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A Unique Dermatan Sulfate-like Glycosaminoglycan from Ascidian

ITS STRUCTURE AND THE EFFECT OF ITS UNUSUAL SULFATION PATTERN ON ANTICOAGULANT ACTIVITY*

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A dermatan sulfate, similar to the mammalian glycosaminoglycans but not identical with any of them, has been isolated from the body of the ascidian *Ascidia nigra*. Degradation with chondroitin ABC lyase, analysis of the disaccharide products by digestion with chondro-4- and -6-sulfatases, and ^{1}H and ^{13}C NMR data confirm that the predominant structure is [4-α-L-IdoA-(2SO_4)-1→3-β-D-GalNAc(6SO_4)-1]$_n$. Mammalian dermatan sulfate is an anticoagulant due to its ability to potentiate inhibition of thrombin by heparin cofactor II. The structure in dermatan sulfate which binds to heparin cofactor II is [4-α-L-IdoA-(2SO_4)-1→3-β-D-GalNAc(4SO_4)-1]$_n$, where n 3. We have compared the ascidian dermatan sulfate with mammalian dermatan sulfate and with chemically oversulfated mammalian dermatan sulfate for anticoagulant activity as measured by the activated partial thromboplastin time assay and for its ability to potentiate heparin cofactor II. In spite of its high content of 2-*O*-sulfated α-L-iduronic acid residues, the ascidian compound had no discernible anticoagulant activity and had low ability to potentiate heparin cofactor II. These results suggest that 4-*O*-sulfation of the *N*-acetyl-β-D-galactosamine residues is essential for the anticoagulant activity of dermatan sulfate.

The glycosaminoglycans are a group of structurally related polysaccharides found as the carbohydrate moieties of proteoglycans and sometimes as free polysaccharides (1). Their functions are diverse. Dermatan sulfate, chondroitin sulfate, and heparan sulfate may all be components of connective tissue (2) or cell surface carbohydrates involved in the cell's interaction with and response to its surroundings (3). The anticoagulant glycosaminoglycan heparin is an important therapeutic agent used in the prophylaxis and treatment of thrombosis (4); dermatan sulfate is also an anticoagulant, although of lower potency than heparin (5–7).

Most interest, understandably, centers on the structures and functions of mammalian glycosaminoglycans, but these polysaccharides are widely distributed throughout the animal kingdom (8), and invertebrate species are a rich source of sulfated polysaccharides with novel structures (9–15). The ascidians (Chordata-Tunicata) are covered by an external supportive tissue called the tunic. This tissue contains large amounts of sulfated polysaccharides different from all previously described mammalian glycosaminoglycans and from the sulfated polysaccharides from marine algae (9, 10, 12, 14). The preponderant polysaccharide in the ascidian tunic is a high molecular weight sulfated α-L-galactan (10, 12, 16, 17). Recently, we reported the occurrence of glycosaminoglycans in the body of the ascidian *Styela plicata* (18). Small amounts of heparan sulfate and a large quantity of a dermatan sulfate-like glycosaminoglycan were found in the body of this ascidian. Preliminary analysis of this dermatan sulfate using degradation with chondroitin ABC lyase suggested structural differences when compared with mammalian dermatan sulfate (18).

In the present work, we report the isolation and structural characterization of a dermatan sulfate-like glycosaminoglycan from the body of the ascidian *Ascidia nigra* and a comparison of its structure and anticoagulant activity with those of mammalian dermatan sulfate and a chemically oversulfated mammalian dermatan sulfate. The ascidian polysaccharide has a distinctive structure, composed of repetitive disaccharide units of 6-*O*-sulfo-2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-α-L-idopyranosyluronic acid)-β-D-galactose. It differs from mammalian dermatan sulfate in its sulfation at both the 2-position of the iduronic acid unit and the 6-position of the *N*-acetylgalactosamine unit, and the absence of sulfation at position 4 of the hexosamine residue. This unique glycosaminoglycan may help to determine the structural requirement for the anticoagulant activity of dermatan sulfate and specifically for the binding to heparin cofactor II.

EXPERIMENTAL PROCEDURES

Materials—Heparan sulfate from human aorta was extracted and purified as described previously (19). Dermatan sulfate was extracted

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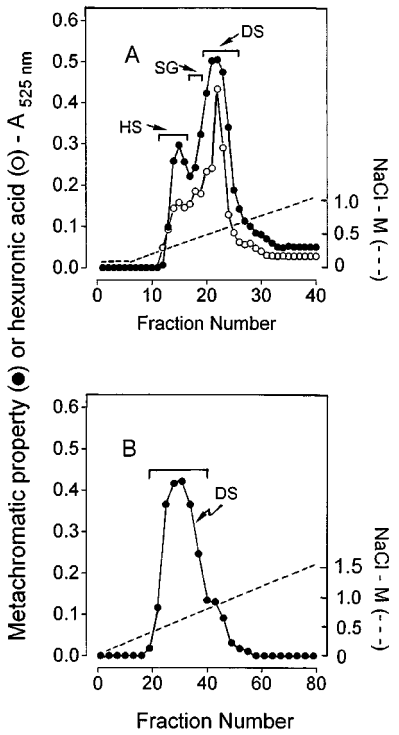

FIG. 1. **Purification of the ascidian dermatan sulfate on DEAE-cellulose.** In *A*, the crude polysaccharides from the ascidian body (~100 mg) were purified on a DEAE-cellulose column (10 × 1.5 cm) as described under "Experimental Procedures." Fractions were assayed by the carbazole reaction (○), for metachromasia (●), and NaCl concentration (– – –). Three groups of fractions were pooled separately as indicated by the *horizontal bars* in the figure, designated *HS* (heparan sulfate), *SG* (sulfated glucan), and *DS* (dermatan sulfate), dialyzed against distilled water, and lyophilized. In *B*, the DEAE-purified dermatan sulfate (~40 mg) was rechromatographed on a new DEAE-cellulose column, and fractions were assayed for metachromasia (●) and NaCl concentration (– – –). Those corresponding to the purified ascidian dermatan sulfate, as indicated by the *horizontal bar* in the panel, were pooled, dialyzed against distilled water, and lyophilized.

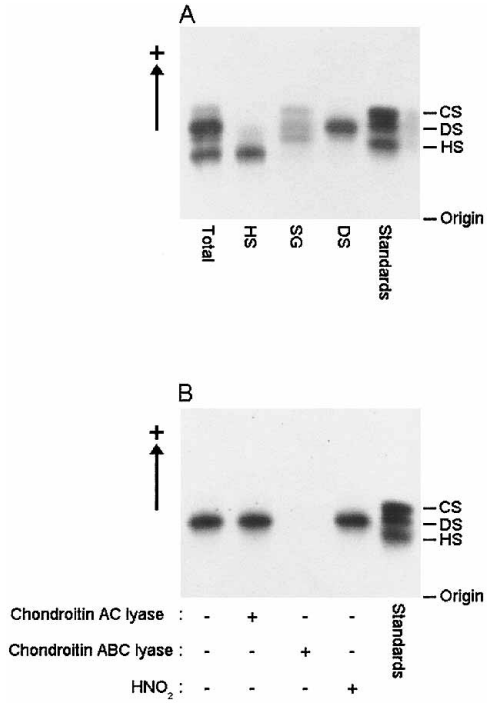

FIG. 2. **Agarose gel electrophoresis of the purified sulfated polysaccharides from ascidian body.** In *A*, the purified sulfated polysaccharides (~20 μg) obtained from the DEAE-cellulose column (see Fig. 1*A*) and a mixture of standard glycosaminoglycans containing 20 μg each of chondroitin 4-sulfate (*CS*), dermatan sulfate (*DS*), and heparan sulfate (*HS*) were applied to a 0.5% agarose gel and run for 1 h at 110 V in 0.05 M 1,3-diaminopropane/acetate buffer (pH 9.0). The sulfated polysaccharides in the gel were fixed with 0.1% *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide solution. After 12 h, the gel was dried and stained with 0.1% toluidine blue in acetic acid/ethanol/water (0.1:5:5, v/v). In *B*, the ascidian dermatan sulfate, repurified on a new DEAE-column (see Fig. 1*B*), before (−) and after (+) treatment with chondroitin AC or ABC lyase, or deaminative cleavage by nitrous acid, were analyzed by agarose gel electrophoresis, as described under *A*.

and purified from bovine lung, and partially oversulfated mammalian dermatan sulfate was prepared by reaction with sulfur trioxide/pyridine, as described (20). Chondroitin 4-sulfate from whale cartilage, chondroitin 6-sulfate from shark cartilage, heparin from porcine intestinal mucosa, chondro-4-sulfatase (disaccharide-4-sulfate sulfatase, EC 3.1.6.9), chondro-6-sulfatase (disaccharide-6-sulfate sulfatase, EC 3.1.6.10), and the disaccharide ΔGlcA($2SO_4$)-GalNAc($6SO_4$)[1] were purchased from Sigma; chondroitin AC lyase (EC 4.2.2.5) from *Arthrobacter aurescens* and chondroitin ABC lyase (EC 4.2.2.4) from *Proteus vulgaris* were from Seikagaku American Inc. (Rockville, MD). Human heparin cofactor II and thrombin were purified as described previously (21). The thrombin substrate tosyl-Gly-Pro-Arg-*p*-nitroanilide acetate (Chromozym TH) was obtained from Boehringer Mannheim. The heparin for the APTT assay was the 4th International Standard (85/502). Antithrombin III neutralizing polyclonal antibody was a gift from Dr. E. Gray (National Institute for Biological Standards and Control, United Kingdom).

Isolation of the Sulfated Polysaccharides—The ascidian *A. nigra* (Chordata-Tunicata) was collected in Angra dos Reis, Rio de Janeiro, Brazil. The animals were immersed immediately in acetone and kept for 24 h at 4 °C. The body was separated from the tunic, cut in small pieces, and dried. The sulfated polysaccharides were extracted from the dried tissues (10 g) using the same methodology described for another tissue (13), yielding ~50 mg (as dry weight).

Purification of the Sulfated Glycans—The glycans extracted from the body of *A. nigra* (~100 mg) were applied to a DEAE-cellulose column (10 × 1.5 cm) equilibrated with 0.5 M sodium acetate buffer (pH 6.0) and washed with 50 ml of the same buffer. The column was developed by a linear gradient of 0–1.5 M NaCl in the same buffer. The flow rate of the column was 8.0 ml/h, and fractions of 1.5 ml were collected and assayed by metachromasia using 1,9-dimethylmethylene blue (22), and by the carbazole reaction (23) for hexuronic acid. The NaCl concentration was estimated by conductivity. The fractions containing the dermatan sulfate (identified by agarose gel electrophoresis and incubation with specific enzymes) were pooled, dialyzed against distilled water, and lyophilized. This sample was reapplied to a newly packed DEAE-cellulose column (10 × 1.5 cm) and repurified as described above. The fractions of this second column containing the glycosaminoglycan were pooled, dialyzed against distilled water, and lyophilized.

TABLE I
Chemical composition and specific optical rotation of the purified ascidian dermatan sulfate and of standard glycosaminoglycans

Sample	Sulfate/Galactosamine molar ratio[a]	$[\alpha]_D^{20\,°C}$
Ascidian dermatan sulfate	1.83	−54 °
Mammalian dermatan sulfate	1.10	−61 °
Vertebrate chondroitin 4-sulfate	1.14	−26 °

[a] Amino sugars were identified in the acid hydrolysates by paper chromatography in butanol:pyridine:water (3:2:1, v/v) for 48 h, visualized by silver nitrate staining, and revealed exclusively galactosamine. Sulfate was estimated by the $BaCl_2$/gelatin method (25), and the molar contents were normalized to total galactosamine, which was estimated by a modified Elson-Morgan reaction (24).

[1] The abbreviations used are: ΔGlcA($2SO_4$)-GalNAc($6SO_4$), 2-acetamido-2-deoxy-3-*O*-(2-sulfo-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-*O*-sulfo-D-galactose; ΔGlcA-GalNAc, 2-acetamido-2-deoxy-3-*O*(α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose; ΔGlcA-GalNAc($4SO_4$), ΔGlcA-GalNAc($6SO_4$), and ΔGlcA-GalNAc(4/6-diSO_4), derivatives of ΔGlcA-GalNAc bearing a sulfate ester at position 4, at position 6, and at both positions, respectively, of the hexosamine moiety; APTT, activated partial thromboplastin time; FPLC, fast protein liquid chromatography; DEPT, distortionless enhancement by polarization transfer; TOCSY, total correlation spectroscopy.

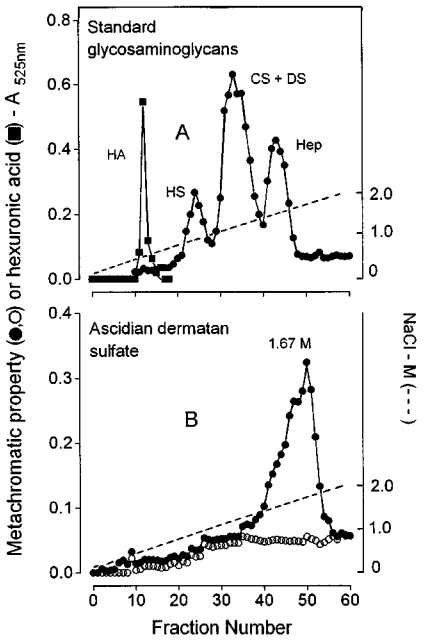

FIG. 3. **Analysis of standard glycosaminoglycans (*A*) and of ascidian dermatan sulfate (*B*) on a Mono Q-FPLC column.** In *A*, a solution (1.0 ml) containing standard hyaluronic acid (250 μg, *HA*), heparan sulfate (250 μg, *HS*), chondroitin sulfate (250 μg, *CS*), dermatan sulfate (100 μg, *DS*), and heparin (250 μg, *Hep*) was applied to a Mono Q-FPLC column (HR 515) equilibrated with 20 mM Tris/HCl buffer (pH 8.0). The column was developed by a linear gradient of 0.15–2.0 M NaCl in the same buffer. The flow rate of the column was 0.45 ml/min, and fractions of 0.5 ml were collected and assayed by metachromasia (●) or by the carbazole reaction (■). In *B*, purified ascidian dermatan sulfate (3 mg from the experiment of Fig. 1*B*), before (●) and after incubation with chondroitin ABC lyase (○), was applied to a Mono Q-FPLC, and the column was developed as described under *A*. The fractions were assayed by metachromasia.

Analysis of the Native and Chondroitinase-treated Ascidian Dermatan Sulfate by Mono Q FPLC—Purified ascidian dermatan sulfate (3 mg), before and after incubation with 0.2 unit of chondroitin ABC lyase in 0.5 ml of 0.05 M ethylenediamine/acetate buffer (pH 8.0) at 37 °C for 12 h, or a solution containing standard hyaluronic acid (250 μg), heparan sulfate (250 μg), chondroitin sulfate (250 μg), dermatan sulfate (100 μg), and heparin (250 μg) was applied to a Mono Q column-FPLC (HR 5/5) from Pharmacia Biotech Inc., equilibrated with 20 mM Tris/HCl buffer (pH 8.0). The column was developed by a linear gradient of 0.15–2.0 M NaCl in the same buffer. The flow rate of the column was 0.45 ml/min, and fractions of 0.5 ml were collected and assayed by metachromasia using 1,9-dimethylmethylene blue (22) and/or by hexuronic acid using the carbazole reaction (23).

Chemical Analysis—After acid hydrolysis (4.0 M HCl, 100 °C for 6 h) of the glycans, total hexosamine and sulfate were estimated for the ascidian dermatan sulfate and for standard mammalian glycosaminoglycans, by a modified Elson-Morgan reaction (24) and by the $BaCl_2$-gelatin method (25), respectively. Standard curves for hexosamine and sulfate were constructed from glucosamine and Na_2SO_4. The hexuronic acid content was estimated by the carbazole reaction (23).The specific optical rotation of the glycans was measured with a Perkin-Elmer digital polarimeter, model 243-B.

Enzymatic Treatments—Glycosaminoglycans (~100 μg) were incubated with chondroitin AC lyase, chondroitin ABC lyase, and chondro-4- and/or -6-sulfatase as described (13). The products formed were separated by paper chromatography, visualized by silver nitrate staining, and quantitated by densitometry using a Quick Scan densitometer (Helena Laboratories, Beaumont, TX).

Chemical Modifications—Desulfation of the purified ascidian dermatan sulfate was performed by solvolysis in dimethyl sulfoxide/methanol (9:1, v/v) at 80 °C for 4 h, as described (26). Desulfation was estimated by the amount of nonsulfated disaccharide (ΔGlcA-GalNAc) formed by degradation of the glycan with chondroitin ABC lyase (see above). Deaminative cleavage by nitrous acid at pH 4.0 was performed as described by Shively and Conrad (27).

NMR Methods—^{1}H spectra were recorded at 500 MHz and ^{13}C spectra at 125 MHz using a Varian Unity 500 spectrometer in the FT mode. About 15 mg of each polysaccharide sample was dissolved in approximately 0.7 ml 99.8% D_2O (Goss Scientific, Ingatestone, United Kingdom) for NMR spectroscopy. All the spectra were recorded at 60 °C, with suppression of the HOD signal by presaturation. ^{13}C spectra were recorded with full proton decoupling using the WALTZ sequence (28); a rapid repetitive rate was used to reduce the intensity of signals from low molecular weight impurities with long relaxation times. Distortionless enhancement by polarization transfer (DEPT) ^{13}C spectra were recorded using the pulse sequence provided by the manufacturer. Two-dimensional double-quantum filtered COSY (29) and TOCSY (30) spectra were recorded in the phase-sensitive mode using the pulse programs supplied by the manufacturer. TOCSY spectra were run with a spin-lock field of about 10 kHz and a mixing time of 80 ms, which was previously determined to give optimum results for these samples. All chemical shifts are relative to internal or external trimethylsilylpropionic acid.

Anticoagulant Action of the Ascidian Dermatan Sulfate Measured by Activated Partial Thromboplastin Time (APTT)—APTT clotting assays were carried out as described previously (31, 32). Normal human plasma (90 μl) was incubated with 10 μl of a solution of glycosaminoglycan (0–100 μg) and 100 μl of kaolin + bovine brain phospholipid reagent (National Institute for Biological Standard reference reagent). After 5 min of incubation at 37 °C, 100 μl of 0.25 M $CaCl_2$ are added to the mixtures, and the clotting time is recorded. The activity was expressed as units/mg using a parallel standard curve based on the International Heparin Standard (193 units/mg).

Inhibition of Thrombin by Heparin Cofactor II in the Presence of Glycosaminoglycans—Incubations were performed in disposable polystyrene cuvettes. The reactants included 68 nM heparin cofactor II, 15 nM thrombin, and 0–1000 μg/ml glycosaminoglycan in 100 μl of 0.02 M Tris/HCl, 0.15 M NaCl, and 1.0 mg/ml polyethylene glycol, pH 7.4 (TS/PEG buffer). Thrombin was added last to initiate the reaction. After a 60-s incubation at room temperature, 500 μl of 100 μM Chromozym TH in TS/PEG buffer was added, and the absorbance at 405 nm was recorded continuously for 100 s. The rate of change of absorbance was proportional to the thrombin activity remaining in the incubation. No inhibition occurred in control experiments in which thrombin was incubated with heparin cofactor II in the absence of glycosaminoglycan, nor did inhibition occur when thrombin was incubated with glycosaminoglycan alone over the range of concentrations tested.

RESULTS AND DISCUSSION

Isolation of a Dermatan Sulfate-like Glycosaminoglycan from Ascidian—The sulfated polysaccharides from the body of *A. nigra* were separated by anion exchange chromatography on a DEAE-cellulose column into two major fractions, which eluted with different NaCl concentrations (*HS* and *DS* in Fig. 1*A*). Additional metachromatic material eluted at ~0.50 M NaCl (*SG* in Fig. 1*A*).

Peak HS was identified as a heparan sulfate-like glycosaminoglycan since it migrates as a single homogeneous band on agarose gel electrophoresis and has the same mobility of standard mammalian heparan sulfate (Fig. 2*A*). In addition, this fraction is resistant to chondroitin AC and ABC lyases, but is cleaved by nitrous acid (not shown). The major fraction of the sulfated polysaccharides from the body of *A. nigra* (*peak DS*) was rechromatographed on another DEAE-cellulose column, now yielding a homogeneous peak (Fig. 1*B*). This polysaccharide migrates on agarose gel electrophoresis as a single homogeneous metachromatic band with the same mobility as standard mammalian dermatan sulfate (Fig. 2*B*). It is not degraded by chondroitin AC lyase[2] or by nitrous acid, but disappears after treatment with chondroitin ABC lyase. These results characterize fraction DS as a dermatan sulfate-like glycosaminoglycan.

The material under *peak SG* (Fig. 1*A*) contains the dermatan sulfate-like glycosaminoglycan, but is contaminated with two other polysaccharides (Fig. 2*A*). These polymers are resistant to

[2] Incubation with chondroitin AC lyase does not reduce the molecular weight of the ascidian dermatan sulfate, as revealed by polyacrylamide gel electrophoresis stained with toluidine blue.

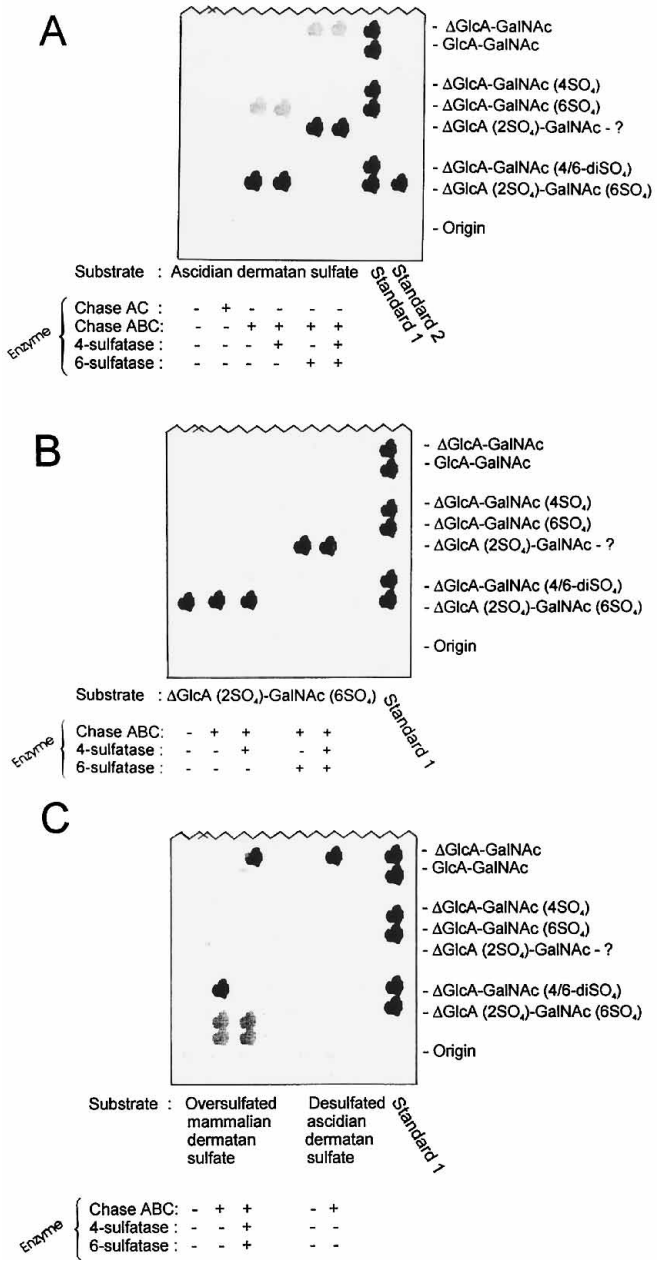

FIG. 4. **Paper chromatograms of the degradation products formed by the action of chondroitin AC or ABC lyases, chondro-4-sulfatase, and chondro-6-sulfatase on the ascidian dermatan sulfate (*A*), standard ΔGlcA(2SO$_4$)-GalNAc(6SO$_4$) (*B*), chemically oversulfated mammalian dermatan sulfate (*C*), and desulfated ascidian dermatan sulfate (*C*).** The various compounds (100 μg of each) were incubated without enzyme (−) or with (+) 10 milliunits each of chondroitin AC lyase (*Chase AC*), chondroitin ABC lyase (*Chase ABC*), chondro-4-sulfatase, and chondro-6-sulfatase, as indicated in the figure. All incubations were for 8 h at 37 °C in 0.05 M ethylenediamine/acetate buffer (pH 8.0) in a final volume of 100 μl. The mixtures were then spotted on Whatman No. 1 paper and subjected to chromatography in isobutyric acid, 1 N NH_4OH (5:3, v/v) for 48 h. The products were located on the chromatograms by silver nitrate staining. *Standard 1* is a disaccharide mixture obtained by degradation of a chemically defucosylated chondroitin sulfate from sea cucumber with chondroitin AC lyase (11); *Standard 2* is the disaccharide ΔGlcA(2SO$_4$)-GalNAc(6SO$_4$) from Sigma. In *B*, the standard ΔGlcA(2SO$_4$)-GalNAc(6SO$_4$) was incubated with chondroitin ABC lyase to exclude the presence of contaminant enzymes that could degrade the disaccharide.

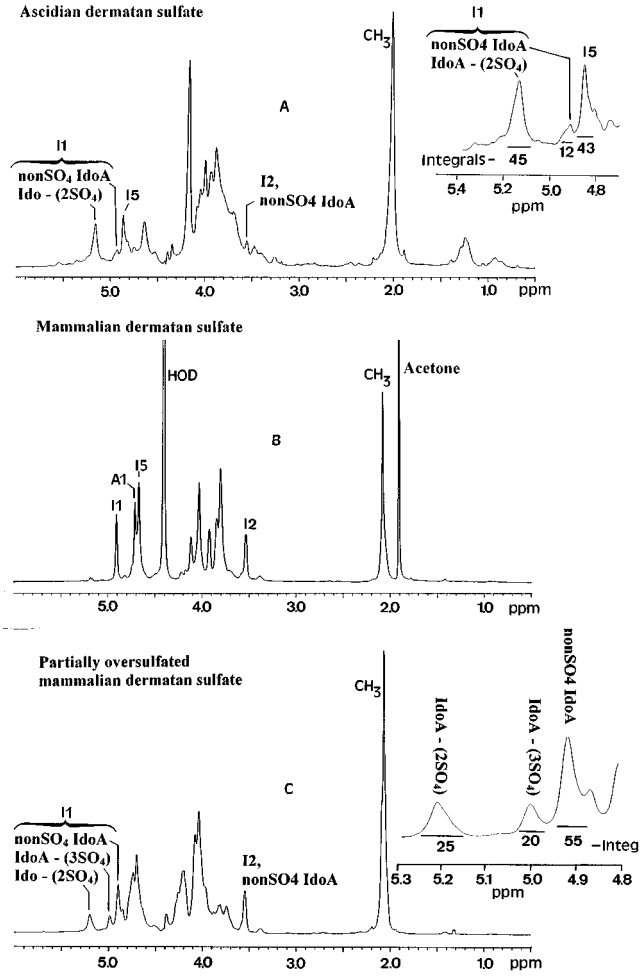

FIG. 5. **^{1}H NMR spectra at 500 MHz of the ascidian dermatan sulfate (*A*), mammalian dermatan sulfate (*B*), and chemically oversulfated mammalian dermatan sulfate (*C*).** All spectra were recorded at 60 °C for samples in D_2O solution. Chemical shifts are relative to internal or external trimethylsilylpropionic acid at 0 ppm. The HOD signal has been suppressed in *A* and *C* by presaturation. Signals designated by *A* refer to those produced by *N*-acetyl-β-D-galactosamine, whereas those of α-L-iduronic acid residues are labeled *I*. Expansions of the 5.5–4.7-ppm and 5.3–4.8-ppm regions of the spectra of ascidian dermatan sulfate and partially oversulfated dermatan sulfate are shown in the *insets* in *A* and *C* respectively. The *integrals* listed under the proton regions of the spectra are normalized to a total of 100 protons. The ^{1}H spectrum of the partially oversulfated dermatan sulfate resembles the spectrum of a similar compound reported in Ref. 40.

chondroitin AC and ABC lyases and to deaminative cleavage by nitrous acid, treatments that specifically degrade chondroitin sulfate, dermatan sulfate, and heparan sulfate. Preliminary analysis indicates the major contaminant is a sulfated glucan.

Table I shows the chemical analysis and the specific optical rotation of the purified ascidian dermatan sulfate. The observation that the sulfate/galactosamine molar ratio is approximately 1.8 suggests that most of the disaccharide units in this glycosaminoglycan are disulfated. The specific rotation of the ascidian dermatan sulfate (−54 °C) is very close to that recorded for a solution of standard dermatan sulfate from the mammalian source (−61 °C).

Analysis of the ascidian dermatan sulfate by anion exchange chromatography on a Mono Q-FPLC column (Fig. 3) confirms the high negative charge density of this glycosaminoglycan. Thus, the ascidian dermatan sulfate (*closed circle* in Fig. 3*B*) was eluted from the column at a higher NaCl concentration than mammalian glycosaminoglycans (Fig. 3*A*), as expected from the data of Table I.[3] The peak corresponding to the ascid-

[3] The electrophoretic migration of glycosaminoglycans in 1,3-diaminopropane/acetate buffer (Fig. 2) depends on the structure of the polysaccharide, which forms a complex with the diamine group (33, 34). Thus,

TABLE II

Proton chemical shifts for residues of α-L-iduronate in ascidian and mammalian dermatan sulfates

The abbreviations used are: NR, not reported; ND, not determined; TSP, trimethylsilylpropionic acid; DS, dermatan sulfate. Values in boldface indicate positions bearing sulfate ester.

GAG	Unit	% of total	Chemical shift				
			H1	H2	H3	H4	H5
					ppm		
This work[a]							
Ascidian DS	IdoA-(2-SO_4)	80	5.14	**4.14**	4.32	4.03	4.83
	IdoA	20	4.90	3.52	4.06	4.06	4.80
Oversulfated	IdoA-(2-SO_4)	25	5.15	**4.12**	ND	ND	ND
mammalian Ds	IdoA-(3-SO_4)	20	4.94	3.68	**4.72**	4.30	4.75
	IdoA	55	4.84	3.50	3.92	4.03	4.73
Mammalian DS	IdoA	>90	4.91	3.53	3.92	4.12	4.71
Literature values							
Oversulfated	IdoA	NR	4.90	3.56	3.97	4.11	4.74
mammalian DS[b]	IdoA-(2-SO_4)	NR	5.22	**4.19**	4.23	ND	ND
	IdoA-(3-SO_4)	NR	4.99	3.75	**4.77**	4.35	4.78
	IdoA-(2,3-diSO_4)	NR	5.23	**4.37**	**4.96**	4.29	4.89
Mammalian DS[b]	IdoA	NR	4.88	3.53	3.90	4.10	4.72
	IdoA-(2-SO_4)	NR	5.16	**4.17**	4.23	4.06	4.85
	GlcA	NR	4.47	3.38	3.58	3.78	3.66
Mammalian DS[c]	IdoA	NR	4.90	3.54	3.92	4.11	4.72

[a] ^{1}H spectra were recorded at 500 MHz, 60 °C in D_2O. Chemical shifts are referenced to internal TSP at 0 ppm in the mammalian dermatan sulfate sample.

[b] Bossennec *et al.* (36). Chemical shifts relative to internal TSP; 37 °C.

[c] Sanderson *et al.* (35).

ian dermatan sulfate is resistant to chondroitin AC lyase (not shown), but disappears after incubation with chondroitin ABC lyase (*open circles* in Fig. 3*B*).

Overall, these results indicate the presence of a dermatan sulfate-like glycosaminoglycan in ascidian. The sulfate/galactosamine molar ratio (Table I) and the elution from a Mono Q-FPLC column (Fig. 3) suggest higher content of sulfate esters in this glycosaminoglycan than in the mammalian dermatan sulfate.

The Products Formed by Degradation with Chondroitin ABC Lyase Indicate That the Ascidian Dermatan Sulfate Has an Unusual Sulfation Pattern—The products of the action of chondroitin lyases, chondro-4-sulfatase and chondro-6-sulfatase on the ascidian dermatan sulfate and on standard compounds were analyzed by paper chromatography (Fig. 4). The action of chondroitin ABC lyase on the ascidian dermatan sulfate produces two products of different mobility on paper chromatography.[4] The one with low mobility has the same migration as the standard disulfated disaccharide ΔGlcA(2SO_4)-GalNAc-(6SO_4) and accounts for ~90% of the reacted material. The high mobility product has the same migration as the standard ΔGlcA-GalNAc(6SO_4) and represents ~10% of reacted material. No product is formed by chondroitin AC lyase.

Further characterization of the disulfated disaccharide was obtained by incubation with specific sulfatases. It resists chondro-4-sulfatase degradation but when digested with chondro-6-sulfatase gives rise to small amounts of nonsulfated disaccharide (ΔGlcA-GalNAc), which originated from the 6-sulfated disaccharide, and a product with a slightly lower chromatographic mobility than ΔGlcA-GalNAc(6SO_4), possibly the disaccharide ΔGlcA(2SO_4)-GalNAc.

Digestion of the standard disaccharide ΔGlcA(2SO_4)-GalNAc(6SO_4) with chondro-4- and -6-sulfatase (Fig. 4*B*) confirms the assignment of the same disaccharide obtained from ascidian dermatan sulfate. The standard disaccharide resists chondro-4-sulfatase but, when digested with chondro-6-sulfatase, it gives rise to ΔGlcA(2SO_4)-GalNAc, with the same chromatographic mobility as that obtained from the ascidian dermatan sulfate.

Chondroitin ABC lyase action on the desulfated ascidian dermatan sulfate produces exclusively the nonsulfated disaccharide ΔGlcA-GalNAc (Fig. 4*C*), as expected.

These experiments demonstrate that the ascidian dermatan sulfate has a backbone of repeating unit [4-α-L-IdoA-1→3-β-D-GalNAc-1] as mammalian dermatan sulfate, but the α-L-iduronate residues are sulfated at the 2-position, and the β-D-galactosamine units are sulfated at the 6-position. However, the chondroitin lyase and sulfatase experiments cannot exclude completely the presence of sulfation at position 3 of the α-L-iduronate residues.

Interestingly, the action of chondroitin ABC lyase on a chemically oversulfated mammalian dermatan sulfate (Fig. 4*C*) produces a major product with the same chromatographic mobility as the disulfated disaccharide ΔGlcA-GalNAc(4/6-diSO_4). Upon the action of chondro-4-sulfatase + chondro-6-sulfatase, it gives rise to the nonsulfated disaccharide (ΔGlcA-GalNAc). These results indicate that on the chemically oversulfated dermatan sulfate, most of the β-D-galactosamine residues are sulfated at both 4- and 6- positions and linked to a nonsulfated α-L-iduronate residue. Thus, both the ascidian and chemically oversulfated dermatan sulfate have a similar saccharide chain and higher sulfate content than standard mammalian dermatan sulfate, but with different sulfation patterns.

^{1}H NMR Spectra Confirm the Preponderance of 2-O-Sulfo-α-L-idopyranosyluronic Acid Units in the Ascidian Dermatan Sulfate—The ^{1}H spectra of the ascidian dermatan sulfate, mammalian dermatan sulfate, and chemically oversulfated dermatan sulfate, are shown in Fig. 5, *A*, *B*, and *C*, respectively.

The ^{1}H spectrum of mammalian dermatan sulfate was in agreement with published results (35). Integration of the resonances from H1 of unsulfated (4.9 ppm) and 2-*O*-sulfated (~5.2 ppm) iduronic acid residues demonstrates that not more than 5% of the iduronic acid units are 2-*O*-sulfated. The assignments of the ^{1}H spectra of ascidian dermatan sulfate and of

the similar electrophoretic mobility of the ascidian and mammalian dermatan sulfate in this buffer is not totally unexpected, besides the different charge densities of these two glycosaminoglycans.

[4] The products formed by chondroitin ABC lyase action on the ascidian dermatan sulfate were also analyzed by gel filtration on Sephadex G-50. The fractions were assayed by the carbazole reaction (25) for hexuronic acid. The predominant product was eluted from the column as disaccharide. A small amount of tetrasaccharide was also detected, possibly as a product of incomplete action of the lyase.

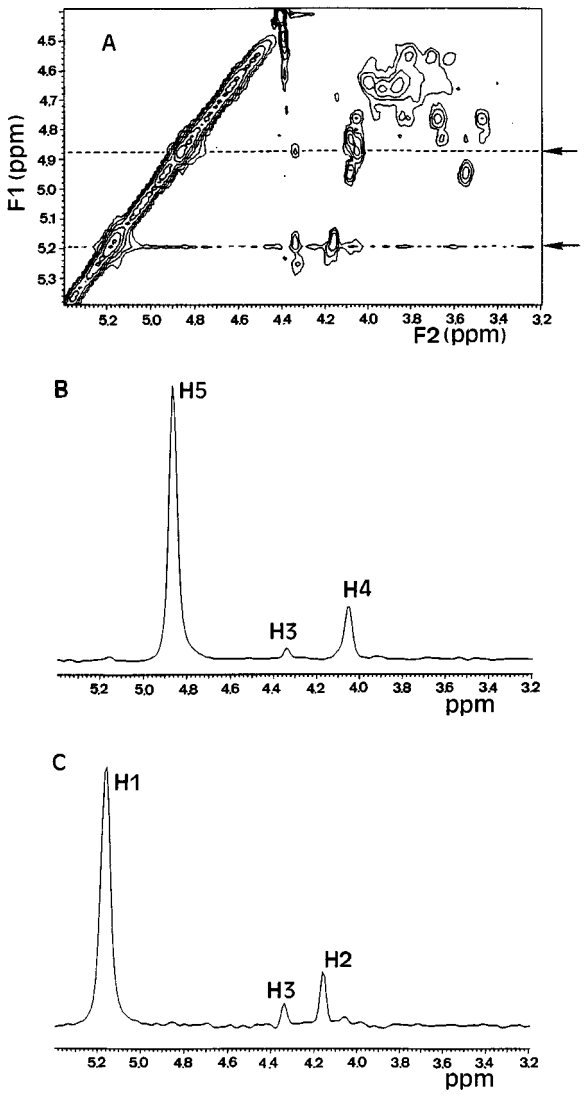

FIG. 6. **Part of the TOCSY spectrum of the ascidian dermatan sulfate.** The *dotted lines* (*A*) show the points at which the two slices of the same spectrum are shown in *B* and *C*. These two slices are taken at the chemical shifts of IdoA-($2SO_4$) H5 and IdoA-($2SO_4$) H1, respectively. Cross-peaks from H5 to H4 and H3, and from H1 to H2 and H3 are displayed clearly in the slices. These peaks allow complete assignment of the IdoA-($2SO_4$) residue in this polysaccharide.

oversulfated mammalian dermatan sulfate are based on the results of COSY and TOCSY spectra and on comparison with published results for a different sample of oversulfated dermatan sulfate (36) and for chondroitin 6-sulfate (37). These assignments and literature values are summarized in Table II.

In both COSY and TOCSY spectra, α-L-iduronate spin systems can be traced from their characteristic H1 resonances. Iduronic acid H1 signals have chemical shifts in the region 4.9–5.23 ppm and small $J_{H1–H2}$ values, unresolved in the broad lines present in the spectra of these heterogeneous and polydisperse polysaccharides (Table II, Literature values). Bossennec *et al.* (36) identify four such systems in the ^1H spectrum of a sample of oversulfated mammalian dermatan sulfate, corresponding to unsulfated IdoA, IdoA-($2SO_4$), IdoA-($3SO_4$), and IdoA-(2,3-diSO_4).

We were able to recognize the first three of these systems in the TOCSY spectrum of our sample of partially oversulfated mammalian dermatan sulfate, but did not see a spin system for IdoA-(2,3-diSO_4). Integration of H1 resonances in the one-dimensional spectrum (Fig. 5*C*, *inset*) indicated that the three types of residues are in the proportion IdoA/IdoA-($2SO_4$)/IdoA-($3SO_4$) of 55:25:20. Assignments for the IdoA-($2SO_4$) residue are incomplete both in this work and in Ref. 35, because of overlap with more intense galactosamine peaks in both one- and two-dimensional spectra, but the H2 signal at 4.12 ppm, 0.62 ppm downfield of H2 in unsulfated iduronic acid residue, is diagnostic of sulfation at the 2-position. The spin system for IdoA-($3SO_4$) is complete in both this work and in Ref. 36; for this residue, H2 is at 3.68 ppm and H3 at 4.72 ppm, 0.8 ppm downfield of H3 in unsulfated iduronic acid units.

In the TOCSY spectrum of the ascidian dermatan sulfate (Fig. 6*A*), it is possible to see two spin systems attributable to α-L-iduronic acid residues, in the proportion ~80:20 (Fig. 5*A*, *inset*). The less abundant residue has chemical shifts which resemble those of unsulfated iduronic acid in mammalian and partially oversulfated mammalian dermatan sulfate, although the H3 signal at 4.06 is 0.14 ppm downfield of H3 in the mammalian compounds. The more abundant residue (assigned using the TOCSY spectrum; see Fig. 6, *B* and *C*) has chemical shifts which are close to those for IdoA-($2SO_4$) in mammalian dermatan sulfate and which agree with the chemical shifts for IdoA-($2SO_4$) in oversulfated mammalian dermatan sulfate in so far as they can be determined. In particular, the H2 signal at 4.14 ppm indicates sulfation at this position; it can therefore be said that most of the α-L-iduronate residues in the ascidian

TABLE III

Proton chemical shift of N-acetyl-β-D-galactosamine residues in ascidian and mammalian dermatan sulfates

The abbreviations used are: ND, not determined; TSP, trimethylsilylpropionic acid; DS, dermatan sulfate. Values in boldface indicate positions bearing sulfate ester.

GAG	Unit	Chemical shift							
		H1	H2	H3	H4	H5	H6	H6′	CH_3
					ppm				
This work[a]									
Ascidian DS	GalNAc-(6-SO_4)	4.64	4.02	3.86	4.15	3.86	**4.15**	**4.15**	2.02
Oversulfated mammalian DS	GalNAc-(4,6-diSO_4)	4.70	4.02	4.0–4.05	**4.76**	4.07	**4.25**	**4.21**	2.06
Literature values									
Chondroitin 6-sulfate[b]	GalNAc-(6-SO_4)	4.58	4.02	3.85	4.16	3.95	**4.23**	**4.21**	2.01
Mammalian DS[c]	GalNAc-(4-SO_4)	4.70	4.05	3.95	**4.65–4.7**	3.80	3.75	3.80	2.08
Mammalian DS[d]	GalNAc-(4-SO_4)	4.70	4.05	4.03	**4.69**	3.80	3.79	3.82	2.08
	GalNAc-(4,6-diSO_4)	ND	ND	ND	ND	4.07	**4.29**	**4.25**	ND

[a] ^1H spectra were recorded at 500 MHz, 60 °C in D_2O. The chemical shifts are referenced to internal TSP at 0 ppm in the mammalian dermatan sulfate sample.

[b] Welti *et al.* (37).

[c] Sanderson *et al.* (35); chemical shifts relative to internal TSP; 60 °C.

[d] Bossennec *et al.* (36); chemical shifts relative to internal TSP; 37 °C.

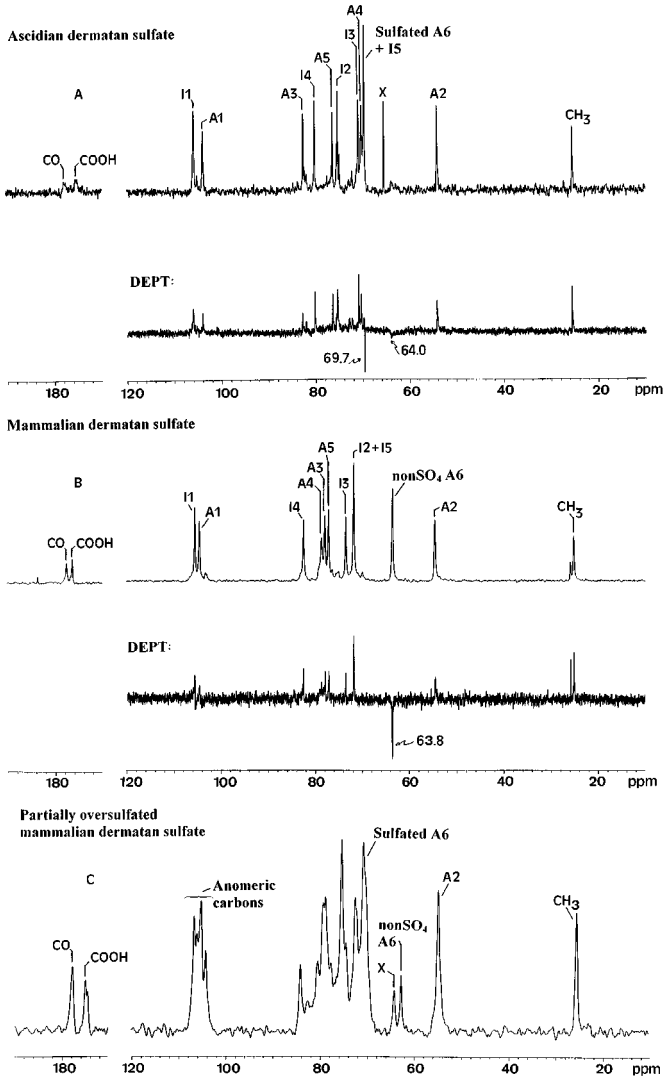

FIG. 7. **^{13}C NMR spectra at 125 MHz of the ascidian dermatan sulfate (*A*), mammalian dermatan sulfate (*B*), and chemically oversulfated mammalian dermatan sulfate (*C*), all at 60 °C in D_2O solution.** Chemical shifts are relative to internal or external trimethylsilylpropionic acid at 0 ppm. Signals designated by *A* refer to those produced by *N*-acetyl-β-D-galactosamine, whereas those of α-L-iduronic acid residues are labeled *I*. Signals marked *X* arise from contaminants. The DEPT spectra of ascidian and mammalian dermatan sulfates are shown in *A* and *B*, respectively, and the chemical shifts of C6 are indicated in the figure.

dermatan sulfate is 2-*O*-sulfated.

The spin systems of *N*-acetyl-β-D-galactosamine residues in the ^{1}H spectra are traced from the H1 signal at about 4.58–4.74 ppm through the COSY and TOCSY spectra. For the ascidian dermatan sulfate, the assignment given for GalNAc(6SO_4) in Table III is consistent with the TOCSY spectrum, but for H4, H5, H6, and H6′ it must be tentative as the cross-peaks (all between resonances at 3.86 and 4.15 ppm) overlap heavily. Although there is no sign of a signal for sulfated H4 at about 4.65–4.70 ppm, the possibility cannot be ruled out that H1 and H4 resonances are coincident.

A TOCSY spectrum of the partially oversulfated mammalian dermatan sulfate allowed complete assignment of the ^{1}H spectrum of the doubly sulfated residue GalNAc-(4,6-diSO_4), partially assigned in Ref. 36. Both these data and the spectrum of GalNAc-(6SO_4) in chondroitin 6-sulfate (37) indicate that the downfield shift for H6 and H6′ on sulfation at C6 is about 0.4 ppm. Although on this basis the H6 and H6′ chemical shifts of ascidian dermatan sulfate are consistent with 6-*O*-sulfation of the galactosamine residues, the difficulties surrounding assignment are too great to make this deduction with certainty from the ^{1}H NMR data.

^{13}C NMR Spectra Confirm 6-O-Sulfation of the N-Acetyl-β-D-galactosamine Residues—The ^{13}C NMR spectra of ascidian dermatan sulfate, mammalian dermatan sulfate, and chemically oversulfated mammalian dermatan sulfate, are illustrated in Fig. 7, *A*, *B*, and *C*, respectively, with the corresponding DEPT spectra. Chemical shifts for mammalian dermatan sulfate were similar to literature values (35) as summarized in Table IV. With the exception of the C6 signals, the assignments of the ^{13}C spectra of the ascidian and partially oversulfated mammalian dermatan sulfates summarized in Table IV are based on comparisons with literature values for mammalian dermatan sulfate (35), an oligosaccharide fragment of mammalian dermatan sulfate (SD-DS BM1/2) (38), and chondroitin 6-sulfate (39).

The DEPT spectra have been recorded in such a way that methine and methyl carbon signals are both 180° out of phase with methylene carbon signals, allowing the identification of C6 of galactosamine, which is the only methylene carbon in the dermatan sulfate structure. In the DEPT spectrum of mammalian dermatan sulfate (Fig. 7*B*), in which galactosamine is sulfated at C4 and linked at C3, the unsubstituted C6 resonating at 63.8 ppm is inverted. The DEPT spectrum of ascidian dermatan sulfate (Fig. 7*A*) has an inverted resonance at 69.7 ppm, shifted downfield by 5.9 ppm. This chemical shift is similar to that of C6 of chondroitin 6-sulfate (69.0 ppm) (39) and is confirmation that most of the galactosamine in the ascidian dermatan sulfate is sulfated at C6. A minor inverted signal in the ^{13}C spectrum of ascidian dermatan sulfate at 64 ppm can be attributed to unsulfated galactosamine C6. The ^{13}C spectrum of the oversulfated mammalian dermatan sulfate (Fig. 7*C*) was complex and overlapping and was not assigned, but the signal at 64.3 ppm from unsulfated C6 is minor in comparison to other peaks. The resonance from the majority of C6 of galactosamine must be in the group of overlapping peaks near 70.7 ppm and so indicates that C6 is largely substituted with sulfate.

Other signals in the ^{13}C spectrum of ascidian dermatan sulfate correspond well with the IdoA-(2SO_4) shifts from the mammalian dermatan oligosaccharide fragment or the GalNAc-(6SO_4) shifts from chondroitin sulfate, and all the signals in the spectrum can be accounted for on this basis. Thus, the spectrum confirms the linkage positions and configurations of the constituent sugars of the ascidian dermatan sulfate.

Overall, the proposed structure for the ascidian dermatan sulfate (Fig. 8*A*) and for the chemically oversulfated mammalian dermatan sulfate (Fig. 8*C*) is supported by degradation with chondroitin ABC lyase, analysis of the disaccharide products by digestion with chondro-4- and -6-sulfatases, and by ^{1}H and ^{13}C NMR data. Both compounds have the same backbone structure but have different patterns of sulfate substitution.

The Ascidian Dermatan Sulfate Has No Anticoagulant Activity and Reduced Capacity to Potentiate Heparin Cofactor II—The results of APTT assays in the absence, and in the presence, of an antithrombin III neutralizing antibody are listed for ascidian dermatan sulfate, mammalian dermatan sulfate, and chemically oversulfated mammalian dermatan sulfate, in Table V. The presence of the antithrombin III neutralizing antibody has the effect that the test measures only anticoagulant activity not mediated by antithrombin III; in practice, this indicates activity mediated by heparin cofactor II. Two extremes for the scale of APTT activity are the heparin standard used for the test (193 units/mg) and chondroitin 6-sulfate (<0.1 units/mg). Mammalian dermatan sulfate has a spe-

TABLE IV
Carbon chemical shifts for ascidian and mammalian dermatan sulfates

The abbreviations used are: ND, not determined; TSP, trimethylsilylpropionic acid; DS, dermatan sulfate. Values in boldface indicate positions bearing sulfate ester.

GAG	Unit	Chemical shift							
		C1	C2	C3	C4	C5	C6	Acetyl-CH_3	Acetyl-CO
						ppm			
This work[a]									
Ascidian DS	IdoA-(2-SO_4)	106.0	**75.4**	71.0	80.3	69.7	175.4		177.8
	GalNAc-(6-SO_4)	104.0	54.2	82.5	70.4	76.5	**69.7**	25.6	
Mammalian DS	IdoA	105.7	72.1	73.7	82.7	72.1	176.6		
	GalNAc-(4-SO_4)	104.8	54.8	78.2	78.9	77.4	63.8	25.4	177.8
Literature values									
Mammalian DS[b]	IdoA	105.7	72.0	73.7	82.7	72.0	176.6		
	GalNAc-(4-SO_4)	104.7	54.8	78.1	**78.9**	77.3	63.7	25.4	177.8
Mammalian DS fragments	IdoA-(2-SO_4)[c]	101.2	**73.8**	69.0	79.2	68.0	ND		
Chondroitin 6-sulfate	GalNAc-(6-SO_4)[d]	102.7	**54.4**	81.3	69.0	75.4	**69.0**	24.0	176.3

[a] ^{13}C spectra were recorded at 125 MHz, 60 °C in D_2O. Chemical shifts relative to internal TSP in the mammalian dermatan sulfate sample.
[b] Sanderson *et al.* (35). Chemical shifts relative to internal TSP; 60 °C.
[c] Mascellani *et al.* (38).
[d] Hamer and Perlin (39). Chemical shifts relative to methanol at 50.7 ppm; 45 °C.

cific activity of 4 units/mg, 71% of which is retained when antithrombin III is neutralized. Oversulfation of mammalian dermatan sulfate, with the introduction of extra 2-*O*-sulfation of the iduronate residues and extra 6-*O*-sulfation of the *N*-acetylgalactosamine residues, increases the specific activity in the APTT assay to 13 units/mg, 83% of which is retained when antithrombin III is neutralized. Analogous increase in the anticoagulant activity of oversulfated dermatan sulfate using APTT assay was reported previously (40).

The high affinity binding sequence in porcine skin dermatan sulfate for heparin cofactor II consists of [4-α-L-IdoA-(2SO_4)-1→3-β-D-GalNAc(4SO_4)-1]$_n$ where $n \geq 3$ (41). In the chemically oversulfated material, about 20% of the iduronate residues are 2-*O*-sulfated (Fig. 5*C*), compared with ≤5% in the starting material, so the increase in specific activity is not unexpected. It is not possible to estimate the contribution of the introduced 6-*O*-sulfation of galactosamine residues in the oversulfated mammalian dermatan sulfate to activity, except to say that it clearly does not inhibit the expression of APTT activity.

The ascidian dermatan sulfate has no detectable APTT activity, in spite of its high degree of sulfation. It can be inferred from this that 4-*O*-sulfation of the galactosamine residues is essential for the anticoagulant activity of dermatan sulfate, as the high activity of the oversulfated mammalian dermatan sulfate shows that 6-*O*-sulfation does not inhibit activity. In addition to the position of the sulfate esters on the disaccharide units, chain size or arrangement of the repeating disaccharide units on the polymer may also influence the anticoagulant activity of dermatan sulfate. These aspects require future investigation using chemically modified molecules.

Direct measurement of inhibition of thrombin by heparin cofactor II in the presence of the various glycosaminoglycans confirms the results obtained using the APTT assays. Thus, the IC_{50} for thrombin inhibition is 2.2 and 1.2 μg/ml for mammalian dermatan sulfate and partially oversulfated dermatan sulfate, respectively, whereas the IC_{50} for ascidian dermatan sulfate is 320 μg/ml (Fig. 9). The relatively low activity of ascidian dermatan sulfate with heparin cofactor II could reflect nonproductive binding of the glycosaminoglycan to the inhibitor. If this were the case, ascidian dermatan sulfate might interfere with the interaction of mammalian dermatan sulfate with heparin cofactor II. As shown in Fig. 9, however, the IC_{50} for mammalian dermatan sulfate was unchanged in the presence of 20 μg/ml ascidian dermatan sulfate.

CONCLUSIONS

Ascidian dermatan sulfate has the same degree of sulfation as the sequence in porcine skin dermatan sulfate with high affinity for heparin cofactor II differing only in the position of sulfation of the galactosamine residues. In spite of this, it has lower activity with heparin cofactor II than mammalian dermatan sulfate (Fig. 9) and no measurable anticoagulant activity (Table V), thus establishing that 4-*O*-sulfation of *N*-acetyl-β-D-galactosamine residues is essential for interaction with heparin cofactor II and consequently for the anticoagulant activity of dermatan sulfate.

The degree of specificity of the interaction between heparin cofactor II and glycosaminoglycan has been the subject of argument. Sié *et al.* (42) concluded that for heparin there is no dependence of sulfation, but only on the degree of sulfation, for the interaction with heparin cofactor II. Our results indicate that the structural requirement of dermatan sulfate for the catalysis of thrombin inhibition by heparin cofactor II is not due to sulfate density alone, which is consistent with the observation that the binding sites in heparin cofactor II for heparin and dermatan sulfate are not identical (21). Ascidian dermatan sulfate is probably the most highly sulfated natural glycosaminoglycan reported to have undetectable APTT activity and very low effect on thrombin inhibition by heparin cofactor II (Table V and Fig. 9). Therefore, we conclude that the interaction of dermatan sulfate and heparin cofactor II is specific and that 4-*O*-sulfation of *N*-acetyl-β-D-galactosamine residues (as well as 2-*O*-sulfation of the α-L-iduronic acid units) is essential.

Other highly sulfated polysaccharides with different sugar backbones, such as pentosan polysulfate, also potentiate heparin cofactor II (43). Studies of the anticoagulant activity of artificially oversulfated dermatan sulfates have all indicated increased activity on extra sulfation (40, 44, 45). The dependence of dermatan sulfate affinity for heparin cofactor II on sulfation of the iduronate residues has now been well established (41), but the importance of the sulfate group at the 4-position of the galactosamine units has not been explored as fully. Comparison of the activities of polysaccharides resembling dermatan sulfate extracted from hagfish demonstrate that sulfation at both the 4- and 6-positions of the galactosamine units does not confer heparin cofactor II affinity unless the iduronate residue is also sulfated (46).

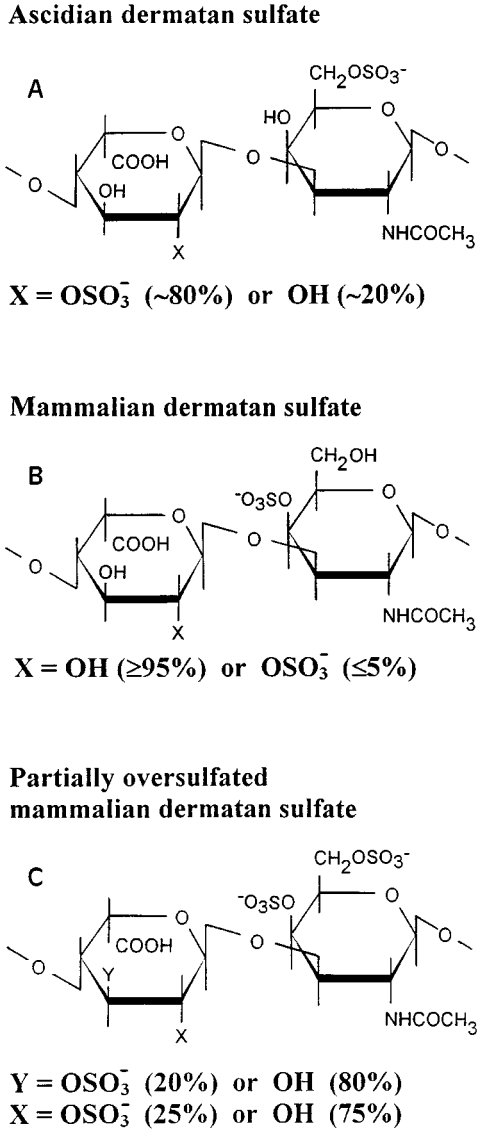

FIG. 8. **Major repetitive disaccharide units of ascidian dermatan sulfate (*A*), mammalian dermatan sulfate (*B*), and chemically oversulfated mammalian dermatan sulfate (*C*).** These glycosaminoglycans have the same backbone structure [4-α-L-IdoA-1→3-β-D-GalNAc-1]$_n$, but have different patterns of sulfate substitutions. The ascidian dermatan sulfate (*A*) is sulfated at both the 2-position of the α-L-iduronic acid and the 6-position of the *N*-acetyl-β-D-galactosamine units. On the partially oversulfated dermatan sulfate (*C*), most of the galactosamine residues are sulfated at both 4- and 6-positions; the nonsulfated, 2-sulfated, and 3-sulfated α-L-iduronic acid residues are in the proportion of 55:25:20. The repetitive disaccharide units of mammalian dermatan sulfate (*B*) are sulfated at carbon 4 of the hexosamine moiety; small amounts (≤5%) of 2-*O*-sulfated α-L-iduronic acid residues are also found in this mammalian glycosaminoglycan.

Our observation on the anticoagulant activity of the ascidian dermatan sulfate suggests that the particular disposition in space of the sulfate groups at the 2-position of α-L-iduronic acid and 4-position of the *N*-acetyl-β-D-galactosamine residues is recognized by heparin cofactor II. As addition sulfate substitution does not appear to hinder the interaction, it may be possible for heavily sulfated polysaccharides such as pentosan polysulfate (with 4-sulfates per disaccharide) to mimic this spatial array, the high density of sulfation overwhelming any specificity of the binding site.

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TABLE V
Anticoagulant properties of ascidian dermatan sulfate and of standard glycosaminoglycans

Glycan	APTT[a]	APTT without antithrombin III[b]
	units/mg	*% original*
Mammalian dermatan sulfate	4	71
Ascidian dermatan sulfate	<0.1	
Oversulfated mammalian dermatan sulfate	13	83
Chondroitin 6-sulfate	<0.1	
Heparin	193	34

[a] The clotting times were recorded as described under "Experimental Procedures." The activity is expressed as units/mg using a parallel standard curve based on the International Heparin Standard (193 units/mg).

[b] APTT assay after the addition of antithrombin III neutralizing antibody.

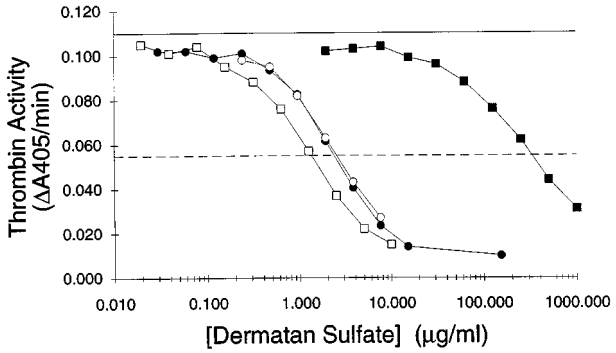

FIG. 9. **Activity of dermatan sulfate with heparin cofactor II.** Heparin cofactor II (68 nM) was incubated with thrombin (15 nM) in the presence of various concentrations of mammalian dermatan sulfate (●), ascidian dermatan sulfate (■), chemically oversulfated dermatan sulfate (□), or mammalian dermatan sulfate plus a fixed concentration of ascidian dermatan sulfate (20 μg/ml) (○). After 60 s, the remaining thrombin activity was determined with a chromogenic substrate (A_{405}/min).

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