. Consequently, a solution of the *N*-resulfated material (80 mg) was applied to a column of IR-120 (H+) ion-exchange resin (4 mL), the combined eluant and washing was titrated to pH 9.5 with 0.1M NaOH, dialyzed against distilled water for 36 h with stirring, and the solution was lyophilized (yield, 72 mg).

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