Carbohydrate Research, 228 (1992) 29–36
Elsevier Science Publishers B.V., Amsterdam

Adverse effects of alkali and acid on the anticoagulant potency of heparin, evaluated with methyl 2-deoxy-2-sulfamino-α-D-glucopyranoside 3-sulfate as a model compound*

Zhengchun Liu and Arthur S. Perlin
Department of Chemistry, McGill University, Montreal, Que. H3A 2A7 (Canada)

(Received January 19th, 1991; accepted for publication, April 22nd, 1991)

ABSTRACT

A variety of chemical modifications can induce a reduction in the anticoagulant activity of heparin. Among such modifications are the removal in alkaline solution of the 2-*O*-sulfonate group of α-L-idopyranosyluronic acid 2-sulfate residues (**1**) and, in a weakly acidic medium, of the *N*-sulfonate group of residues of 2-deoxy-2-sulfamino-α-D-glucopyranose 6-sulfate (**2**). This study examined the possibility that the losses in anticoagulant potency are related to a concomitant removal of the 3-*O*-sulfonate group of residues of 2-deoxy-2-sulfamino-α-D-glucopyranose 3,6-disulfate (**6**) in the AT-III binding site. It entailed a synthesis of methyl 2-deoxy-2-sulfamino-α-D-glucopyranoside 3-sulfate (**7**), as a model compound that was subjected to both the strongly alkaline and weakly acidic conditions appropriate for the modification of residues **1** and **2**, respectively. The 3-sulfate group of **7** was found to be highly stable in both environments. This indicated that the adverse effects that these conditions have on the anticoagulant properties of heparin are not specifically associated with the 3-sulfate substituent of residues of **6** in the polymer.

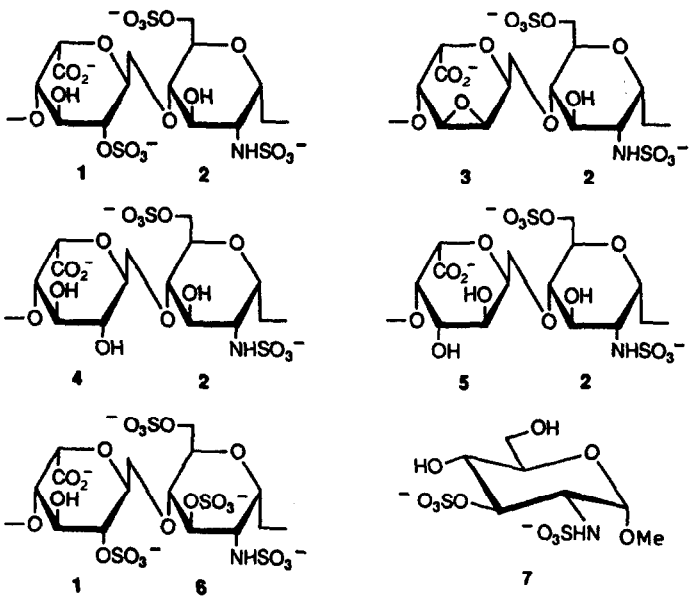
INTRODUCTION

Of the three sulfate substituents on the major residues of heparin, *i.e.*, α-L-idopyranosyluronic acid 2-sulfate (**1**) and 2-deoxy-2-sulfamino-α-D-glucopyranose 6-sulfate (**2**), the 2-sulfate group of **1** is uniquely unstable in alkaline solution. This was attributed[1,2] to a facile intramolecular displacement of the group by the neighboring O-3 anion, whereby **1** is converted into a residue of 2,3-anhydro-α-L-gulopyranosyluronic acid (**3**). By stereoselective hydrolysis of the oxirane ring of **3** to give either the α-L-*ido* (**4**) or α-L-*galacto* (**5**) diastereomeric product, two other modified heparins have been obtained as well[1–3]. According to the n.m.r. spectra of these polymers, the residues of **2** that each contains remain unaltered. Consequently, as these chemical transformations were accompanied[1,2] by an almost complete loss of the USP and anti-Xa activities exhibited by the parent heparin, intact residues of **1** appear to be essential for the expression of heparin's anticoagulant properties.

One other type of residue that merits consideration in this context is the 3-sulfate

* Dedicated to Professor Serge David on the occasion of his 70th birthday.

0008-6215/92/$ 05.00

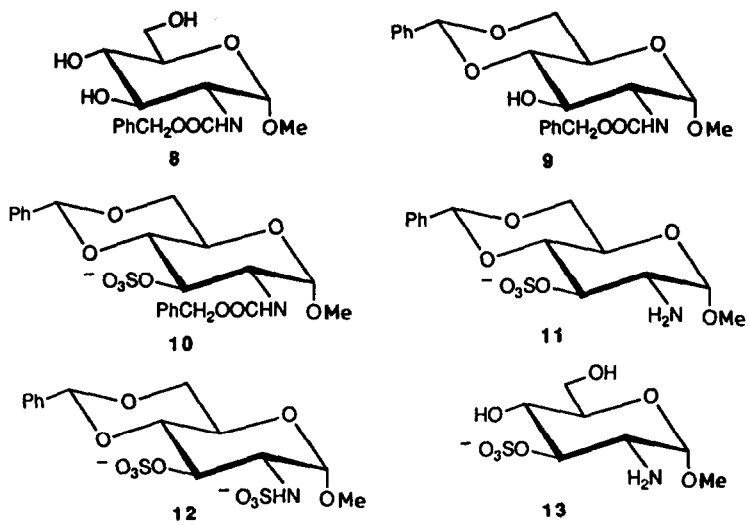


analog of **2**, *i.e.*, residue **6**, because it is a critical component of the antithrombin-III (AT-III) binding site[4] involved in heparin's role as an anticoagulant. Our n.m.r. measurements[1-3] did not enable us to determine whether or not residues of **6** were among those altered during preparation of the modified polymers*. Nevertheless, little change was expected[2,7] in view of the marked stability of both the 6-sulfate and 2-sulfamino groups of closely-related residue **2**[1-3], as well as the fact that the equatorial 3-sulfate group of **6** is not suitably oriented for internal base-catalyzed displacement (as is [1-3] the axial 2-sulfate group of **1**). That expectation now receives support from indirect evidence provided here. It is shown that methyl 2-deoxy-2-sulfamino-α-D-glucopyranoside 3-sulfate (**7**), synthesized and tested as a model for residue **6**, is stable under the experimental conditions used in the preparation of the modified heparins. The 3-sulfate group of **7** was also shown to be stable under acidic conditions that effect hydrolysis of its sulfamino group, which is consistent with the long-held view[8] that the sulfamino group is critical for anticoagulant activity.

RESULTS AND DISCUSSION

Synthesis of glucoside **7**. — A synthesis of methyl 2-deoxy-2-sulfamino-α-D-glucopyranoside 3-sulfate (**7**) was carried out by the following sequence of reactions. Methyl 2-benzyloxycarbonylamino-2-deoxy-α-D-glucopyranoside[9] (**8**) was converted

* The detection of residues of **6** in heparin by n.m.r. spectroscopy has been demonstrated[5,6] with fractions enriched in such residues through binding to AT-III; an analogous enrichment was not feasible with the present polymers because they have little affinity for AT-III.



into the 4,6-*O*-benzylidene derivative **9** with benzaldehyde–zinc chloride. Sulfation of **9** with sulfur trioxide–trimethylamine in *N*,*N*-dimethylformamide afforded the 3-sulfate **10**, which has recently been described by Leder[10]. Selective removal of the *N*-benzyloxycarbonyl substituent by catalytic-transfer hydrogenation with palladium–cyclohexene yielded **11**, which was sulfated as described above to give the 2-deoxy-2-sulfamino derivative **12**. The *O*-benzylidene group of **11** was removed by hydrolysis with 80% acetic acid, and the amine produced (**13**) was selectively sulfated in aqueous hydrogen carbonate solution to afford **7**. The disodium salt of the latter compound, as well as that of **12**, proved to be highly hygroscopic, although both were obtained as analytically pure, hydrated forms. It is worth noting that the chemical shift of H-3 of **7** in D_2O is δ 4.33 (see Fig. 1C), as compared with a value of δ 4.37 for this proton in a residue of **6** contained in a synthetic pentasaccharide model compound[11].

An examination of the stability of glycoside **7** *under alkaline conditions.* — An aqueous solution containing a mixture of **7** and heparin was adjusted to pH 11.8 and then lyophilized. These conditions corresponded to those[1] whereby heparin (with its normal content of residues of **6**) is efficiently converted into a polymer consisting mainly of residues of the 2,3-anhydride **3** and intact residues of the major amino sugar (**2**). The product (see Fig. 1A) of lyophilization, and of the exposure to concentrated alkali accompanying it, was then subjected to dialysis, to facilitate examination of the polymeric and low-molecular-weight components of the mixture as separate entities. As seen in Fig. 1B, the ^{1}H-n.m.r. spectrum of the polymer fraction is closely similar to that[1] of the product obtained from heparin alone, which verified that the reaction conditions used had been appropriate for testing the effect of alkali on glucoside **7**. The dialyzate was found to consist essentially of intact **7**, which was identified by its ^{1}H-n.m.r. spectrum (Fig. 1C). Similarly, **7** was found to be stable when it was subjected to lyophilization at pH 12.5, *i.e.*, the slightly more alkaline conditions[2] under which the

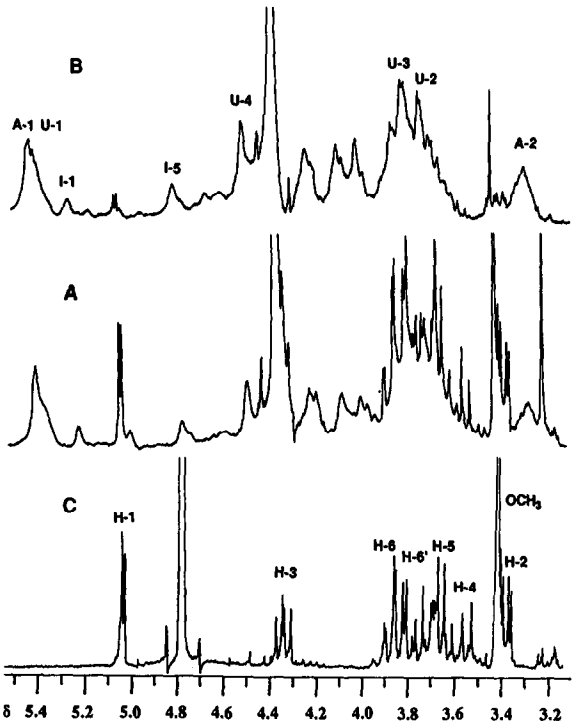


Fig. 1. ^{1}H-N.m.r. spectra (300 MHz; solvent, D_2O) of: (A) the product recovered after lyophilization of a mixture of heparin and methyl 2-deoxy-2-sulfamino-α-D-glucopyranoside 3-sulfate (**7**) at an initial pH of 11.8 (temp., 65°); (B) the fraction of the product (in (A)) retained within the dialysis membrane (temp., 65°); and (C) the dialyzate (temp., 25°). A and U refer to protons of residues **2** and **3**, respectively, of the modified heparin (for more detailed assignments, see Fig. 2A of ref. 1), and I to protons of unmodified residues of **1**. The ^{1}H designations given in Fig. 1C are based on an analysis of the corresponding spectrum for pure **7**, which this spectrum closely resembles.

α-L-iduronic acid 2-sulfate residue (**1**) is converted ultimately into its desulfated form (**4**).

Clearly, then, the 3-sulfate group of **7** was not significantly affected by these alkaline media. The results also showed that the sulfate group had not been activated by the neighboring 2-sulfamino substituent, a remote possibility that we had entertained. Consequently, if the analogy drawn between **7** and **6** is valid, the loss in anticoagulant potency by heparin is not due to a reaction between the alkali and residues of **6**, but to the base-catalyzed desulfation of residues of α-L-iduronic acid 2-sulfate (**1**).

An examination of the stability of glycoside **7** *under acidic conditions.* — Glycoside **7** also served as a model for testing the stability of the 3-sulfate group of residue **6** towards acid. A sulfamino group is highly acid-labile[8], and selective acid hydrolysis or solvolysis of the 2-sulfamino group of residue **2** in heparin can be readily effected under mild conditions without other apparent changes in the polymer. As the 2-amino analog produced is devoid of anticoagulant activity, the *N*-sulfonate function is regarded[8] as a

key factor in the interaction between heparin and AT-III. However, by analogy with the foregoing considerations about alkali stability, the effect of acidity on the 3-sulfate group of residue **6** in the AT-III binding site also should be taken into account. To our knowledge, this had not already been done.

Consequently, **7** was converted into the (weakly acidic) pyridinium salt, which was subjected to solvolysis[12] in 1:19 water–dimethyl sulfoxide at 55° for 1.5 h. These conditions are particularly efficient for selective hydrolysis of the 2-sulfamino group of residues of **2** in heparin, and cause a marked decrease in anticoagulant activity. Examined by ^{1}H-n.m.r. spectroscopy, the solvolysis product consisted almost entirely of methyl 2-amino-2-deoxy-α-D-glucopyranoside 3-sulfate (**11**), showing that only the 2-sulfamino group of **7** had been affected. It is likely, therefore, that the 3-sulfate group of residues of **6** in heparin would survive the mild reaction conditions that are designed to selectively hydrolyze the 2-sulfamino group, and that the loss of anticoagulant potency is attributable to the latter alteration in the heparin structure, as proposed[8] originally. However, as the 2-sulfamino group of **6** is required for binding to AT III, the extent to which residues of **2** may be involved in the total adverse effect of the acid hydrolysis remains to be determined.

In summary, when heparin is subjected to appropriate treatment in an alkaline or a weakly acidic medium, it suffers a reduction in anticoagulant activity due to removal, respectively, of the 2-*O*-sulfonate group of residues of **1**, or of the *N*-sulfonate group of residues of **2** (and also, likely, of **6**). This loss of activity is not caused by a concomitant removal of the 3-sulfate group of residues of **6** which, in fact, is a relatively stable substituent under both sets of conditions.

EXPERIMENTAL

General methods. — Melting points were determined with a Fisher–Johns apparatus, and are uncorrected. Optical rotations were determined at 25° with a Jasco Dip 140 digital polarimeter. N.m.r. spectra were recorded with Varian XL300, XL200, or Gemini 200 spectrometers operating at 300 or 200 MHz for ^{1}H-n.m.r. and 50 MHz for ^{13}C-n.m.r spectrometry, each equipped with a 5-mm ^{1}H 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) for D_2O solutions, and of internal tetramethylsilane for organic solutions. The 2D (^{1}H, ^{1}H) COSY experiments were performed with the Varian pulse-sequence programs, and utilized for verifying most of the spectral assignments given. Samples (as the sodium salt) were treated with D_2O by repeated addition and evaporation of their solutions prior to the n.m.r. analysis, and these ^{2}H-exchanged products were dissolved in D_2O to give solutions containing ~ 25 mg per 0.5 mL of deuterium oxide. Mass spectra were recorded at the Biomedical Mass Spectrometry Unit, McGill University, Montreal. T.l.c. was carried out with Silica Gel plates (F_{250}, Merck), and the spots were detected by charring with 8% H_2SO_4. Column chromatography was performed on Silica Gel Merck (230–400 mesh ASTM), the loadings being in the range of 1:25–1:100. Solvents were dried over molecular sieves or anhydrous

$MgSO_4$. Solutions were evaporated under diminished pressure at 40°, or by lyophilization. Elemental analyses were performed with a CEC Model 240 XA CHN Analyzer.

Methyl 2-benzyloxycarbonylamino-2-deoxy-α-D-glucopyranoside (**8**). — 2-Benzyloxycarbonylamino-2-deoxy-D-glucose (0.6 g, 1.92 mmol) was dissolved in methanol (30 mL) and the solution was refluxed for 6 h with Amberlite IR 120 (H^+) (3 g) cation-exchange resin as the catalyst. After removal of the ion-exchange resin, the solution was concentrated to dryness. The white powder crystallized from ethanol to yield **8** (6.39 g, 62%), m.p. 152–154°, $[\alpha]_D^{20}$ + 77°(*c* 1.16, pyridine); lit.[9] m.p. 154–155°, $[\alpha]_D$ + 80° (pyridine)].

Methyl 4-6-O-benzylidene-2-benzyloxycarbonylamino-2-deoxy-α-D-glucopyranoside (**9**). — Methyl 2-benzyloxycarbonylamino-2-deoxy-α-D-glucopyranoside (**8**) (0.4 g, 1.22 mmol) and $ZnCl_2$ (anhydrous, 0.17 g, 1.22 mmol) were stirred in redistilled benzaldehyde (5 mL) at 40° for 3 h. The mixture was partitioned between chloroform and ice–water, and the organic phase was washed with water, dried ($MgSO_4$), and concentrated. Crystallization from dichloromethane–petroleum ether gave **9** (0.40 g, 80%), m.p. 205–208°, $[\alpha]_D^{20}$ +28° (*c* 1.0, *N,N*-dimethylformamide); lit.[10] m.p. 214–215°, $[\alpha]_D$ +47° (chloroform).

Methyl 4,6-O-benzylidene-2-benzyloxycarbonylamino-2-deoxy-2-D-glucopyranoside 3-sulfate, sodium salt (**10**). — A mixture of **9** (0.3 g, 0.72 mmol) and sulfur trioxide–trimethylamine complex (0.21 g, 1.45 mmol) in *N,N*-dimethylformamide (3 mL) was stirred at room temperature for 24 h. Methanol (1 mL) was added and the mixture was chromatographed on a column of silica gel. Elution successively with 1:1 (v/v) chloroform–ethyl acetate and 18:1 (v/v) ethyl acetate–methanol gave a purified product which was dissolved in water, treated with cation-exchange resin Chelex 100 (Na^+), and lyophilized to yield **10** (0.25 g, 68%), m.p. 181–185°, $[\alpha]_D^{20}$ +45° (*c* 1.6, ethanol); ^{1}H-n.m.r. (CD_3OD): δ 5.61 (s, 1 H, PhCH), 5.10 (dd, 2 H, CH_2), 4.92 (d, 1 H, $J_{1,2}$ 3.42 Hz, H-1), 4.68 (dd, 1 H, $J_{2,3}$ = $J_{3,4}$ 9.79 Hz, H-3), 4.23 (dd, 1 H, $J_{5,6a}$ 5.62 Hz, H-6a), 3.88–3.68 (m, 4 H, H-2,5,6b,4), and 3.40 (s, 3 H, OCH_3); lit.[10] potassium salt of **10**, m.p. 192–193°, $[\alpha]_D$ 0° (water).

Methyl 2-amino-4,6-O-benzylidene-2-deoxy-α-D-glucopyranoside 3-sulfate, sodium salt (**11**). — A mixture of **10** (0.10 g, 0.19 mmol), 5% Pd–C (50 mg), cyclohexene[13] (2 mL), and ethanol (4 mL) was refluxed with stirring for 4 h. After filtration and evaporation, the residue was purified by chromatography on a silica gel column (3:1, v/v, ethyl acetate–methanol) to give a product which crystallized from methanol to give crystalline **11** (0.065 g, 88%), m.p. 238–241°, $[\alpha]_D^{20}$ +45° (*c* 0.66, methanol); ^{1}H-n.m.r. (CD_3OD): δ 5.57 (s, 1 H, PhCH), 4.88 (d, 1 H, H-1), 4.62 (dd, 1 H, $J_{2,3}$ = $J_{3,4}$ 9.84 Hz, H-3), 4.24 (dd, 1 H, $J_{5,6a}$ 5.4 Hz, H-6a), 3.82–3.62 (m, 3 H, H-4,5,6b), 3.45 (s, 3 H, OCH_3), and 3.24 (dd, 1 H, $J_{1,2}$ 3.18 Hz, H-2).

Anal. Calc. for $C_{14}H_{18}O_8NNaS$: C, 43.86; H, 4.74; S, 8.36. Found: C, 43.68; H, 4.60; S, 8.25.

Methyl 4,6-O-benzylidene-2-deoxy-2-sulfamino-α-D-glucopyranoside 3-sulfate, disodium salt (**12**). — A solution of **11** (0.1 g, 0.261 mmol) and sulfur trioxide–trimethylamine complex (0.14 g, 0.78 mmol) in *N,N*-dimethylformamide (2 mL) was

stirred at room temperature for 30 h. The mixture was chromatographed on a column of silica gel. Elution successively with 1:1 (v/v) chloroform–methanol and 1:3 (v/v) ethyl acetate–methanol gave a purified product which was dissolved in water, treated with cation-exchange resin Chelex 100 (Na^+), and lyophilized to yield **12** (0.089 g, 71%), m.p. 221–222° (dec.), $[\alpha]_D^{20}$ + 22° (*c* 0.57, water); ^{1}H-n.m.r. (D_2O): δ 5.78 (s, 1 H, PhCH), 5.14 (d, 1 H, $J_{1,2}$ 3.46 Hz, H-1), 4.55 (dd, 1 H, $J_{3,4}$ 9.38 Hz, H-3), 4.36 (dd, 1 H, $J_{5,6a}$ 5.96 Hz, H-6a), 3.96–3.91 (m, 3 H, H-4,5,6b), 3.58 (dd, 1 H, $J_{2,3}$ 10.3 Hz, H-2), and 3.47 (s, 3 H, OCH_3); f.a.b.m.s. (glycerol): *m/z* 485 (M), 486 (M + 1), and 508 (M + 1 + Na).

Anal. Calc. for $C_{14}H_{19}O_{11}NNa_2S_2 \cdot H_2O$: C, 33.40; H, 3.81; S, 12.74. Found: C, 33.37; H, 3.91; S, 12.91.

Methyl 2-amino-2-deoxy-α-D-glucopyranoside 3-sulfate (**13**). — A solution of **12** (0.3 g, 0.783 mmol) in 80% acetic acid (10 mL) was stirred at room temperature for 36 h when t.l.c. (1:1, v/v, ethyl acetate–methanol) indicated the reaction was completed. After evaporation, the residue was filtered through a short silica gel column (4 mL), the filtrate was adjusted with 0.1M NaOH to pH 7 and lyophilized to give **13** as an amorphous solid (0.19 g, 83%), $[\alpha]_D^{20}$ + 72° (*c* 0.8, water); ^{1}H-n.m.r. (CD_3OD): δ 4.90 (d, 1 H, $J_{1,2}$ 3.43 Hz, H-1), 4.43 (m, 1 H, $J_{3,4}$ 4.69 Hz, H-3), 3.83 (s, 2 H, H-6a,6b), 3.64 (d, 2 H, H-4,5), 3.46 (s, 3 H, OCH_3), and 3.11 (dd, 1 H, $J_{2,3}$ 9.91 Hz, H-2); lit.[10], m.p. 208–210°, $[\alpha]_D$ + 124° (water).

Methyl 2-deoxy-2-sulfamino-α-D-glucopyranoside 3-sulfate, disodium salt (**7**). — Compound **13** (0.10 g, 0.339 mmol) was dissolved in water and Na_2CO_3 (0.072 g, 0.678 mmol) was introduced. The solution was stirred for 1 h and added to sulfur trioxide–trimethylamine complex (0.07 g, 0.51 mmol). The mixture was stirred at 55° for 24 h, cooled, and filtered through a short silica gel column (4 mL). The filtrate was applied to a column of Amberlite IR-120 (H^+) cation-exchange resin (6–7 mL) to remove ammonium ions, titrated to pH 9.2 with 0.1M NaOH, and lyophilized to give **7** as a white, hygroscopic, and fluffy powder (0.094 g, 70%), $[\alpha]_D^{20}$ + 29° (*c* 2.57, water); ^{1}H-n.m.r. (D_2O): δ 5.03 (d, 1 H, $J_{1,2}$ 3.42 Hz, H-1), 4.33 (dd, 1 H, $J_{3,4}$ 8.25 Hz, H-3), 3.87 (dd, 1 H, $J_{5,6a}$ 2.25 Hz, $J_{6a,6b}$ 12.21 Hz, H-6a), 3.78 (dd, 1H, $J_{5,6b}$ 4.45 Hz, H-6b), 3.70 (m, 1 H, H-5), 3.64 (dd, 1 H, $J_{4,5}$ 8.06 Hz, H-4), 3.41 (s, 3 H, OCH_3), and 3.37 (dd, 1 H, $J_{2,3}$ 10.69 Hz,H-2); f.a.b.m.s. (glycerol): *m/z* 397 (M) and 420 (M + Na).

Anal. Calc. for $C_7H_{13}O_{11}NNa_2S_2 \cdot H_2O$: C, 20.24; H, 3.65; S, 15.44. Found: C, 20.16; H, 3.36; S, 15.69.

Effect on the 3-sulfate **7** *of alkaline conditions used to desulfate heparin.* — A solution consisting of a mixture of **7** (25 mg) and heparin (40 mg) in water (10 mL) was adjusted to pH 11.8 with 0.1M NaOH, and then lyophilized. The powdery residue was dissolved in water (20 mL) and subjected to dialysis (Spectrapor dialysis membrane, Spectrum Medical Industries, Los Angeles, CA; mol. wt. cut off, 3500), following which the dialyzate and the solution inside the membrane were examined by ^{1}H-n.m.r. spectroscopy in D_2O (deuterium-exchanged) (see Fig. 1).

Solvolysis of the pyridinium salt of the 3-sulfate (**7**). — A solution of **7** (25 mg) in water (10 mL) was passed through a column of IR-120 (H^+) cation-exchange resin. The acidic effluent and washings were combined, neutralized (pH 6.5) with pyridine, and

lyophilized. Dissolved in dimethyl sulfoxide containing 5% water (3 mL), the pyridinium salt was subjected to *N*-desulfation conditions for 90 min at 55°; water (3 mL) was introduced, the solution was lyophilized, and the residue in D_2O (deuterium-exchanged) was examined by ^1H-n.m.r. spectroscopy.

ACKNOWLEDGMENTS

The authors thank the Natural Sciences and Engineering Research Council of Canada for generous support.

REFERENCES

1. M. Jaseja, R. N. Rej, F. Sauriol, and A. S. Perlin, *Can J. Chem.*, 67 (1989) 1449–1456.
2 R. N. Rej, M. Jaseja, and A. S. Perlin, *Thromb. Haemostas.*, 61 (1989) 540.
3 R. N. Rej and A. S. Perlin, *Carbohydr. Res.*, 200 (1990) 437–447.
4 U. Lindahl, G. Backstrom, M. Hook, L. Thunberg, L.-A. Fransson, and A. Linker, *Proc. Natl. Acad. Sci. U.S.A.*, 76 (1979) 3198–3202; U. Lindahl, G. Backstrom, L. Thunberg, and I. G. Leder, *Proc. Natl. Acad. Sci. U.S.A.*, 77 (1980) 6551–6555.
5 G. Torri, B. Casu, G. Gatti, M. Petitou, J. Choay, J. C. Jacquinet, and P. Sinaÿ, *Biochem. Biophys. Res. Commun.*, 128 (1985) 134–140.
6 B. Mulloy and E. A. Johnson, *Carbohydr. Res.*, 170 (1987) 151–165.
7 J. R. Turvey, *Adv. Carbohydr. Chem.*, 20 (1966) 183–218.
8 A. B. Foster, E. F. Martlew, and M. Stacey, *Chem. Ind. (London)*, (1953) 899–901.
9 A. Neuberger and R. Pitt Rivers, *J. Chem. Soc.*, (1939) 122–126.
10 I. G. Leder, *J. Carbohydr. Chem.*, 7 (1988) 583–592.
11 M. Petitou, P. Duchaussoy, I. Lederman, J. Choay, P. Sinaÿ, J. C. Jacquinet, and G. Torri, *Carbohydr. Res.*, 147 (1986) 221–236.
12 Y. Inoue and K. Nagasawa, *Carbohydr. Res.*, 46 (1976) 87–95.
13 G. M. Anantharamaih and K. M. Sivanandaiah, *J. Chem. Soc., Perkin Trans. 1*, (1977) 490–491.