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Conversion of *N*-sulfated glucosamine to *N*-sulfated mannosamine in an unsaturated heparin disaccharide by non-enzymatic, base-catalyzed C-2 epimerization during enzymatic oligosaccharide preparation

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Abstract

A novel disaccharide was isolated beside the predominant trisulfated disaccharide, ΔHexA(2-*O*-sulfate)(α1-4)GlcN(2-*N*-,6-*O*-disulfate) (ΔHexA and GlcN represent 4-deoxy-α-L-*threo*-hex-4-ene-pyranosyluronic acid and D-glucosamine, respectively) after treatment of porcine intestinal heparin with *Flavobacterium* heparinase. It accounted for 18% of total disaccharides. The structure was characterized by secondary ion mass spectrometry, enzymatic digestions, amino sugar analysis, and 500 MHz one- and two-dimensional ^{1}H NMR spectroscopy as ΔHexA(2-*O*-sulfate)(α1-4)ManN(2-*N*-,6-*O*-disulfate), where ManN represents D-mannosamine. The C-2 epimerization from ΔHexA(2-*O*-sulfate)(α1-4)GlcN(2-*N*-,6-*O*-disulfate) to ΔHexA(2-*O*-sulfate)(α1-4)ManN(2-*N*-,6-*O*-disulfate) was also demonstrated to take place *in vitro* under very mild alkaline conditions. Hence, the latter compound is not a biosynthetic product, but is most likely an artifact generated by non-enzymatic, base-catalyzed C-2 epimerization during enzymatic preparation of heparin oligosaccharides. The present results warn that the formation of the C-2 epimerized compound has to be circumvented in the structural analysis of heparin/heparan sulfate.

Keywords: C-2 Epimerization; Heparin; Heparinase; *N*-Sulfated mannosamine

1. Introduction

Heparin has been widely used as a blood anticoagulant in therapy and also possesses other potentially exploitable therapeutic properties. A wide range of biological activities of heparin, such as inhibition of blood coagulation [1], modulation of cellular proliferation [2,3], and potentiation of angiogenesis [4], result from its ability to interact with various proteins causing their activation, deactivation or stabilization. Structurally, heparin is a highly sulfated co-polymer of glucosamine and uronic acid residues that are alternatively

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(1→4)-linked. Most of the heparin molecule is made up of the major trisulfated disaccharide repeating unit, -4)IdoA2*S*(α1-4)GlcN*S*6*S*(α1-, where IdoA, 2*S*, 6*S* and N*S* stand for L-iduronic acid, 2-*O*-sulfate, 6-*O*-sulfate and 2-*N*-sulfate, respectively. This repeating sequence forms highly sulfated regions and represents at least 75% of heparin from porcine intestine [5]. Since molecular interactions between heparin and proteins depend at least in part on the structural variability of heparin, microanalysis of the biologically active domains of heparin is essential for better understanding of the mechanism of action of heparin.

In the course of preparation of structurally defined oligosaccharides from porcine intestinal heparin after exhaustive digestion with *Flavobacterium* heparinase [6], we encountered an unidentified compound in addition to the major structurally-established trisulfated disaccharide when the resultant disaccharide fraction was analyzed by HPLC. In the present study we isolated and characterized this compound, which turned out to be an unreported disaccharide containing *N*-sulfated ManN at the reducing terminus. *In vitro* conversion of an *N*-sulfated GlcN residue into an *N*-sulfated ManN residue by non-enzymatic, base-catalyzed C-2 epimerization was also demonstrated, and probably the chemical basis of the conversion observed during enzymatic preparation of the disaccharide.

2. Results

In an earlier work, stage 14 porcine intestinal heparin was digested exhaustively with *Flavobacterium* heparinase and the digest was fractionated into disaccharides and larger oligosaccharides by gel filtration on Sephadex G-25. Then, the oligosaccharide fraction was separated into Fractions 1–8 by gel filtration on Bio-Gel P-10. Fractions 6 and 7 were further fractionated into ten and eleven oligosaccharides, respectively, and characterized [6,7]. When the disaccharide fraction was analyzed by HPLC on an amine-bound silica column, an unknown peak was detected at the elution position shortly after the authentic trisulfated disaccharide, ΔHexA2*S*(α1-4)GlcN*S*6*S*, where ΔHexA stands for 4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid (Fig. 1).

Isolation of the unknown compound.—Subfractionation of the disaccharide fraction was carried out by HPLC on an amine-bound silica column as described in Experimental and the peaks were designated as compounds I and II as shown in Fig. 1. The major compound I was identified as ΔHexA2*S*(α1-4)GlcN*S*6*S* based upon the elution position by comparing with those of standard disaccharides. The structure of the unknown compound II was studied by secondary ion mass spectrometry (SIMS), enzymatic digestion and 500 MHz ^{1}H NMR analysis as described below.

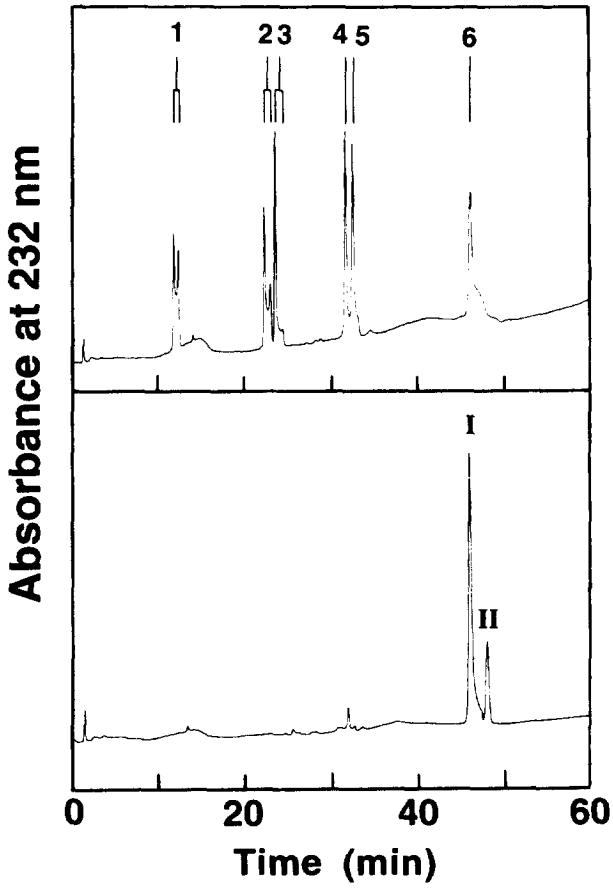

Fig. 1. HPLC analysis of the disaccharide fraction. The disaccharide fraction (0.75 nmol as ΔHexA) prepared by heparinase digestion of porcine intestinal heparin was analyzed by HPLC on an amine-bound silica column as described in the Experimental section (lower panel). The chromatogram of heparin/heparan sulfate-derived standard unsaturated disaccharides (0.3 nmol each) is also shown in the upper panel. 1, ΔHexA(α1-4)GlcNAc; 2, ΔHexA(α1-4)GlcNAc6*S*; 3, ΔHexA-(α1-4)GlcN*S*; 4, ΔHexA(α1-4)GlcN*S*6*S*; 5, ΔHexA2*S*(α1-4)-GlcN*S*; 6, ΔHexA2*S*(α1-4)GlcN*S*6*S*.

SIMS analysis.—SIMS analysis of the underivatised compound II defined its molecular weight. In the negative ion mode SIMS spectrum, pseudo-molecular ions of the type $[M-(x+1)H+xNa]^-$ (M represents the fully protonated acid form of the sulfated oligosaccharide) were preferentially

observed. The molecular ion signal clusters at *m/z* 620 and 642 afforded by authentic ΔHexA2*S*(α1-4)-GlcN*S*6*S* (results not shown) corresponded to di- and tri-sodiated molecular ions of $\Delta HexA_1$-$HexNS_1$ (HexN*S* represents *N*-sulfated hexosamine) with 2 additional *O*-sulfate groups ($[M-3H+2Na]^-$ and $[M-4H+3Na]^-$), respectively. The assignment of the molecular ion signals afforded by compound II is shown in Fig. 2. Molecular ion signal clusters at *m/z* 620 and 642 were observed, indicating that the unknown compound and ΔHexA2*S*(α1-4)GlcN*S*6*S* were isomers with the common composition $\Delta HexA_1HexNS_1$ with 2 additional *O*-sulfate groups.

Enzymatic analysis.—The structure of the nonreducing side of compound II was analyzed using $\Delta^{4,5}$-glycuronate-2-sulfatase, abbreviated as 2-sulfatase, in conjunction with HPLC analysis of the digest. 2-Sulfatase acts only on the 4,5-unsaturated uronic acid 2-*O*-sulfate structure at the nonreducing end, and resulted in a peak shift by 12 min of the parent compound II on HPLC (data not shown), indicating that a ΔHexA(2-sulfate) structure was present on the nonreducing side of compound II. The structure of the hexosamine residue on the reducing side was characterized by ^1H NMR spectroscopy and amino sugar analysis (see below).

500 MHz ^1H NMR analysis.—Determination of the isomer type of the amino sugar residue in compound II was accomplished by 500 MHz ^1H NMR analysis. The one-dimensional spectrum of compound II is depicted in Fig. 3 and the NMR data are summarized with those of compound I in Table 1. The resonances between 5.4 and 5.6 ppm are characteristic of anomeric protons, whereas those at around 6 ppm are characteristic of H-4 of ΔHexA [8]. The other proton chemical shifts were assigned using two-dimensional homonuclear Hartmann-Hahn (data not shown) and correlation spectroscopy (COSY) analysis (Fig. 4) as reported for the sulfated oligosaccharides isolated previously from heparin [6,7,9–12]. Beginning with the H-1 proton signal of ΔHexA at δ 5.461, a cross-peak showing a connectivity to the H-2 resonance at δ 4.549 was found in the COSY spectrum. Continuation of this process allowed localization of the H-3 and H-4 resonances at δ 4.345 and 5.952, respectively, as indicated in Fig. 4. In a similar fashion, starting with the H-1 resonance at δ 5.208, all other proton signals of the α-anomer of the hexosamine residue were assigned. The chemical shifts and coupling constants are given in Table 1. The proton signals of the β-anomer of the hexosamine residue were not observed.

The spectral data of compound II were compared with those of compound I. No significant differences were observed except for the chemical shifts of the H-2 and H-3 signals and the coupling constants $J_{1,2}$ and $J_{2,3}$ belonging to the reducing hexosamine residue, suggesting that the configuration at C-2 or C-3 of the hexosamine residue is

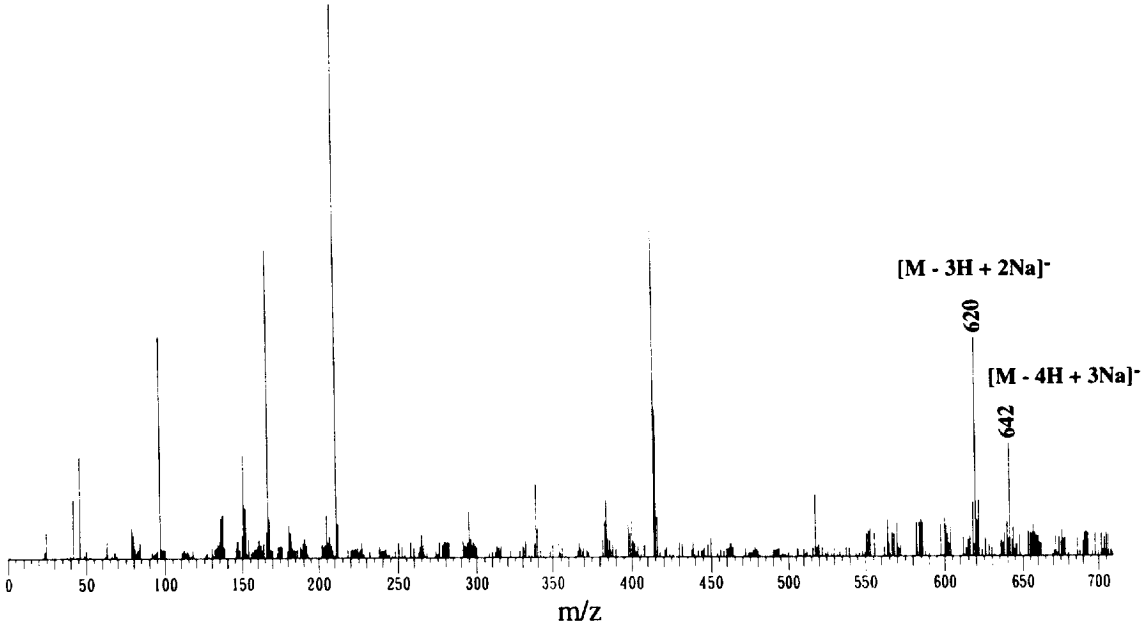

Fig. 2. SIMS spectrum of compound II recorded in a negative ion mode.

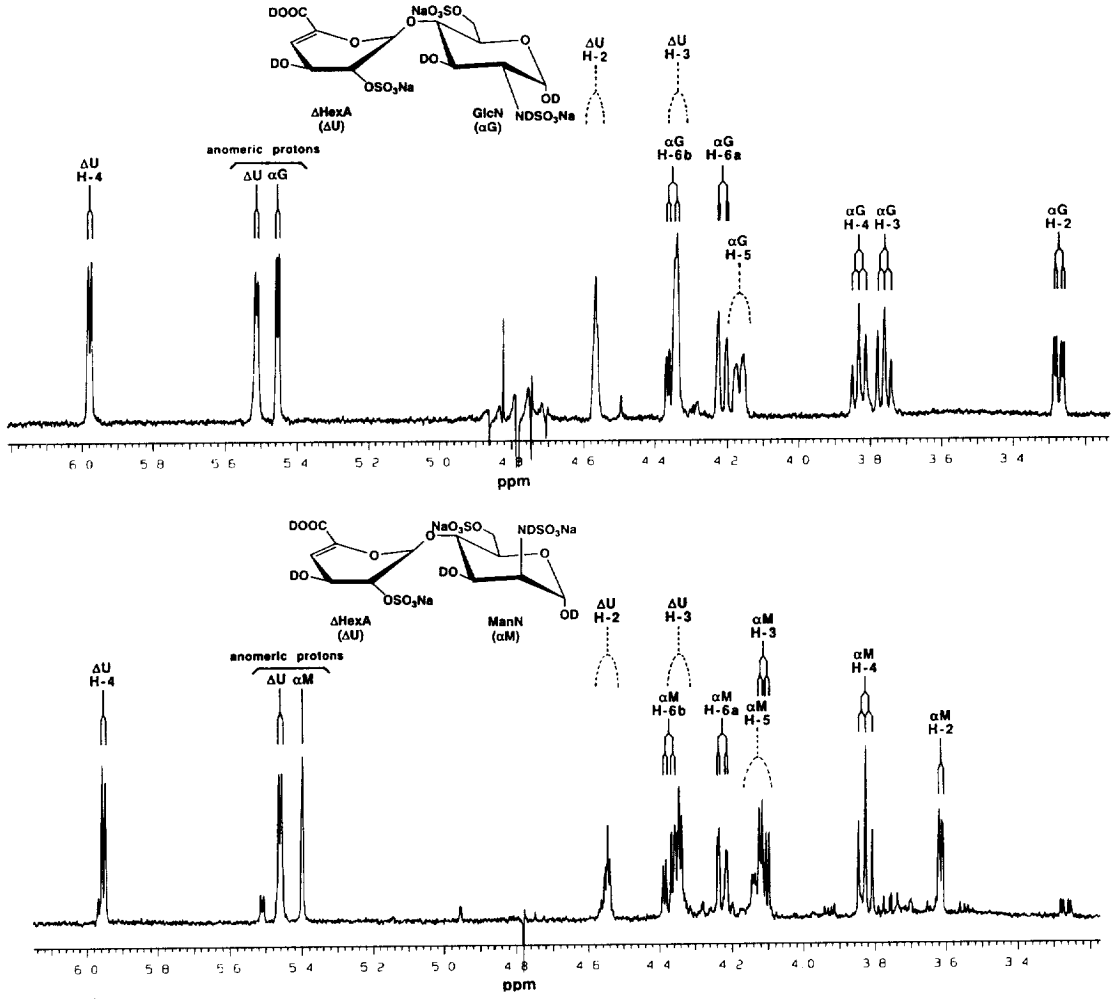

Fig. 3. 500 MHz ^{1}H NMR spectra of ΔHexA2*S*(α1-4)GlcN*S*6*S* and compound II recorded in D_2O at 26 °C. Upper panel, ΔHexA2*S*(α1-4)GlcN*S*6*S*; lower panel, compound II. The numbers and letters in the spectra refer to the corresponding residues in the structures.

different from that of GlcN. The very small coupling constant (1.5 Hz) obtained for $J_{1,2}$ is characteristic for ManN [13]. Thus, we propose the following structure for compound II: ΔHexA2*S* (α1-4)ManN*S*6*S*. The identity of the hexosamine residue as ManN was confirmed by amino sugar analysis of the compound in an amino acid analyzer after acid hydrolysis.

Disaccharides containing an *N*-sulfated GlcN residue at the reducing terminus have been shown to give proton signals of the α-, but not of the β-anomer [9,14,15]. Since the proton signals of the β-anomer of the *N*-sulfated ManN residue were not observed in the ^{1}H NMR spectrum of ΔHexA2*S*-(α1-4)ManN*S*6*S*, sulfation of the amino group of both GlcN and ManN at the reducing terminus may fix these saccharides in an α-configuration. In the case of β-D-mannopyranose, the arrangement of oxygen atoms around C-1 and C-2 results in increased conformational instability (Δ2) [16]. Such a Δ2-like-condition involving the amino group at C-2 may occur to *N*-sulfated ManN so that the α-form predominates in the equilibrium mixture through electrostatic repulsion.

Base-catalyzed conversion of GlcN to ManN.—It is well known that treatment of D-Glc with dilute alkali yields an equilibrium mixture of D-Glc, D-Fru, and D-Man intermediating enol forms, called enediols (Lobry de Bruyn transformation) [17]. It is also established that D-GlcNAc and D-ManNAc are reversibly epimerized in the presence of a base [13,18,19]. Hence, the *N*-sulfated ManN residue at the reducing terminus of compound II may have been formed by base-catalyzed C-2 epimerization of *N*-sulfated GlcN during the enzymatic preparation of oligosaccharides. To clarify whether ΔHexA2*S*(α1-4)GlcN*S*6*S* is converted to ΔHexA2*S* (α1-4)ManN*S*6*S* under alkaline conditions,

Table 1
^{1}H Chemical shifts and coupling constants of the constituent monosaccharides of the isolated disaccharides derived from heparin[a]

Residue		Compound I	Compound II
		Reporter groups	
αHexN	H-1	5.455	5.399
	H-2	3.274	3.624
	H-3	3.761	4.110
	H-4	3.834	3.833
	H-5	4.167	4.122
	H-6a	4.214	4.224
	H-6b	4.353	4.371
ΔHexA	H-1	5.513	5.461
	H-2	4.567	4.549
	H-3	4.343	4.345
	H-4	5.980	5.952
		Coupling constants	
αHexN	$J_{1,2}$	3.5	1.5
	$J_{2,3}$	10.0	4.5
	$J_{3,4}$	9.0	10.0
	$J_{4,5}$	10.0	10.0
	$J_{5,6a}$	1.5	2.0
	$J_{5,6b}$	4.0	4.0
	$J_{6a,6b}$	11.5	11.5
ΔHexA	$J_{1,2}$	3.5	3.5
	$J_{2,3}$	2.0	3.5
	$J_{3,4}$	4.5	4.5

[a] Chemical shifts and coupling constants are expressed in ppm and Hz, respectively. The estimated error for chemical shifts was ± 0.002 ppm. Since the resonances of the β-anomer of HexN were not observed, only the values of the α-anomer of HexN are shown.

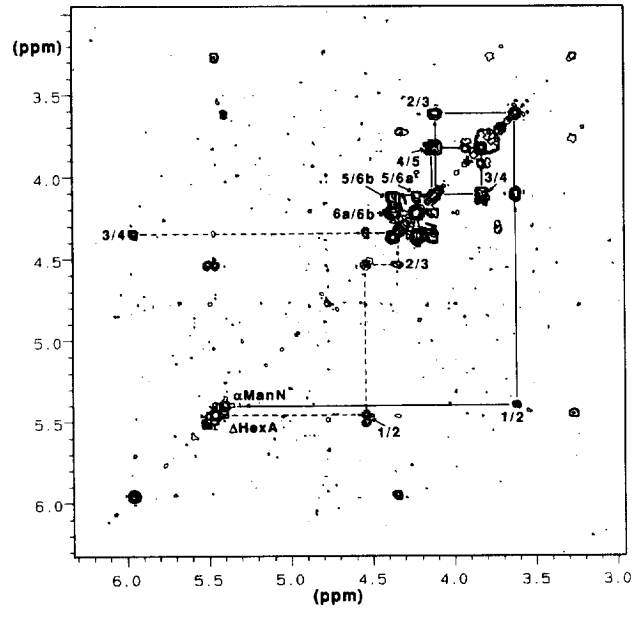

Fig. 4. Two dimensional COSY spectrum of compound II recorded in D_2O at 26 °C. Proton signals of ΔHexA and ManN were assigned starting with the H-1 resonances at δ 5.461 (- - -) and 5.399 (——), respectively.

ΔHexA2*S*(α1-4)GlcN*S*6*S* was dissolved at 1 mM in 0.25% ammonium hydroxide, and incubated at 30 °C. Aliquots were withdrawn at 1, 2 and 10 days to monitor the reaction by HPLC analysis. Two peaks at different retention times are seen corresponding to ΔHexA2*S*(α1-4)GlcN*S*6*S* and ΔHexA2*S*(α1-4)ManN*S*6*S* with recoveries of 97 and 3%, 95 and 5%, and 59 and 19% (Fig. 5, panel A), respectively, taking the UV absorbance of the control reaction mixture without incubation as 100%. The newly formed minor peak, which eluted later than the original peak, was also demonstrated to co-elute with ΔHexA2*S*(α1-4)ManN*S*6*S* by co-chromatography (Fig. 5, panel B). Although the reaction mixture at 30 days also yielded two peaks (data not shown), their recoveries were only 18 and 9%, respectively, suggesting that the disaccharides were decomposed by the long incubation under basic conditions. To further confirm the structure of the newly formed peak, amino sugar analysis was also conducted after acid hydrolysis, which showed two peaks corresponding to GlcN and ManN (results not shown). When the acid hydrolysate of the reaction mixture was co-chromatographed with that of ΔHexA2*S*(α1-4)ManN*S*6*S*, the minor peak which had accounted for 13% was quantitatively augmented (results not shown). The conversion of GlcN to ManN was also demonstrated by alkaline treatment of the trisulfated disaccharide at the compound concentration of 0.1 and 0.01 mM or at 25 °C, to a degree similar to that observed under the above conditions. These results suggest that the C-2 epimerization takes place under mild alkaline conditions.

3. Discussion

In this study, we determined the structure of a disaccharide containing a novel *N*-sulfated ManN residue isolated from the heparin disaccharide fraction, which was prepared by the extensive digestion of porcine intestinal heparin with *Flavobacterium* heparinase [6]. We also demonstrated that the heparin trisulfated disaccharide containing *N*-sulfated GlcN was epimerized at C-2 being converted to the disaccharide containing *N*-sulfated ManN *in vitro* under very mild alkaline conditions, suggesting that the ΔHexA2*S*(α1-4)ManN*S*6*S* structure had been formed by C-2 epimerization from ΔHexA2*S*(α1-4)GlcN*S*6*S* during the preparative manipulation for the disaccharides:

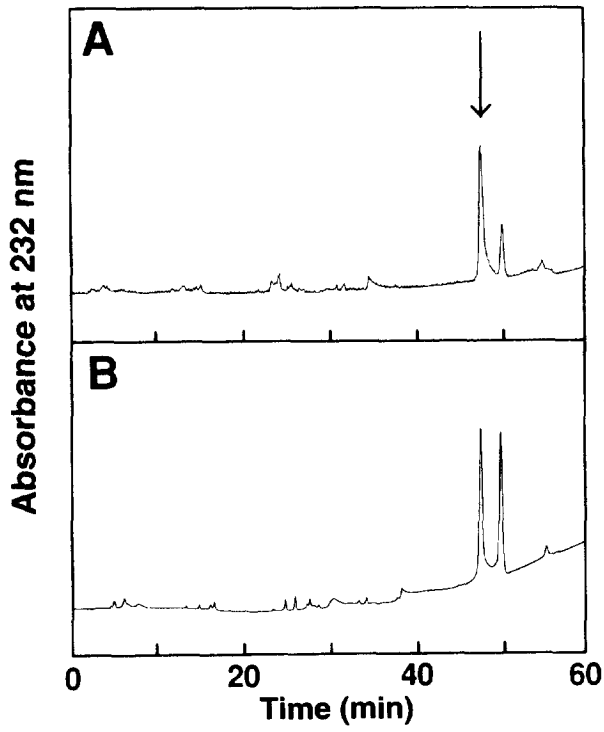

Fig. 5. HPLC analysis of the products formed by the alkaline treatment of ΔHexA2*S*(α1-4)GlcN*S*6*S*. ΔHexA2*S*(α1-4)GlcN*S*6*S* (1.25 nmol) was treated with aq 0.25% NH_4OH at 30 °C for 10 days and analyzed by HPLC as described for Fig. 1 (panel A). A mixture of alkali-treated ΔHexA2*S*(α1-4)-GlcN*S*6*S* shown in panel A and 0.3 nmol of compound II obtained in the experiments shown in Fig. 1 was analyzed (panel B). The elution position of the untreated ΔHexA2*S*-(α1-4)GlcN*S*6*S* is indicated in the upper panel by an arrow.

enzymatic fragmentation of heparin or gel filtration of the digest. Toida et al. [13] also observed the conversion of a heparan sulfate-derived tetrasaccharide ΔHexA-GlcNAc-GlcA-GlcNAc to ΔHexA-GlcNAc-GlcA-ManNAc under basic conditions, and proposed the following mechanism for the conversion, which can be applied to the present case. The reducing terminal GlcN residue is in equilibrium with the pyranose form and the ring-opened aldehyde form. While treatment with base leads to abstraction of the acidic proton at C-2 of GlcN in the ring-opened aldehyde form, the C-2 carboanion is generated. Then, a simple C-2 epimerization takes places where the *gluco*- and *manno*-epimers are in dynamic equilibrium.

Although C-2 epimerases which reversibly epimerize GlcNAc and ManNAc at the free monosaccharide and the nucleotide sugar level have been found in some bacteria and mammalian organs [20–26], no *N*-sulfated ManN residue in native heparin or heparan sulfate polysaccharides nor C-2 epimerase that epimerizes *N*-sulfated GlcN to *N*-sulfated ManN at the polymeric level has been reported to our knowledge. In conclusion, the disaccharide containing *N*-sulfated ManN residue is most likely an artifact. When unsaturated sulfated disaccharides derived from heparin/heparan sulfate were analyzed by HPLC after alkali treatment, some of them yielded an unidentified minor peak which eluted near the respective parent disaccharides (results not shown). Thus, continual exposure of oligosaccharides from heparin/heparan sulfate to alkaline conditions for a long time has to be avoided to circumvent the C-2 epimerization of GlcN residues. However, this type of epimerization would take place only at the reducing terminal GlcN residues of oligosaccharides as expected from the mechanism proposed above. A number of sulfated oligosaccharides have been isolated from heparin/heparan sulfate after digestion with *Flavobacterium* eliminases (for a review, see [27]). Besides the major well-characterized oligosaccharides, many unidentified minor peaks have been observed when the oligosaccharide fractions were analyzed by HPLC (for example, see Fig. 1 in [7]). These variabilities may depend not only upon the types of internal uronic acid residues and sulfation pattern but also C-2 epimerization of a reducing terminal amino sugar residue.

4. Experimental

Materials.—Stage 14 heparin (Na salt) of porcine intestine was purchased from American Diagnostica, New York; D-mannosamine hydrochloride was from Nacalai Tesque, Kyoto; heparinase (EC 4.2.2.7) was obtained from Seikagaku Corp., Tokyo; $\Delta^{4,5}$-Glycuronate-2-sulfatase (EC 3.1.6.-) [28] was a gift from Dr. Keiichi Yoshida (Seikagaku Corp.).

Preparation of the disaccharides.—The disaccharide fraction was prepared from stage 14 heparin after heparinase digestion as described [6]. Briefly, the heparin preparation was purified by anion-exchange chromatography and digested exhaustively with *Flavobacterium* heparinase. The digest was fractionated by gel filtration on Sephadex G-25 and the disaccharide fraction was subfractionated by HPLC on an amine-bound silica column using a linear gradient of NaH_2PO_4 from 16 to 530 mM over 60 min at a flow rate of 1 mL/min [29,30]. Each peak was purified by rechromatography under the same conditions as the first

step, and desalted by gel filtration through a column of Sephadex G-25.

2-Sulfatase digestion of the isolated compound.—2-Sulfatase digestion was carried out by treating 1.0 nmol of the isolated compound with 1 mIU of the enzyme for 2 h at 37 °C in a total volume of 20 μL of 10 mM imidazole-HCl buffer, pH 6.5.

Mild alkali treatment of the heparin-derived trisulfated disaccharide.—ΔHexA2*S*(α1-4)GlcN*S*6*S* was dissolved at 0.01–1 mM in a total volume of 0.1 mL 0.25% NH_4OH (pH 11) and the sample was incubated at 25 or 30 °C for 0–30 days. Aliquots (5 μL) of the reaction mixture were withdrawn at 1, 2, 10 and 30 days and monitored by HPLC as described above. The reactions were terminated by dilution with 16 mM NaH_2PO_4.

Mass spectrometry and 500 MHz ^1H NMR spectroscopy.—Sugar and sulfate compositions of the isolated compound were determined by SIMS. SIMS was carried out with a Hitachi M-4100 mass spectrometer equipped with a caesium gun. Mass spectra of native disaccharides were recorded in a negative ion mode. Samples were dissolved in aq 5% HOAc and approximately 0.5 nmol each was loaded directly onto the probe precoated with a monothioglycerol matrix. Disaccharides for NMR analysis were fully-sodiated using a Dowex 50-X8 (Na^+ form) column (7×18 mm), and then repeatedly exchanged in D_2O with intermediate lyophilization. 500 MHz ^1H NMR spectra of disaccharides were measured on a Varian VXR-500 instrument at a probe temperature of 26 °C as reported [9]. Chemical shifts are given relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured indirectly relative to acetone (δ 2.225) in D_2O [31].

Analysis of uronic acid and amino sugars.—Uronic acid was determined by the carbazole method [32]. Unsaturated uronic acid was spectrophotometrically quantified based on an average millimolar absorption coefficient of 5.5 at 232 nm [33]. Amino sugars were quantified after acid hydrolysis in 3 M HCl at 100 °C for 16 h in a Beckman 6300E amino acid analyzer [34].

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