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# Regioselectivity in the sulfation of dermatan sulfate and methyl 4,6-*O*-benzylidene-α-D-idopyranoside

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ABSTRACT

The sulfation of dermatan sulfate by $SO_3$–trimethylamine in *N,N*-dimethylformamide led to substitution initially at HO-6 of residues of 2-acetamido-2-deoxy-β-D-galactopyranosyl 4-sulfate (**1**), to produce the 4,6-disulfate (**6**). When this step reached a level of > 50%, sulfation occurred with equal facility at HO-2 and HO-3 of residues of α-L-idopyranosyluronic acid (**2**), giving rise to a mixture of 2-, 3-, and 2,3-disulfates. An analogous substitution pattern was observed for HO-2 and -3 of a simpler idopyranose unit, in the sulfation of methyl 4,6-*O*-benzylidene-α-D-idopyranoside (**12**). This lack of regioselectivity in the reaction of **2** (and **12**) contrasts markedly with the high affinity of the reagent for HO-3 of residues of α-L-idopyranosyluronic acid present in a modified form of heparin. It is attributed to a difference between the two polymers in the relative orientation of their neighboring amino sugar residues, whereby there is an unobstructed access of the reagent in one instance, and hindrance of HO-2 selectively in the other. Enzymolysis by chondroitinase ABC was found to yield unsaturated disaccharide containing residues of 4,6-disulfate, as well as larger fragments containing unsaturated glycosyl groups derived from L-idopyranosyluronic acid 2-sulfate, evidence of a relatively broad enzyme specificity. The presence of extra sulfate groups in dermatan sulfate did not enhance its weak antithrombotic activity, as measured by anti Xa assay, in disagreement with earlier reports.

## INTRODUCTION

The importance of heparin as an anticoagulant and antilipiemic agent has stimulated an interest in the biological properties of many other polysaccharide sulfates, as well as in the preparation and properties of sulfated forms of a wide variety of polysaccharides. In comparison with heparin, the related glycosaminoglycan, dermatan sulfate, is a weak anticoagulant[1–4], although its effectiveness was reported[3] to be enhanced by the introduction of an additional 1.4 sulfate group per disaccharide unit. Considering the main repeating disaccharide unit of the polymer (**1**→**2**), this increase would be expected to have involved more rapid substitution of HO-6 of the 2-acetamido-2-deoxy-β-D-galactopyranosyl 4-sulfate residue (**1**) than either of the secondary 2- or 3-OH groups of the α-L-iduronic acid unit (**2**). As the sites of substitution on the dermatan sulfate had not been indicated, we have examined this question for comparison with results obtained[5] on the sulfation of a partially-desulfated form of heparin. The latter polymer also contains[6] a residue of α-L-idopyranosyluronic acid (**4**, together with amino sugar unit **3**) which was found[5] to give rise selectively to the 3-sulfate unit **5**.

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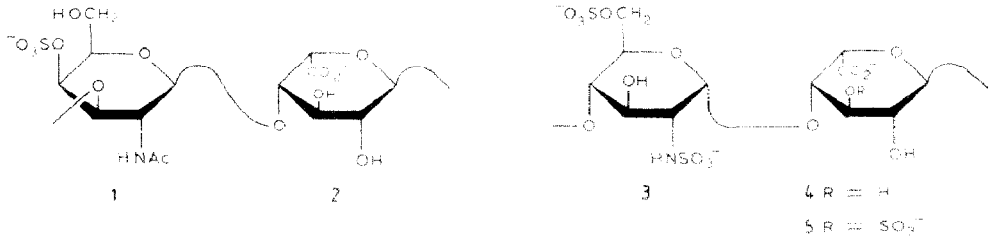
Accordingly, the point addressed here is how closely analogous are the residues of L-idopyranosyluronic acid (**2** and **4**) in dermatan sulfate and the modified heparin with respect to regioselectivity in sulfation reactions. Also examined was the sulfation of methyl 4,6-*O*-benzylidene-α-D-idopyranoside, because it appeared to afford a relatively simple model of how HO-2 and -3 of an α-idopyranosyl residue might undergo substitution under the experimental conditions used for the polymers.




## RESULTS AND DISCUSSION

*Sulfation* of dermatan sulfate.* — The conditions reported[3] for the sulfation of dermatan sulfate entailed the use of the sodium salt of the polymer in reaction with sulfur trioxide and pyridine in *N*,*N*-dimethylformamide at 60° for 5 h. We chose conditions more closely comparable to those applied[5] to the modified heparin, *i.e.*, the dermatan sulfate was converted into the pyridinium salt, and then treated with sulfur trioxide·trimethylamine complex in *N*,*N*-dimethylformamide at 5, 25, or 55° for various periods.

On sulfation of the polymer at 5° for 20 h, its $^1$H-n.m.r. spectrum (Fig. 1A) was altered primarily (see Fig. 1B) in the region of the A-6,6′ protons of residue **1**. That is, there was a marked decrease in the relative intensity of the broad group of signals for A-5, 6,6′ at $\delta$ 3.8, and the emergence of new A-6,6′ signals at $\delta$ 4.2. This deshielding effect of 0.4 p.p.m. was consistent[7] with the anticipated, selective, sulfation initially of the primary hydroxyl group of **1**, and formation of the 4,6-disulfate (**6**).

With an increase in the level of substitution, by reaction at 25° for 2.5 h, a number of additional spectral changes were observed** (Fig. 1C). New peaks at $\delta$ 5.1 and ~5.25 are especially noteworthy. A $^1$H $^1$H correlation (COSY) version[8] of the spectrum indicated that the latter signal is due to the anomeric proton (U-1) of a residue of L-idopyranosyluronic acid 2-sulfate (**7**), and that at $\delta$ 5.1 to U-1 of the 3-sulfate (**8**). These assignments are based upon the deshielding expected[7] of U-1 of **2** ($\delta$ 4.9, Fig. 1A), which amounts to a value of 0.24 p.p.m. when a 2-sulfate substituent is introduced, and by 0.15 p.p.m. due to a 3-sulfate (see Table I). The presence of a minor shoulder at $\delta$ 5.25

* The term "sulfation" is used in the sense that sulfate groups are formed. More exactly, the *sulfonation* of a hydroxyl group occurs to give a sulfate (or *O*-sulfo) substituent.

** *Note added in press:* These observations support those on the sulfation of dermatan sulfate, reported in an article[8] that appeared after the present work was submitted for review.

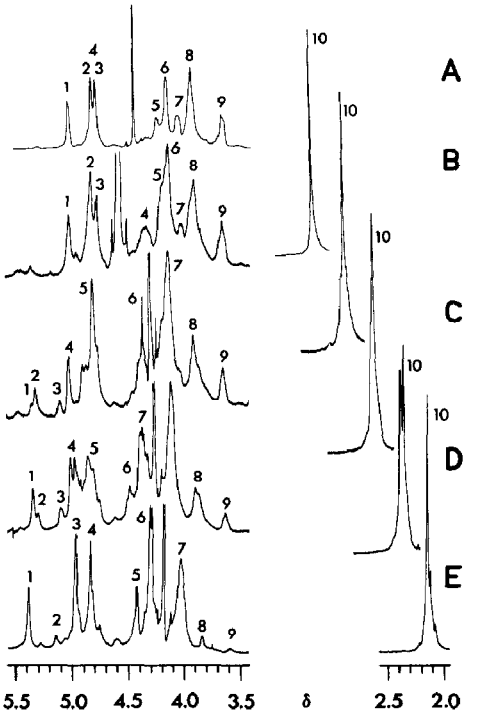
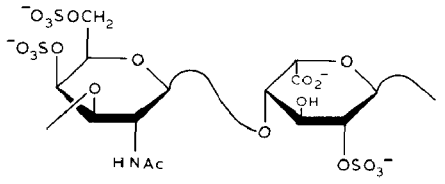
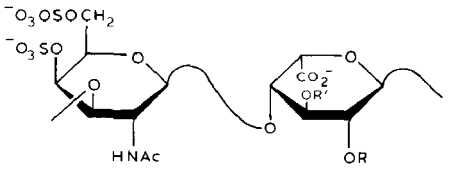



Fig. 1. $^1$H-N.m.r. spectra (300 MHz; solvent, $D_2O$) of: (A) Dermatan sulfate (at 70°); signal designations: 1, I-1; 2, I-5; 3, A-4; 4, A-1; 5, I-4; 6, A-2, -3; 7, I-3; 8, A-5, -6,6′; 9, I-2; and 10, $COCH_3$ (A = **1**; I = **2**). (B) Product of sulfation at 5° for 20 h (at 55°); signal designations: 1, I-1; 2, I-5, $A_s$-4; 3, A-1, 4; 4, $A_s$-6,6′; 5, I-4; 6, A-2, -3, $A_s$-5; 7, I-3; 8, A-5, -6,6′; 9, I-2; and 10 $COCH_3$ (I = **2**; $A_s$ = **6**). (C) Product of sulfation at 25° for 2.5 h (at 85°); signal designations: 1, U-1, 2, $U_2$-1; 3, $U_3$-1; **4**, I-1; 5, contains $U_3$-3,5; 6, contains $A_s$-6,6′, $U_2$-2; 7, contains $A_s$-5; 8, contains $U_3$-2; 9, I-2; and 10, $COCH_3$ (I = **2**; $A_s$ = **6**; $U_2$ = **7**; $U_3$ = **8**; U = **9**). (D) Product of sulfation at 25° for 20 h (at 85°); signal designations: 1, U-1; 2, $U_2$-1; 3, $U_3$-1; 4, contains U-3, -5; 5, contains $U_3$-3,-5; 6, contains U-2; 7, contains U-4; 8, contains $U_3$-2; 9, I-2; and 10, $COCH_3$ ($U_2$ = **7**; $U_3$ = **8**; U = **9**). (E) Product of sulfation at 55° for 20 h (at 85°); signal designations: 1, U-1; 2, $U_3$-1; 3, U-3, -5; **4**, $A_s$-1, -4; 5, U-2; 6, $A_s$-6,6′, U-4; 7, $A_s$-2, -3, -5; 8, $U_3$-2; 9, I-2; and 10, $COCH_3$ (I = **2**; $A_s$ = **6**; $U_3$ = **8**; U = **9**). The chemical shift ($\delta$) of the offset $COCH_3$ signals (No. 10) in each spectrum (A–D) is approximately the same as that in spectrum E.

(Fig. 1C) is commensurate, in the COSY spectrum, with a small proportion of substitution at both HO-2 and -3 (as in **9**). A $^{13}$C-n.m.r. spectrum of this product showed a level of sulfation at C-6 of ~ 75%, from the greatly reduced intensity of the A-6 signal of **1** remaining at $\delta$ 63.8.




8 R = H, R′ = $SO_3^-$

9 R = R′ = $SO_3^-$

TABLE I

Chemical shift displacements[a] of $^1H$ of the L-idopyranosyluronic acid residue (**2**) of dermatan sulfate, and of methyl 4,6-*O*-benzylidene-α-D-idopyranoside (**12**) and -altropyranoside (**16**), caused by the introduction of sulfate groups

| | *Residue or compound* | | | | | | |
|---|---|---|---|---|---|---|---|
| | **2** | *2-sulfate* (**7**) | | *3-sulfate* (**8**) | | *2,3-disulfate* (**9**) | |
| | δ | δ | Δδ | δ | Δδ | δ | Δδ |
| H-1 | 4.97 | 5.21 | 0.24 | 5.12 | 0.15 | 5.36 | 0.39 |
| H-2 | 3.58 | 4.18 | 0.60 | 3.82 | 0.24 | 4.40 | 0.82 |
| H-3 | 3.97 | 4.35 | 0.38 | 4.82 | 0.85 | 4.95 | 0.98 |
| H-4 | 4.12 | 4.28 | 0.16 | 4.28 | 0.16 | 4.29 | 0.17 |
| H-5 | 4.75 | 4.72 | −0.03 | 4.82 | 0.07 | 4.95 | 0.20 |
| | *Residue or compound* | | | | | | |
| | **12** | *2-sulfate* (**14**)[b] | | *3-sulfate* (**15**)[b] | | *2,3-disulfate* (**13**) | |
| H-1 | 4.79 | 5.07 | 0.28 | 4.89 | 0.10 | 5.07 | 0.28 |
| H-2 | 3.70 | 4.29 | 0.59 | 3.89 | 0.19 | 4.47 | 0.77 |
| H-3 | 3.86 | 4.12 | 0.26 | 4.47 | 0.61 | 4.68 | 0.82 |
| H-4 | 4.22 | 4.12 | −0.10 | 4.39 | 0.17 | 4.36 | 0.14 |
| H-5 | 4.01 | 4.00 | −0.01 | 4.00 | −0.01 | 4.02 | 0.01 |
| | *Residue or compound* | | | | | | |
| | **16** | *2-sulfate* (**17**) | | | | *2,3-disulfate* (**18**) | |
| H-1 | 4.73 | 4.94 | 0.21 | | | 4.93 | 0.20 |
| H-2 | 3.97 | 4.49 | 0.52 | | | 4.86 | 0.89 |
| H-3 | 4.12 | 4.35 | 0.23 | | | 4.93 | 0.81 |
| H-4 | 4.12 | 4.05 | −0.07 | | | 4.21 | 0.09 |
| H-5 | 3.91 | 3.90 | −0.01 | | | 3.92 | 0.01 |

[a] For a solution in $D_2O$ at 85°; values of Δδ are in p.p.m. relative to the nonsulfated analog. Other chemical shift parameters, and data for **12**–**18** recorded for solutions in $CD_3OD$, are given in the Experimental section. [b] From the spectrum of the mixture.

By prolonging the reaction at 25° to 20 h, the 2,3-disulfate (**9**) became far more prominent a product than either the 2- or 3-monosulfate, as seen from the strength of its U-1 signal (δ 5.36) in Fig. 1D. Corresponding to this change is the observation that a much weaker signal for U-2 of **2** remains (δ 3.58), being balanced by the U-2 signal of **9**, located downfield by 0.82 p.p.m., owing to the influence of the 2-sulfate and the adjacent 3-sulfate group. A COSY spectrum helped to locate the signals of various other protons that experienced deshielding to a greater or lesser extent through substitution vicinally or more remotely, *e.g.*, the signals for U-2 and U-3 of **9**, and A-4 and A-5 of **8**. As the A-6 signal of **1** in the $^{13}C$-n.m.r. spectrum of the product (Fig. 2) was only a minor one, almost complete substitution at the 6-position[10] of the aminodeoxyhexose residues had taken place at this stage. Also worthy of note in the $^{13}C$-spectrum is the splitting of the

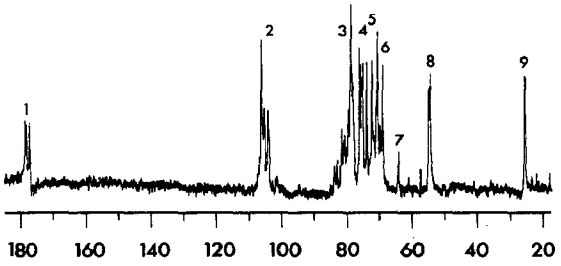



Fig. 2. $^{13}$C-N.m.r. spectrum (75.4 MHz; solvent, $D_2O$; 25°) of the product of sulfation of dermatan sulfate at 25° for 20 h; signal designations: 1, CO, I-6, U-6; 2, A-1, $A_s$-1, I-1, U-1; 3, contains $A_s$-4, U-4; 4, I-3; 5, I-2; 6, $A_s$-6; 7, A-6; 8, $A_s$-2; and 9, $CH_3$ (A = **1**; I = **2**; $A_s$ = **6**; U = **9**).

signal of the acetamidomethyl group ($\delta$ 26) into almost equal components [which is matched by an analogous pair of $^1$H signals ($\delta$ 2.1–2.2)], as well as of the $^{13}$C-resonance ($\delta$ 55) for A-2 of the amino sugar residues. This is taken to be a reflection of inhomogeneity among their neighboring uronic acid residues, which comprise an approximately 1:1 mixture of **2** and **9** in the polymer, as is the splitting of the anomeric ($\delta$ 102–104) and carbonyl ($\delta$ 178–180) $^{13}$C-resonances.

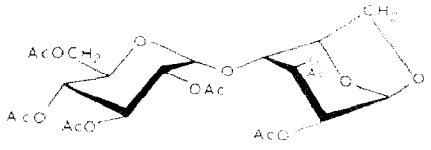
Finally, a modified dermatan sulfate made up almost entirely of residues of **6** and **9** was obtained by reaction at 55° for 20 h. The high degree of structural homogeneity of this material is evident from the relative simplicity of its $^1$H-n.m.r. spectrum (Fig. 1E, the assignments given being confirmed from a COSY version), as well as of its $^{13}$C-spectrum which, as required, consists mainly of 14 resonances.

Increments in the sulfate content of these products, over that in dermatan sulfate, were also monitored by i.r. spectroscopy, which showed a progressive increase in the intensity of the sulfate absorption band ($\nu$ 1240 $cm^{-1}$) relative to that of the carboxyl band ($\nu$ 1420 $cm^{-1}$).

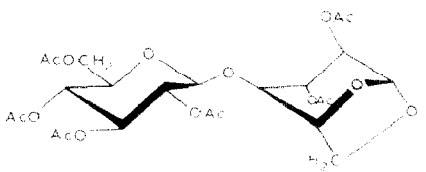
*Contrast between the sulfation of the* L-*idopyranosyluronic residue (***2***) of dermatan sulfate and that (***4***) of a modified heparin.* — The foregoing observations showed that in the reaction of dermatan sulfate with sulfur trioxide·trimethylamine, the primary alcohol group of residue **1** is most readily substituted, as expected. Subsequently, HO-2 and -3 of residue **2** are sulfated at about the same rate, leading progressively to the 2,3-disulfate (**8**). This lack of regioselectivity is in marked contrast to the finding that the α-L-idopyranosyluronic acid residue (**4**) in a partially-desulfated heparin (**3**→**4**) undergoes substitution with high selectivity for HO-3. Hence, despite their close identity, residues **2** and **4** differ widely in the case of substitution of the HO-2 group.

Possibly, this distinction is related to the observation[11,12] that residue **2** of dermatan sulfate is more highly susceptible to periodate oxidation than are the (minor) constituent residues of **4** in heparin. In accounting for the difference, it has been proposed[11] that the conformations of these residues – and hence the relative orientations of their 2,3-diol groups – are not the same. However, the fact that their $^1$H- and $^{13}$C-n.m.r. parameters are closely similar[5,8,13–15] indicates that **2** and **4** have analogous conformations (or mixtures of conformations; see ref. 16).

Another possibility is that the chemical reactivities of **2** and **4** are influenced by differences in the way they are aligned with respect to the adjacent aminodeoxyhexose constituents (**1** and **3**). If HO-2 of **4** were to be engaged in interresidue hydrogen bonding, for example, but not HO-2 or -3 of **2**, then the selectivity for position-3 of **4** might be understandable. Support for this proposal appears to be available from conformational studies[17] on oligosaccharides related to the types of structures involved here. In several oligosaccharides related to heparin, the 2-NHR group of the aminodeoxy-α-D-hexose residue attached (1→4) to the α-L-idopyranosyluronic acid residue is relatively close (more or less, depending on the pattern of sulfate substituents) to O-2 of the acid residue. Another indication of a close interresidue proximity effect of this kind is found[18], even in the absence of hydrogen-bonding, in the molecular conformation of 1,6-anhydro-β-maltose hexaacetate. As seen in partial formula **10**, it serves as a model of a 1,2-*a,e* D-glycosyl unit attached (1→4) to a 2,3,4-*a,a,a* α-L-glycose moiety. The orientation of the two rings with respect to each other appears to favor a near approach of O-2 and O-2′.



**10**



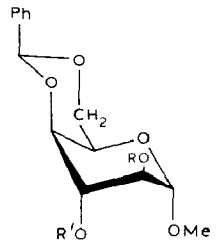
**11**

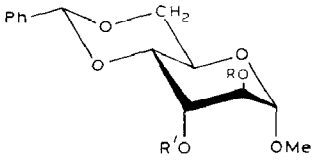
Although comparable information about oligosaccharides in the dermatan sulfate series is not available, the β anomer of **10** (represented by **11**, a partial structure of 1,6-anhydro-β-cellobiose hexaacetate) may be regarded as a model for the 1,2-*e,e* β-D-glycosyl type of residue (**1**). It is worth noting, therefore, that O-2 of the cellobiose derivative appears[19] to be sufficiently remote from O-2′ and O-3′ as to render any interresidue interaction relatively unlikely.

According to these conjectures, therefore, residue **2** of dermatan sulfate need not be expected to undergo substitution with as high a degree of regioselectivity as residue **4** of the modified heparin. The resistance to substitution of HO-2 in the latter may not be caused by an interresidue hydrogen-bond *per se*, inasmuch as some hydrogen-bonded hydroxyl groups exhibit[20] *enhanced* rates of esterification, but as a result of hindered access to HO-2 in the conformation anticipated for the repeating unit (**3**→**4**).

*Sulfation of methyl 4,6-O-benzylidene-α-D-idopyranoside.* — If regioselectivity in the sulfation of L-idopyranosyluronic acid residues (**2**) of dermatan sulfate is not materially influenced by the adjacent residues **1**, then an analogous sulfation pattern might be found with a "simpler" α-idopyranoside. Accordingly, methyl 4,6-*O*-benzylidene-α-D-idopyranoside (**12**) was examined. Its reaction with sulfur trioxide-trimethylamine in *N,N*-dimethylformamide at temperatures ranging from −40° to 50° was found (by t.l.c. examination) to afford mainly the 2,3-disulfate (**13**), accompanied by minor proportions of monosulfated material. In preparative runs, the latter was

shown to be a 1:1 mixture of the 2- and 3-monosulfate (**14** and **15**), which were not adequately separated, but readily identified in admixture by n.m.r. spectroscopy (see Table I). Data for the disulfate are also given.




**12** R = R′ = H
**13** R = R′ = $SO_3^-$
**14** R = $SO_3^-$, R′ = H
**15** R = H, R′ = $SO_3^-$

**16** R = R′ = H
**17** R = $SO_3^-$, R′ = H
**18** R = R′ = $SO_3^-$

Clearly, therefore, the sulfation characteristics of HO-2 and -3 of idoside **12** are analogous to those observed for residue **2** of dermatan sulfate, in that there is no detectable difference in reactivity between the hydroxyl groups. In both the polymer and the monosaccharide derivative, these groups appear to be unimpeded in reaction with the sulfating reagent, which reinforces the likelihood that the regioselectivity observed for HO-3 of the L-idopyranosyluronic acid residue (**4**) in the modified heparin is due to hindered access of the reagent to HO-2.

*Sulfation of methyl 4,6-O-benzylidene-α-D-altropyranoside.* — Another pair of diaxially-oriented 2,3-*trans* hydroxyl groups is found in methyl 4,6-*O*-benzylidene-α-D-altropyranoside (**16**). In this instance, substitution occurred more readily at HO-2 than HO-3. An approximately 4:3 mixture of the 2-sulfate (**17**) and 2,3-disulfate (**18**) was obtained, whereas the 3-sulfate was not detected. Although this may reflect some shielding of HO-3 by the *cis-eq.* O-4 substituent, in contrast to the *trans-ax.* orientation of O-4 in the *ido* diastereomer, nevertheless both of the hydroxyl groups are found, once again, to react in substantial measure with the sulfating reagent.

Chemical shift data utilized for the characterization of the sulfation products **13**–**15**, and **17** and **18** are grouped in Table I. They uniformly reflect well-known deshielding influences of the sulfate groups on adjacent and neighboring protons, and are seen to be closely comparable to the data included in the Table for the products of sulfation of dermatan sulfate.

*Enzymolysis of sulfated dermatan sulfate by chondroitinase ABC.* — It was of interest to determine the effect of additionally introduced sulfate groups on the polymers' susceptibility towards chondroitinase ABC. This readily-available mixture of enzymes degrades dermatan sulfate almost completely to α,β-unsaturated disaccharide[21] **19**. Whereas the most highly sulfated product (represented by Fig. 1 E) appeared to be unaffected by the enzyme, substantial degradation was observed with the less

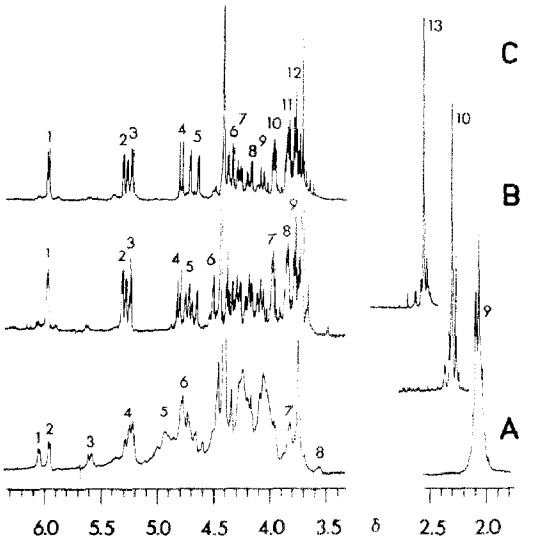
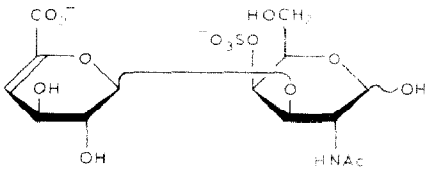
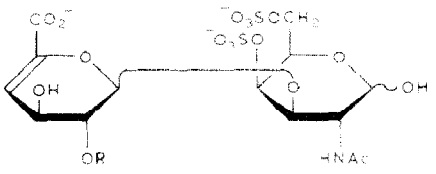



Fig. 3. $^1$H-N.m.r. spectra (300 MHz; solvent, $D_2O$; 60°) of: (A) The disaccharide (**19**) produced from dermatan sulfate by chondroitinase ABC; signal designations: 1, U-4; 2, U-1; 3, A-1α; 4, A-1β; 5, A-4α, β; 6, A-2α; 7, A-3α, -5α; 8, A-3β; 9, A-2β; 10, U-3; 11, U-2; 12, A-6,6′α, β, A-5β; and 13, $COCH_3$ (U, uronic acid residue; A, aminodeoxyhexose residue). (B) The mixture of disaccharides **19** and **20** produced from a sulfated (2.5 h at 25°) dermatan sulfate by chondroitinase ABC (dialyzate); signal designations: 1, U-4; 2, U-1; 3, A-1α; 4, A-1β; 5, A-4α, β, $A_s$-4α, β; 6, $A_s$-6; 7, U-3; 8, U-2; 9, A-6, A-5β; and 10, $COCH_3$ (U, uronic acid residue; A, aminodeoxyhexose residue in **19**; $A_s$, aminodeoxyhexose residue in **20**. (C) The nondialyzable component corresponding to (B); signal designations: 1, $U_3$-4; 2, U-4; 3, $U_3$-1; 4, U-1, $U_3$-2; 5, contains I-1, $U_3$-1; 6, contains A-4, A-1, I-5; 7, contains U-2; 8, I-2; and 9, $COCH_3$ (U, uronic acid residue, as in **20**; $U_s$, uronic acid 2-sulfate residue as in **21**; A = **1**; I = **2**). The sharp singlet at δ 3.7 is due to the presence of an enzyme contaminant.

substituted materials that gave the spectra in Figs. 1B and 1C. The enzymolysis product corresponding to the latter was separable into dialyzable (mol. wt. cut-off, 3500) and nondialyzable fractions, represented by the $^1$H-n.m.r. spectra shown in Figs. 3B and 3C, respectively. The former is closely similar in many respects to the spectrum in Fig. 3A, which is that of the product (**19**) of enzymolysis of unmodified dermatan sulfate. It differs mainly in having signals attributable to more strongly deshielded A-6,6′ protons (δ ~4.5). As the polymer had been heavily sulfated at HO-6, this shows that the dialyzable product is disaccharide **20**, and hence that the enzyme specificity is compat-



**19**



**20** R = H

**21** R = $SO_3^-$

TABLE II

Anticoagulant activity of sulfated modifications of dermatan sulfate

| *Substrate* | *Anti Xa assays*[a] *at a concentration of (μg/mL)* | | | | *aPTT assays*[b] *[clotting time(s)]* |
|---|---|---|---|---|---|
| | 5 | 12.5 | 25 | 50 | |
| Dermatan sulfate (unmodified) | 23 | 46 | 83 | 155 | 31 |
| Sulfated at 5° for 20 h | 17 | 36 | 74 | 129 | 39 |
| Sulfated at 25° for 2.5 h | 16 | 26 | 58 | 109 | 72 |
| Sulfated at 25° for 20 h | 16 | 33 | 65 | 127 | 72 |
| Sulfated at 55° for 20 h | 12 | 25 | 49 | 98 | 49 |

[a] In units/mg, relative to a standard curve based on porcine mucosal heparin (160 units/mg). [b] Relative to a serum clotting time for porcine mucosal heparin (165 IU/mg) of > 500 s.

ible with the presence of the 4,6-disulfate residue (**6**), which is also a constituent of **20**, as well as the 4-sulfate (**1**) present initially. The spectrum (Fig. 3C) of the nondialyzable material provided evidence that some residues of L-idopyranosyluronic acid 2-sulfate (**7**) also had been involved in the enzymolysis*. This is seen from the $\delta$ 6.05 signal assigned to an olefinic U-4 proton; its location 0.1 p.p.m. downfield of the U-4 signal of disaccharides **19** or **20** is compatible with deshielding by a 2-sulfate substituent (as in **21**). Also worth noting is the marked reduction in the intensity of the U-2 signal (in Fig. 3C) of residues of unsubstituted **2** (at $\delta$ 3.58 in spectra of Fig. 1), which is here accounted for by the corresponding proton of dialyzable disaccharide **20**.

*Anticoagulant properties of the products of sulfation of dermatan sulfate.* — Measurements of anticoagulant activity were carried out, based on the facility with which the sulfation products inactivate Factor Xa (anti Xa assay), or prolong clot formation [activated partial thromboplastin time (aPPT)]. The data in Table II show that over a 10-fold range of concentration the anti Xa activity in all instances is reduced with respect to the unmodified dermatan sulfate. Therefore, the results are at variance with the earlier indication[3] that an increase in the level of sulfation of the polymer increases its anti Xa potency**.

According to the aPTT assays, the introduction of extra sulfate groups does promote a slight enhancement of the clotting time (Table II), although the maximum value was only 72 s as compared with that of > 500 s for beef lung heparin at the same level.

## EXPERIMENTAL

*General methods.* — Melting points were determined with a Fisher–Johns appara-

* Although residues of **7** in heparin undergo elimination[22] catalyzed by heparinase, this latter enzyme was found to have no effect on the sulfated dermatan sulfate.

** *Note added in press:* It now appears[23] that the assay procedure used earlier[3] gave deceptively high potency values.

tus, and are uncorrected. Optical rotations were determined at 25° with a Jasco Dip140 digital polarimeter. N.m.r. spectra were recorded with a Varian XL300 spectrometer operating at 300 MHz for $^1$H-, and 75.4 MHz for $^{13}$C-spectroscopy, 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 ($\delta$ 0.0) for solutions in $D_2O$, and of internal tetramethylsilane for organic solutions. The 2D ($^1$H,$^1$H) COSY and ($^{13}$C,$^1$H) HETCOR experiments were performed with the Varian pulse-sequence programs, and utilized for verifying most of the spectral assignments given. Samples (as the sodium salts) 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 3–4% (w/w) of polysaccharide for $^1$H-, and 10–20% (w/w) for $^{13}$C-spectroscopy. Thin-layer chromatography 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°.

*Sulfation of dermatan sulfate.* — The reactions were carried out at either 5, 25, or 55° for varying periods. A representative experiment was the following: Dermatan sulfate (Hepar) (92.7 mg) in water was converted into the acid form by ion-exchange, then into the pyridinium salt by neutralization (pH 6.5) of the solution with pyridine, followed by lyophilization. The solid residue was dispersed in dry *N*,*N*-dimethylformamide (20 mL), sulfur trioxide·trimethylamine complex (1.8 g) was introduced, and the mixture was stirred at 25° for 20 h. Water (60 mL) was added, the solution was dialyzed against 0.2% $NaHCO_3$ solution for 4 h, then against water for 24 h, and lyophilized. Further purification was carried out by dissolving the product in water (2 mL), introducing ethanol (5 vols.), and recovering the precipitated solid by centrifugation, then treating an aqueous solution of the precipitate with Chelex ion-exchange resin, followed by lyophilization (yield, 88.5 mg); the $^1$H- and $^{13}$C-n.m.r. spectra of the sulfation product are shown in Fig. 1D and Fig. 2, respectively. Elemental analysis gave a C:S ratio (%) of 1.00:0.52, in comparison with a ratio of 1.00:0.23 for the original dermatan sulfate, which indicated that an *extra* 1.5 mol of sulfate per mol had been introduced.

*Examination of the sulfation products by i.r. spectroscopy*[24]. — Solid state i.r. spectra of dermatan sulfate and its sulfated modifications were recorded with an FT-IR spectrometer (Mattson Instruments, Model IR10100) equipped with a photoacoustic sample cell (EG + G, Princeton Applied Research, Model 6003 with preamplifier Model 6005). Ratios of the absorbance of the band at 1240 cm$^{-1}$ ($\nu_{max}^{solid}$, SO) relative to that at 1420 cm$^{-1}$ ($\nu_{max}^{solid}$, $CO_2^-$) were as follows: dermatan sulfate, 1.10:1.00; sulfation products, 1.19:1.00 (5°, 20 h), 1.33:1.00 (25°, 2.5 h), 1.35:1.00 (25°, 20 h), and 1.47:1.00 (55°, 20 h).

*Treatment of sulfated dermatan sulfate with chondroitinase.* — A solution of the product (35.5 mg) in phosphate buffer (5.0 mL; pH 8.0) was treated with chondroitinase ABC (5 units; Sigma) at 37° for 24 h. (These conditions were selected following

preliminary $^1$H-n.m.r. observations on smaller-scale reactions conducted with solutions in $D_2O$). The mixture was heated at 100° for 1 min, then dialyzed against distilled water (3 × 300 mL), which was followed by lyophilization of the inner and outer solutions; yields, 22 and 20 mg, respectively.

*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).

*Methyl 4,6-O-benzylidene-α-D-idopyranoside* (**12**). — The method of Sorkin and Reichstein[25] was used to convert sequentially methyl α-D-galactopyranoside into methyl 4,6-*O*-benzylidene-α-D-galactopyranoside and methyl 2,3-anhydro-4,6-*O*-benzylidene-α-D-gulopyranoside. Dimethyl sulfoxide was employed in place of methanol for the latter step[26], which avoided the formation of a 3-*O*-methyl derivative of **12** as a byproduct[27], and afforded a higher yield of the anhydride (77%). Product **12** was then obtained as usual (yield 81%), m.p. 149–150°, $[\alpha]_D$ +51° (*c* 2.0, chloroform); lit.[25] m.p. 148–149°, $[\alpha]_D$ + 49° (chloroform).

*Methyl 4,6-O-benzylidene-α-D-idopyranoside 2,3-disulfate* (**13**), *2-sulfate* (**14**), *and 3-sulfate* (**15**). — A mixture of **12** (0.21 g) and sulfur trioxide·trimethylamine complex (0.24 g) in *N,N*-dimethylformamide (2 mL) was stirred at 25° for 20 h, and then applied to a column of silica gel. Elution successively with 1:1 chloroform–methanol and 2:1 ethyl acetate–methanol afforded two solid products. Each was converted in water into the sodium salt with Chelex ($Na^+$) ion-exchange resin, affording the 2,3-disulfate (**13**) (0.25 g, 72%), m.p. 190–194° (dec.), $[\alpha]_D$ +56° (*c* 1.84, water), $\nu_{max}^{solid}$ 1240 $cm^{-1}$ (S=O); and a mixture of the 2-sulfate (**14**) and 3-sulfate (**15**) (24 mg, 8%); $^1$H-n.m.r. data for H-1 to H-5 for solutions in $D_2O$ are given in Table I; other protons: $\delta$ 5.74 (PhCH), 4.30 (H-6,6′), and 3.47 ($OCH_3$) for **12**, virtually constant for **13**–**15**; for solutions in $CD_3OD$ of **13**: $\delta$ 5.60 (s, 1 H, PhCH), 5.02 (dd, 1 H, $J_{1,2}$ 0.8 Hz, H-1), 4.77 (d, 1 H, H-3), 4.51 (dd, 1 H, $J_{2,3}$ 1.9 Hz, H-2), 4.26 (dd, 1 H, $J_{3,4}$ 1.5 Hz, H-4), 4.18 (dd, 2 H, H-6,6′), 3.88 (s, 1 H, H-5), and 3.38 (s, 3 H, $OCH_3$); of **14**: $\delta$ 5.63 (s, 1 H, PhCH), 5.03 (s, 1 H, H-1), 4.35 (dd, 1 H, $J_{2,3}$ 2.5 Hz, H-2), 4.21 (m, 2 H, H-6,6′), 4.11 (dd, 1 H, H-3), 4.02 (s, 1 H, H-4), 3.93 (s, 1 H, H-5), and 3.44 (s, $OCH_3$); of **15**: $\delta$ 5.59 (s, 1 H, PhCH), 4.80 (s, 1 H, H-1), 4.53 (dd, 1 H, $J_{2,3}$ 2.2 Hz, H-3), 4.40 (dd, 1 H, $J_{3,4}$ 2.6 Hz, H-4), 4.21 (m, 2 H, H-6,6′), 3.93 (s, 1 H, H-5), 3.82 (dd, 1 H, $J_{1,2}$ 0.8 Hz, H-2), and 3.44 (s, $OCH_3$).

*Methyl 4,6-O-benzylidene-α-D-altropyranoside 2-sulfate* (**17**) *and 2,3-disulfate* (**18**). — A mixture of methyl 4,6-*O*-benzylidene-α-D-altropyranoside (0.3 g) and sulfur trioxide·trimethylamine complex (0.36 g) in *N,N*-dimethylformamide (2 mL) was stirred at 25° for 20 h, diluted with methanol (1 mL), and applied to a column of silica gel. Elution successively with 1:1 chloroform–methanol and 3:1 ethyl acetate–methanol gave two products. Each was converted in water into the sodium salt with Chelex ($Na^+$) ion-exchange resin, giving the 2-sulfate (**17**) (0.22 g, 42%), m.p. 191–193° (dec.), $[\alpha]_D$ +47° (*c* 1.4, methanol), $\nu_{max}^{solid}$ 1240 $cm^{-1}$ (S=O); and the 2,3-disulfate (**18**) (0.13 g, 33%), m.p. 222–225° (dec.), $[\alpha]_D$ +43° (*c* 0.5, methanol), $\nu_{max}^{solid}$ 1240 $cm^{-1}$ (S=O). The $^1$H-n.m.r. data for H-1 to H-5 for solutions in $D_2O$ are given in Table I; other protons: $\delta$ 3.43 ($OCH_3$), 4.24 (H-6′), 4.37 (H-6), 5.84 (PhCH) for **16**, virtually constant for **17** and **18**.

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