Pergamon

Biochemical Pharmacology, Vol. 47, No. 7, pp. 1241–1252, 1994.

0006-2952/94 $6.00 + 0.00

0006-2952(93)E0068-I

LOW MOLECULAR WEIGHT DERMATAN SULFATE AS AN ANTITHROMBOTIC AGENT

STRUCTURE–ACTIVITY RELATIONSHIP STUDIES

ROBERT J. LINHARDT,*† UMESH R. DESAI,* JIAN LIU,* AZRA PERVIN,* DEBRA HOPPENSTEADT‡ and JAWED FAREED‡

*Division of Medicinal and Natural Products Chemistry, College of Pharmacy, University of Iowa, Iowa City, IA 52242; and ‡Departments of Pathology and Pharmacology, Loyola University Medical Center, Maywood, IL 60153, U.S.A.

(*Received* 26 *March* 1993; *accepted* 6 *October* 1993)

Abstract—A structure–activity relationship of low molecular weight dermatan sulfate was undertaken to understand better this new non-heparin, glycosaminoglycan-based antithrombotic agent. A dermatan sulfate prepared from bovine intestinal mucosa [average molecular weight (MWavg) 25,000], and currently in clinical trials as an antithrombotic agent, was used in this study. Dermatan sulfate was partially depolymerized using hydrogen peroxide and copper(II) as catalyst to MWavg 5600 to obtain a low molecular weight dermatan sulfate. This low molecular weight dermatan sulfate was then fractionated by gel permeation chromatography to obtain four subfractions having MWavg 7800, 5500, 4200 and 1950. The dermatan sulfate, low molecular weight dermatan sulfate and its subfractions showed substantially different optical rotations. The ^{1}H-NMR spectroscopic analysis of dermatan sulfate samples showed some differences including increased content of GalpNAc4S6S residues and improved resolution in ring resonances for low molecular weight dermatan sulfate fractions, primarily the result of reduced molecular weight and lowered heterogeneity. Saccharide compositional analysis relied on chondroitin ABC lyase treatment followed by capillary electrophoresis. Polyacrylamide gel-based oligosaccharide mapping was also performed by treating dermatan sulfate samples with chondroitin B, AC and ABC lysases. These analyses showed increased amounts of sulfation as the MWavg decreased. *In vitro* bioassay showed maximum anti-Xa activity in the 4.2 kDa fraction and maximum heparin cofactor II-mediated anti-IIa activity in the 5.5 kDa fraction. The *in vivo* antithrombotic activity of these fractions was measured using a modified Wessler stasis thrombosis model. The 4.2 kDa fraction showed greater antithrombotic activity than the other low molecular weight dermatan sulfate fractions, dermatan sulfate, and low molecular weight dermatan sulfate. This enhanced activity may result from several structural features of the 4.2 kDa fraction including: a high content of 4,6- and 2,4-disulfated disaccharide sequences; the requirement of specific chain length; a change in the ratio of iduronic to glucuronic acid; and the presence of chondroitin ABC lyase resistant material.

Key words: dermatan sulfate; low molecular weight; antithrombotic; glycosaminoglycan

DS§ is a polydisperse, micro-heterogeneous sulfated copolymer of *N*-acetyl-D-galacytopyranose (*N*-acetylgalactosamine, D-Galp-NAc) and primarily L-idopyranosyluronic acid (iduronic acid, L-IdoAp) having an average molecular weight of 20,000–30,000. DS is found in a wide variety of animal tissues [1] but is prepared commercially from either porcine or bovine mucosa.

Heparin is a related glycosaminoglycan, and it is widely used as a clinical anticoagulant [2, 3]. By potentiating the activity of serine proteinase inhibitors such as ATIII [4] and HCII [5], heparin inhibits serine proteases and blocks the coagulation cascade. Heparin also acts as an antithrombotic agent through its action on platelets and on the endothelium [6], as measured by its *in vivo* blocking of thrombus formation. Heparin exhibits significant side-effects, primarily hemorrhagic complications [7].

DS is currently under clinical investigation as a new antithrombotic agent [8]. Unlike heparin, it does not act through ATIII but instead is believed to act primarily on thrombin through HCII [5, 9]. Because of this selectivity, DS has only a weak anticoagulant effect. Interest in DS has centered on its high *in vivo* antithrombotic activity and negligible hemorrhagic complications [10]. These data suggest that DS may possess a higher therapeutic index than heparin. Recent studies in our laboratory have demonstrated that a DS with increased *in vivo* antithrombotic activities also has an increased content of conformationally flexible iduronic acid residues [11, 12]. Because of its high molecular weight, DS is not well absorbed when administered subcutaneously.

LMW heparins, prepared by the partial depoly-

† Corresponding author. Tel. (319) 335–8834; FAX (319) 335–6634.

§ Abbreviations: DS, dermatan sulfate; ATIII, antithrombin III; HCII, heparin cofactor II; LMW, low molecular weight; CE, capillary electrophoresis; t_R, retention time; AUFS, absorbance units full scale; PT, prothrombin time; APTT, activated partial thromboplastin time; TT, thrombin time; WHO, World Health Organization; and FEIBA, factor VIII inhibitor bypass activator.

Table 1. Physical characteristics and *in vitro* activity of DS and LMW-DS samples*

Sample	Molecular weight	Optical rotation†	Anti-Xa activity‡ (U/mg)	HCII-mediated activity§ (U/mg)	APTT‖ (sec)	TT¶ (sec)
DS OP435	25,000	−57	0	192 ± 8.4	90 ± 4.1	300 ± 0
LMW-DS OP370	5600	−50	9.8 ± 1.2	135 ± 6.5	70 ± 3.5	225 ± 11.2
LMW-DS 105-1	7800	−60	0.39 ± 0.2	115 ± 5.4	65 ± 3.2	100 ± 8.8
LMW-DS 105-2	5500	−70	2.2 ± 1.0	129 ± 5.0	63 ± 3.4	30 ± 2.1
LMW-DS 105-3	4200	−51	4.3 ± 0.8	88.6 ± 3.8	60 ± 3.1	25 ± 1.4
LMW-DS 105-4	1950	−30	0.23 ± 0.1	19.3 ± 1.7	45 ± 2.1	20 ± 1.0

* Values are means ± SD, N = 3.
† Degrees at Na_D line in water at 20°.
‡ Antithrombin III-mediated anti-factor Xa activity.
§ Heparin cofactor II-mediated anti-factor IIa activity.
‖ Activated partial thromboplastin time at 100 μg/mL (plasma only control 30–35 sec).
¶ Thrombin time at 25 μg/mL (plasma only control 18–20 sec).

merization of heparin [13], require ATIII to inhibit enzymes in the coagulation cascade. These agents are currently in clinical studies in an effort to circumvent the problems of heparin and to increase its therapeutic index [14]. The promise of these drugs has not been fully realized. In clinical studies, it appears that their only advantage may be prolonged half-life and improved bioavailability when administered subcutaneously [14]. DS can also be partially depolymerized by either chemical [15] or enzymatic methods to prepare LMW-DS [16, 17]. This paper examines a DS having moderate antithrombotic activity, a LMW-DS prepared from this DS by peroxy-radical based depolymerization using hydrogen peroxide, and various subfractions of this LMW-DS prepared by gel permeation chromatography. The chemical properties and *in vitro* and *in vivo* activities of these agents are examined in an effort to understand the relationship between the molecular size of DS and its antithrombotic activity.

MATERIALS AND METHODS

Materials. Dermatan sulfate OP435(Lot No. 7/91), LMW-DS, OP370, and molecular weight subfractions of OP370, 105-1, -2, -3, and -4 were from Opocrin, Modena, Italy. Chondroitin ABC lyase (EC 4.2.2.4, from *Proteus vulgaris*, contains <0.1 and <0.1% chondroitin-4- and 6-sulfatase activity, respectively), chondroitin AC lyase (EC 4.2.2.5, from *Arthrobacter aurescens*), and chondroitin B lyase (EC 4.2.2.-, from *Flavobacterium heparinum*) were from Seikagaku America, Inc., Rockville, MD. Reference disaccharides (C-kit and D-kit) of known structure were from Seikagaku America, and heparin/heparin sulfate disaccharides were from Grampian Enzymes, Aberdeen, U.K. Their purity was confirmed by CE [18] and their structure by NMR [19] and MS [19, 20] analysis. Heparin lyase I (EC 4.2.2.7) and heparin lyase II (EC 4.2.2.-), both from *F. heparinum*, were prepared in our laboratory [21]. Antithrombin III was obtained from Kabi Vitrum, Molndal, Sweden. Heparin cofactor II was from Diagnostica Stago, Asnieres, France. Human thrombin was from Ortho Diagnostics Systems, Raritan, NJ, and factor Xa from Enzyme Research Laboratories South Bend, IN; chromogenic substrates were from American Diagnostics, Greenwich, CT. All reagents used in electrophoresis were from the Fisher Chemical Co., Fairlawn, NJ.

A Bio-Rad (Richmond, CA) 20 cm vertical Protean™II slab gel unit equipped with a model 1420B power source was used for electrophoresis. Desalting was done with Spectropore dialysis tubing (mol. wt. cut-off 1000) from Spectrum Medical, Houston, TX, or with a P-2 gel desalting column from Bio-Rad. Ultrogel ACA202 with Trisacryl GF were from IBF, Villeneuve sur la Garonne, France. For gel permeation HPLC, we used 4.6 × 250 mm Protein Pak 125 and 300 columns (Waters, Milford, MA) in series or a 4.6 × 600 mm TSK G4000 SW gel column from Toso Haas (Lab Service, Bologna, Italy). A waters 840 liquid chromatography system was used that was equipped with a model 490 multiwavelength UV detector and a Digital 300 series minicomputer running Waters 840 software specifically designed to determine the molecular weight of polymers. CE was performed on a Dionex Capillary Electrophoresis system fitted with a fused silica capillary and equipped with a variable wavelength ultraviolet detector. ^{1}H-NMR spectroscopy was performed at 500 MHz in ^2H_2O (99.996 atom%) with 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, sodium salt (99+ atom%) as the internal reference (Aldrich Chemical Co., Wilwaukee, WI) at 60° on a Varian VXR 500 spectrometer equipped with a Sun Spark work station.

Preparation of DS and LMW-DS. DS was prepared by Opocrin by proteolysis of bovine intestinal mucosa, strong anion exchange chromatography, and ethanol precipitation [15]. LMW-DS was prepared by the controlled depolymerization of DS with hydrogen peroxide in the presence of copper(II) acetate as previously described [15]. LMW-DS was dissolved in water at 3.5% (w/v), calcium chloride 6% (v/w) was added and finally 0.4 vol. of ethanol. The precipitate formed after overnight storage at 5° was collected and converted to the sodium salt

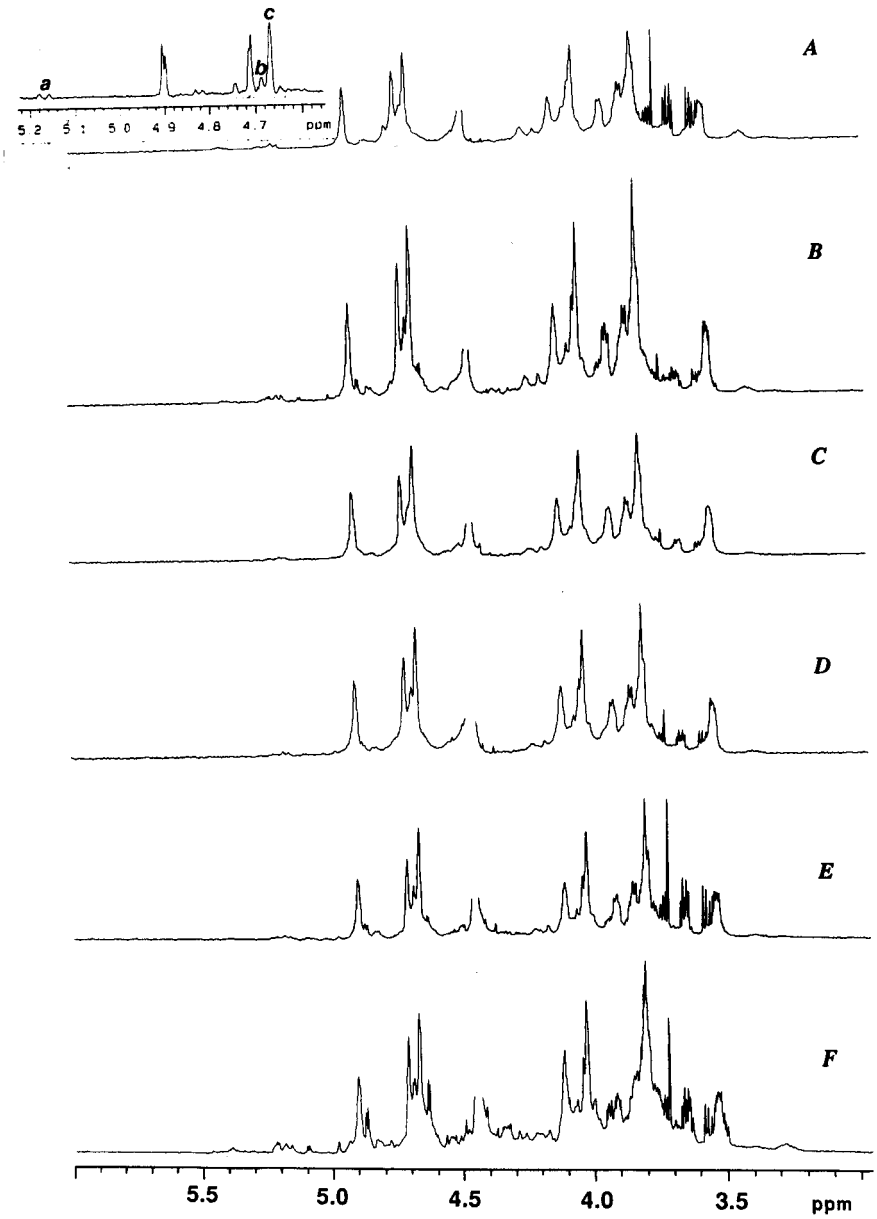
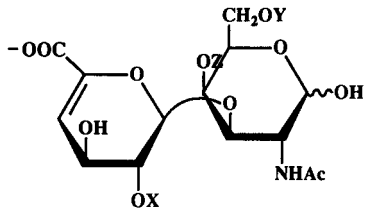


Fig. 1. ^{1}H-NMR spectra of dermatan sulfates at 60°. (A) DS; (B) LMW-DS; (C) LMW-DS 105-1; (D) LMW-DS 105-2; (E) LMW-DS 105-3; and (F) LMW-DS 105-4. The singlet at δ 2.08 assignable to the *N*-CH_3 of the GalNAc residue is not shown. Note the increase in sharpness of peaks as molecular weight decreases. The inset shows the resolution-enhanced spectrum in the region of δ 4.5–5.2 of the DS sample. The IdoAp2S (1-H), GalpNAc4S6S (1-H) and GalpNAc4S (1-H and 4-H) signals are labeled a, b and c, respectively. The HDO signal is observed at 4.45 ppm.

using a cation exchange resin. Gel permeation chromatography of LMW-DS (5%, w/v) was done on Ultrogel AcA202, desalted on Trisacryl GF, and lyophilized. The LMW-DS subfractions 105-1, -2, -3 and -4 were recovered in yields of 15, 42, 23 and 8%, respectively. Stock solutions of dermatan sulfates were prepared by dissolving each sample at 20 mg/mL in distilled water.

Determination of the molecular weight of DS. Prior to their use, each gel permeation column was calibrated with a collection of related low molecular weight heparins serving as molecular weight calibrators [22]. DS or LMW-DS (20 μL, 10 mg/mL) was injected into the column and eluted with 125 mM sodium sulfate containing 2 mM monosodium phosphate at pH 6.0 at a flow rate of 0.9 mL/min; the eluant was monitored at 205 nm. Values for t_R were determined and used along with the known molecular weights of the calibrators to calculate a calibration curve by the third polynomial regression



	X	Y	Z
1	H	H	H
2	H	SO_3^-	H
3	H	H	SO_3^-
4	SO_3^-	H	H
5	H	SO_3^-	SO_3^-
6	SO_3^-	SO_3^-	H
7	SO_3^-	H	SO_3^-
8	SO_3^-	SO_3^-	SO_3^-

Fig. 2. Structures of disaccharides obtained by treating DS and LMW-DS with chondroitin lyases.

with coefficients D_0, D_1, D_2, and D_3. Molecular weight was calculated using the equation: log molecular weight = $D_0 + D_1(t_R) + D_2(t_R)^2 + D_3(t_R)^3$. The total area under the elution curve was determined by integration.

NMR analysis of DS samples. The DS and LMW-DS samples were exchanged with ^2H_2O before being made up at approximately 5% (w/v) for ^{1}H-NMR. The ^{1}H-NMR spectra were recorded with 0.24 Hz digital resolution and at 60° to prevent the HDO signal from overlapping the broad signals from δ 4.64 to 4.74. The ^{1}H-NMR spectrum of each dermatan sulfate showed the following signals: δ 4.90 (br, IdoAp,H-1), 4.72 (br, GalpNAc4S, H-1), 4.68 (br, GalpNAc4S6S, H-1) 4.66 (br, GalpNAc4S,H-1), 4.11 (br, IdoAp,H-4), 4.02 (br, GalpNAc4S,H-2,3), 3.91 (br, IdoAp,H-3 and GalpNAc4S,H-5), 3.80 (br, GalpNAc4S,H-6,6'), 3.53 (br, IdoAp,H-2) and 2.07 (GalpNAc, N-CH_3). The overlapping broad resonances of the H-4 and H-1 of galactopyranose residues were resolved using appropriate sine bell and phase-shifted sine bell window functions. The resolution-enhanced spectra were then multiplied by a spline polynomial to correct for baseline distortions. The integral ratios were calculated from the resolution enhanced spectra.

Saccharide composition and oligosaccharide mapping of DS samples. DS sample (50 μL of 20 mg/mL) was added to 330 μL of 5 mM sodium phosphate and 100 mM sodium chloride buffer, pH 7; chondroitin ABC, AC, or B lyase (20 μL, 20 mIU) was added to make the total solution volume 400 μL. The reactions were run at 30° for chondroitin B lyase and 37° for chondroitin AC and ABC lyases to completion in 24 hr and terminated by adding 50 μL to 1 mL of 30 mM hydrochloric acid. The saccharide composition of each DS sample was analyzed by CE using ultraviolet detection at 232 nm. Separation and analysis were carried out using a fused silica (externally coated except where the tube passed through the detector) capillary tube (75 μm i.d., 375 μm o.d., 68 cm long) from Dionex. The capillary tube was washed extensively with 0.1 M phosphoric acid, 0.5 M sodium hydroxide, and deionized, distilled water and then filled with the operating buffer consisting of 10 mM sodium borate and 50 mM SDS having a pH of 8.8. The sample was injected by gravity injection (15 nL), and electrophoresis was performed at 20 kV using the operating buffer. The elution profile was monitored at 232 nm, 0.02 absorbance units full scale (AUFS). Disaccharide peaks were identified by their coelution with reference disaccharides. Oligosaccharide maps of depolymerized DS were prepared using gradient PAGE by adding sample (1 μL of 20 mg/mL) to a stacking gel of 5% (total acrylamide) with a 12–22% linear gradient resolving gel [23]. After 400 V was applied for 4 hr (running a bromophenol blue marker 20 cm into the resolving gel), the gel was kept at 4° by a refrigerated circulating water bath. The gel was fixed and stained in alcian blue, destained, and then silver stained [23–25].

Examination of DS samples for contamination with heparin or heparin sulfate. Each DS sample (25 μL of 20 mg/mL) was treated with a combination of 15 mU of heparin lyase I and 15 mU of heparin lyase II in 75 μL of 5 mM sodium phosphate buffer containing 200 mM sodium chloride at pH 7.1 for 24 hr at 30°. Following enzymatic treatment, CE was performed under conditions [26] similar to those described for the analysis of DS disaccharides. A mixture of eight heparin and heparan sulfate samples was used as reference material.

Assays of the anticoagulant and antithrombotic activities of dermatan sulfate. DS, LMW-DS and the four fractions were supplemented in normal human plasma at a concentration range of 0–100 μg/mL and measured in various anticoagulant assays. The routine clotting assays, PT, APTT, TT (5 U), and the Heptest®, were performed according to manufacturers' specifications. Thromboplastin (Dade, Miami, FL) was used for PT, APTT reagent (Organon Teknika, Morris Plains, NJ) was used for APTT, Fibrindex was used for TT (5 U) (Ortho Diagnostic Systems), and the Heptest® kit (Heamachem, St. Louis, MO) was used for the Heptest® assay. Calcium TT (5 U) was measured by reconstituting thrombin with 25 mM calcium chloride (in place of sodium chloride) and determining thrombin time. The anti-factor IIa and anti-factor Xa assays were performed as previously described using the 4th International WHO heparin standard having 193.4 IU/mg [27, 28]. Heparin cofator II-mediated anti-factor IIa activity was determined by an amidolytic assay as previously described [23, 29] or using a Stachrom kit [30]. Heparin cofactor II activity, determined by a Stachrom kit, is reported as units per milligram calculated based on a standard curve using the WHO heparin standard. The curves obtained for DS and heparin standard were parallel.

In vivo antithrombotic activity was evaluated by a modified Wessler stasis thrombosis model [31] in rabbits (N = 5) using FEIBA (7.5 U/kg) as a thrombogenic challenge at a s.c. dose of 2.5 mg/kg and an i.v. dose of 1 mg/kg. Antithrombotic effect was also measured in a rat jugular clamping model [32] on rats (N = 3/dose) at an i.v. dose of 5 mg/kg.

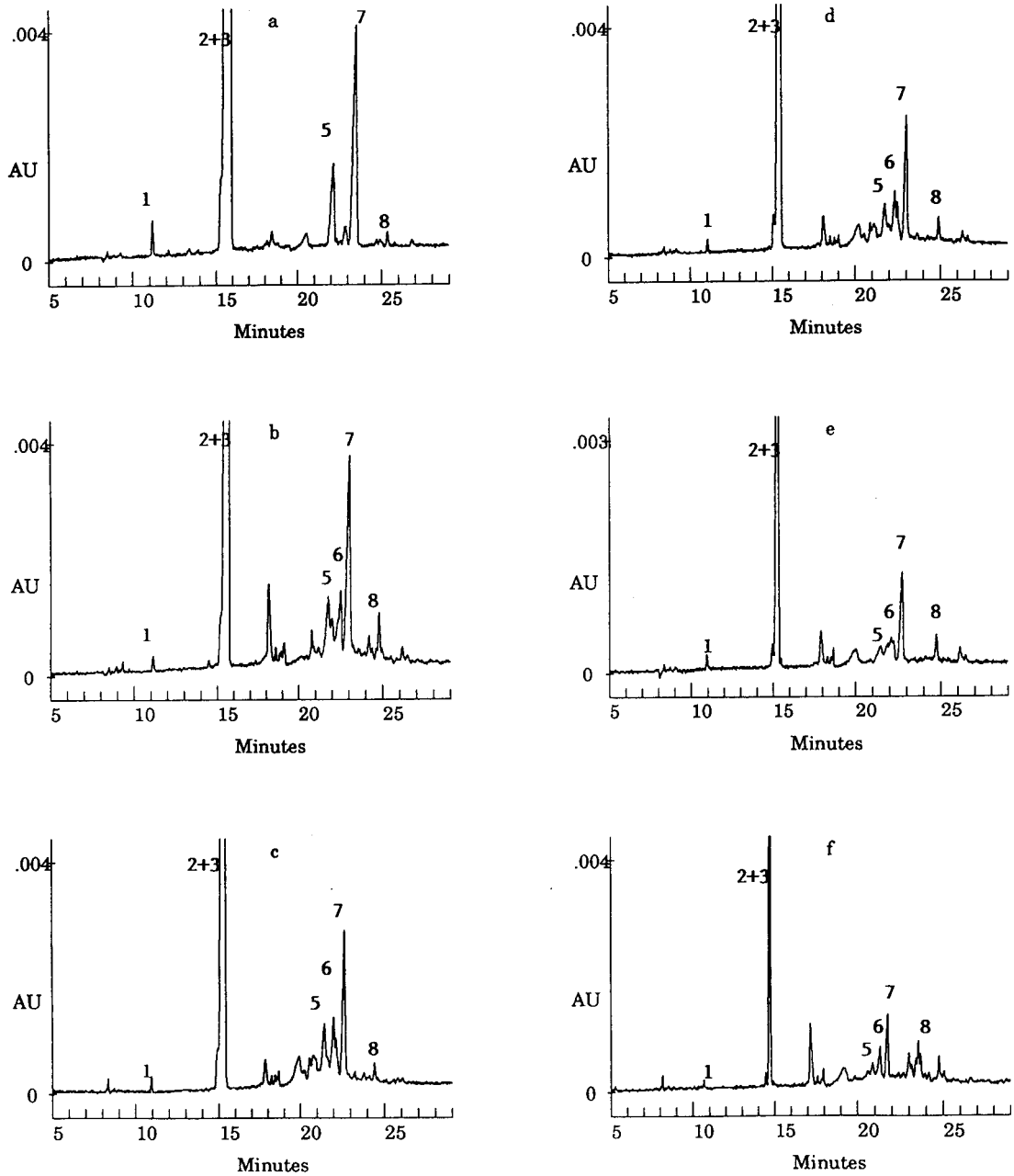


Fig. 3. Capillary electropherograms of DS and LMW-DS samples treated with chondroitin ABC lyase. Electropherograms a–f correspond to DS (grandparent), LMW-DS (parent) and LMW-DS 105-1, -2, -3 and -4 (children), respectively. The peaks are labeled with numbers corresponding to the disaccharide structures shown in Fig. 2.

RESULTS

Bovine mucosal DS was oxidatively depolymerized by treatment with hydrogen peroxide at elevated temperatures to prepare a LMW-DS [15]. The LMW-DS was size fractionated by gel permeation chromatography to obtain four subfractions. The molecular weights of these DS samples were determined by gel permeation HPLC and together with the optical rotations of each DS sample are presented in Table 1.

The ^{1}H-NMR spectra of each DS sample, obtained in ^2H_2O, were very similar (Fig. 1), and the assignments are given in Materials and Methods. The major difference in these spectra was an increase in the percentage of GalpNAc4S6S residues from 22–23% for LMW-DS 105-1 and 105.2 to 35.36% for LMW-DS 105-3 and 105-4 (DS and LMW-DS

Table 2. Disaccharide composition of DS samples treated with chondroitin B lyase and chondroitin ABC lyase

		Percent disaccharide*											
		1	**2†**	**3†**	**4**	Unidentified a‡	Unidentified b‡	**5**	Unidentified c‡	**6**	**7**	Unidentified d‡	**8**
Retention time (min)		11.36	15.12	15.35	15.78	16.44	17.88	22.49	23.22	23.63	23.70	23.99–25.11	27.02
Sample	*Enzyme*§												
DS OP 435	ABC	0.68	2.15	73.80	ND‖	ND	TR¶	3.27	0.63	ND	9.83	9.60	TR
	B	ND	ND	30.16	ND	47.87	ND	ND	2.93	ND	14.78	ND	TR
LMW-DS OP370	ABC	TR	1.71	61.72	ND	ND	3.23	1.40	2.65	TR	9.72	19.62	TR
	B	ND	ND	51.83	ND	23.91	ND	ND	8.79	ND	11.72	ND	TR
LMW-DS 105-1	ABC	TR	1.03	73.50	ND	ND	1.32	2.11	1.52	ND	7.77	12.71	TR
	B	ND	ND	35.95	ND	32.81	ND	ND	15.16	ND	12.11	ND	TR
LMW-DS 105-2	ABC	TR	1.12	72.36	ND	ND	2.03	1.77	1.46	TR	8.53	12.78	TR
	B	ND	ND	31.79	ND	31.91	ND	ND	15.82	ND	13.48	ND	TR
LMW-DS 105-3	ABC	TR	1.17	65.57	ND	ND	3.10	3.10	3.82	ND	10.07	13.21	TR
	B	ND	ND	46.06	ND	28.96	ND	ND	10.69	ND	10.41	ND	TR
LMW-DS 105-4	ABC	TR	0.50	45.73	ND	ND	9.00	2.05	3.46	ND	8.58	27.59	TR
	B	ND	ND	19.74	ND	36.22	ND	ND	17.94	ND	10.36	ND	TR

* See Fig. 2 for structure. Percentage of total absorbance at 232 nm is presented.
† The content of **2** and **3** (not resolved in Fig. 3) was determined using identical CE conditions but on a longer capillary column.
‡ Unidentified peaks probably corresponding to lyase-resistant tetrasaccharides, hexasaccharides, or disaccharides of modified structure.
§ Analysis was performed on DS samples following treatment with chondroitin ABC lyase (ABC) or chondroitin B lyase (B).
‖ None detected.
¶ Trace.

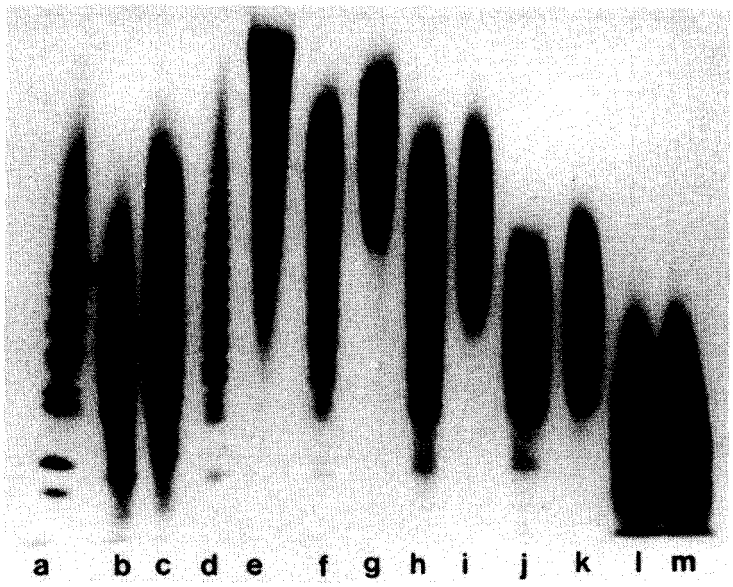


Fig. 4. Gradient PAGE analysis of DS and LMW-DS samples untreated and treated with chondroitin AC lyase. Lane a, oligosaccharide reference materials prepared from partially depolymerized bovine lung heparin; lane b, LMW DS (parent) treated with lyase; lane c, LMW-DS untreated; lane d, DS (grandparent) treated with lyase; lane e, DS untreated; lane f, LMW-DS 105-1 treated with lyase; lane g, LMW-DS 105-1 untreated; lane h, LMW-DS 105-2 treated with lyase; lane i, LMW-DS 105-2 untreated; lane j, LMW-DS 105-3 treated with lyase; lane k, LMW-DS 105-3 untreated; lane l, LMW-DS 105-4 treated with lyase; lane m, LMW-DS 105-4 untreated.

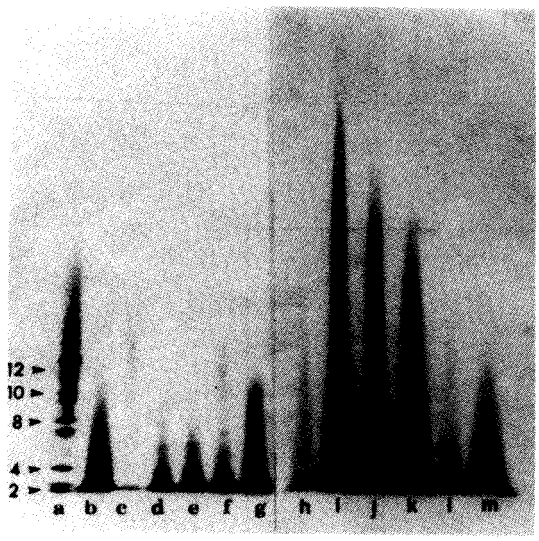


Fig. 5. Gradient PAGE analysis of DS and LMW-DS samples treated with chondroitin ABC and B lyase. Lane a, oligosaccharide reference materials prepared from partially depolymerized bovine lung heparin; lanes b–g are samples treated with chondroitin ABC lyase. Lane b, LMW-DS (parent); lane c, DS (grandparent); lane d, LMW-DS 105-1; lane e, LWM-DS 105-2; lane f, LMW-DS 105-3; and lane g, LMW-DS 105-4. Lanes h–m are samples treated with chondroitin B lyase. Lane h, LMW-DS (parent); lane i, DS (grandparent); lane j, LMW DS 105-1; lane k, LMW-DS 105-2; lane l, LMW-DS 105-3; and lane m, LMW-DS 105-4.

were 22 and 31% GalpNAc4S6S, respectively). The spectral resolution was also enhanced with decreased sample molecular weight.

Each DS sample was depolymerized with chondroitin B lyase, chondroitin AC lyase and chondroitin ABC lyase. Treatment with either chondroitin B or ABC lyase gave extensive depolymerization of the DS samples (Fig. 2), permitting CE (Fig. 3) to be used for the determination of disaccharide composition. The structures of these disaccharides and the amounts in which they were found in each DS sample are given in Fig. 2 and Table 2.

The presence of heparin or heparan sulfate contamination of each DS sample was also assessed. DS samples were treated with an equi-unit mixture lyase I and heparan lyase II. CE failed to detect the formation of any heparin or heparan sulfate derived disaccharides (data not shown) [26].

Each DS sample was analyzed by gradient PAGE preceding enzymatic treatment (Fig. 4, lanes c, e, g, i, k, m). Each DS sample was also analyzed following treatment with: chondroitin AC lyase (Fig. 4, lanes a, d, f, h, j, and l); chondroitin B lyase (Fig. 5, h–m); and chondroitin ABC lyase (Fig. 5, lane, b–g). The molecular weight of DS sample and the oligosaccharides formed following enzymatic treatment can be estimated against a banding ladder (Figs. 4 and 5, lane a) of heparin-derived oligosaccharides prepared by partial heparin lyase I depolymerization of bovine lung heparin [33].

The *in vitro* anticoagulant activity of each DS sample was determined by the clot based APTT and thrombin time assays. The anti-factor Xa activity

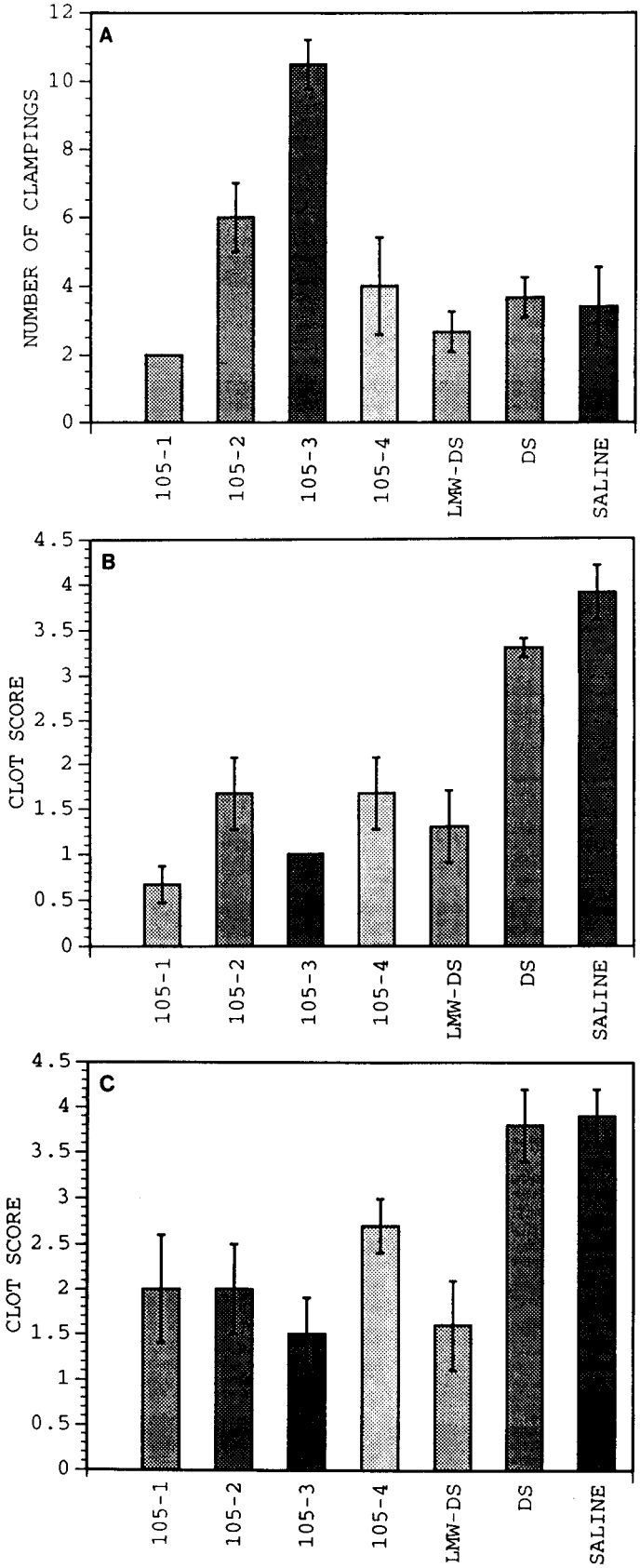


Fig. 6. *In vivo* antithrombotic activity of DS and LMW-DS samples. Panel A: activity as measured by the rat jugular clamping model at an i.v. dose of 5 mg/kg. Panel B: activity as measured by a modified Wessler stasis model at an i.v. dose of 1 mg/kg. Panel C: activity as measured by a modified Wessler stasis model at a s.c. dose of 2.5 mg/kg. Values are means ± SD, N = 3.

and HCII-mediated anti-factor IIa activity were determined using amidolytic methods. The amidolytic assays are presented in units per milligram as calculated from a heparin standard curve, while the clotting times were determined at a single concentration and are expressed in seconds (Table 1).

The *in vivo* antithrombotic activity of each DS sample was measured in both rat and rabbit models. The antithrombotic effect was measured using a rat jugular clamping model [32] following the i.v. administration of each DS sample (Fig. 6A). A modified Wessler stasis thrombosis model [31] was also used to measure the antithrombotic effect following the i.v. and s.c. administration of each DS sample (Fig. 6, B and C).

DISCUSSION

Dermatan sulfate has become the focus of a number of scientific [8, 10] and clinical studies [34, 35] aimed at the discovery of new glycosaminoglycan-derived antithrombotic agents. The goal is to replace heparin (the most widely used clinical anticoagulant [2, 3]) for certain indications thus reducing osteoporosis, heparin-induced thrombocytopenia and hemorrhagic complications [7]. The first attempts to find a heparin replacement focused on the development of low molecular weight heparins [14]. These drugs are prepared by controlled, partial chemical or enzymatic depolymerization of heparin and exhibit significantly improved bioavailability [12], but clinical studies have not clearly demonstrated a decrease in hemorrhagic complications and effects on platelets with the use of these agents [14].

Clinical studies and *in vivo* and *in vitro* research data have suggested that DS may represent an improvement when compared with heparin. Org 10172, a heparinoid drug consisting of a mixture of heparin, heparan sulfate, DS and chondroitin sulfates, has done remarkably well in a series of clinical studies [34, 35]. The reason for the success of this DS containing heparinoid is its high *in vivo* antithrombotic potency with its very low anticoagulant effect in plasma [8, 10].

Tollefsen and coworkers [5, 9] have provided the rationale for DS activity in demonstrating its ability to potentiate HCII inhibition of thrombin. Studies by our group suggest that the disaccharide sequence →4)-α-L-IdoAp (or β-D-glucuronic acid, β-D-GlcAp) (1→3)-β-D-GalpNAc4S6S (1→present in DS is associated with its HCII activity [36]. Studies by Maimone and Tollefsen [37] have implicated a second disaccharide sequence →4)-α-L-IdoAp2S (1→3)-β-D-GalpNAc4S(1→, and suggested it to be responsible for the interaction of DS with HCII. We recently demonstrated that the heparin cofactor II activity of a DS correlated poorly with its *in vivo* antithrombotic activity. One of the structural correlations observed was that as the iduronic acid content of DS increased, so did the *in vivo* antithrombotic activity [36]. This increase in antithrombotic activity may be the result of the high conformational flexibility of the iduronic acid residue [11, 12], promoting its interaction with as yet unidentified proteins that regulate thrombosis.

LMW-DS has been suggested as a potential new antithrombotic agent [16, 17]. The rationale for examining LMW-DS is: (1) the inherent high *in vivo* antithrombotic activity and low hemorrhagic side-effects of DS; (2) the ease of applying the chemical and enzymatic methods, used to prepare LMW heparins, to the preparation of LMW-DS; (3) the expectations that reduction in DS molecular weight will improve its bioavailability and pharmacokinetics; and (4) the possibility that LMW-DS will have improved antithrombotic properties. As part of our continuing study on the antithrombotic activity of DS [25, 36], we decided to examine the structure and activity of one such LMW-DS and its subfractions.

The DS chosen for this study, OP435 (grandparent), is prepared from bovine intestinal mucosa and has an average molecular weight of 25,000 (Table 1). Controlled, partial depolymerization of the grandparent DS with hydrogen peroxide and copper (II) as catalyst led to the parent LMW-DS (OP370) having an average molecular weight of 5600 (Table 1). The parent LMW-DS was fractionated by gel permeation chromatography to prepare LMW-DS 105-1, 105-2, 105-3, and 105-4 (children) having average molecular weights ranging from 7800 to 1950 (Table 1). With the exception of LMW-DS 105-2, the optical rotation increases with decreasing average molecular weight (Table 1). This may be due to either increased glucuronic acid content (chondroitin sulfate A and C have a 2- to 5-fold higher optical rotation than DS [38]) or changes in the secondary structure of DS. The ^{1}H-NMR spectra of the DS samples were very similar (Fig. 1) with the major differences being an increased content of GalpNAc4S6S and an enhancement of signal resolution with decreased average molecular weight.

Disaccharide compositional analysis of the DS samples (Fig. 3, Table 2) showed several interesting features. Chondroitin ABC lyase treatment of DS and LMW-DS resulted in 90 and 75% conversion to disaccharide products, respectively. Hydrogen peroxide-based depolymerization of DS decreased molecular weight, increased the amount of unidentified products, and appreciably altered the digestibility of polysaccharide by chondroitin lyases. These results are consistent with the significantly decreased level of chondroitin ABC lyase digestibility of LMW-DS compared with DS as observed in gradient PAGE mapping (Fig. 5, lanes b and c). This decreased chondroitin lyase digestibility (Figs. 4 and 5) suggests that hydrogen peroxide results in the modification of certain saccharide residues (particularly at the sensitive aldehydic reducing end), affording an increased percentage of unidentified oligosaccharide products (Table 2). Alternatively, DS oligosaccharides, like heparin oligosaccharides [39], may become resistant to lyase treatment due to their reduced size.

Our previous studies [25, 36] pointed to the importance of the iduronic acid content of DS to its antithrombotic activity. Whinna *et al.* [40] also showed that IdoAp rich GAG chains accelerated thrombin inhibition ten times as fast as IdoAp poor chains. The increased optical rotation of LMW-DS suggests that it might contain decreased iduronic

acid content [38]. Thus, two antithrombotic assays were used to evaluate DS and LMW-DS (Fig. 6). The Wessler stasis model is the most widely used thrombosis model. Although it is considered a good screening model, it is not very physiologic because the blood is not flowing. In the recently developed clamping model, blood is allowed to flow through the damaged vessel. This may be more physiologic, mimicking the endothelium damage that occurs in human vascular disease. In the Wessler stasis model [31], the clot is judged by a standardized score [0 (no clot) to +4]. In the jugular clamping assay [32], the vein is clamped for 1 min with a hemostat and released, a Doppler measure of blood flow is made, this procedure is repeated until no blood flow is measured, and the number of clampings required to form a clot is recorded. Contrary to expectations, a modified Wessler stasis model showed enhanced antithrombotic activity for LMW-DS, whereas the rat jugular clamping model showed comparable activity (Fig. 6). DS and LMW-DS samples were also analyzed by oligosaccharide mapping and disaccharide compositional analysis. Chondroitin ABC lyase acts at linkages containing either iduronic or glucuronic acid residues, whereas chondroitin B lyase and chondroitin AC lyase are specific for linkages containing iduronic acid and glucuronic acid, respectively [41–43]. Disaccharide compositional analysis by CE defines the frequency of occurrence of specific disaccharide sequences (underlined below) within the polymer, while the use of both chondroitin B and ABC lyases show the context (flanking sequences) in which these disaccharides are found. Differences in the disaccharide composition of DS (grandparent) and the LMW-DS (parent and children) include a decrease in the percentage of unsulfated and monosulfated saccharide residues following hydrogen peroxide-based depolymerization. This is most clearly demonstrated by the absence of disaccharide **1**, corresponding to the unsulfated sequence →4)UAp(1→3)-β-D-GalpNAc-(1→4)UAp(1→ (where UAp is either α-L-IdoAp or β-D-GlcAp), in all of the LMW-DS samples and the disenrichment of disaccharides **2** and **3**, corresponding to the monosulfated sequences →4)-UAp(1→)-β-D-GalpNAc6S(1→4)UAp(1→ and →4)UAp(1→3)-β-D-GalpNAc4S(1→4)UAp(1→, in all the LMW-DS samples.

Comparison of the LMW-DS size fractionated samples (children) resulted in some intriguing biological data (Table 1). The anti-factor Xa activities of DS and LMW-DS samples were low. This was expected, since DS is believed to exert most of its activity through its inhibition of factor IIa. LMW-DS 105-1, -2 and -3 have comparable HCII-mediated anti-IIa and APTT activities, while LMW-DS 105-4 has substantially lower anti-factor Xa, HCII-mediated anti-IIa and APTT activities. These assays suggest that below 4.2 kDa, corresponding to LMW-DS 105-3, the *in vitro* activity is diminished markedly. This observation is consistent with the report of Tollefsen *et al.* [44] that DS oligosaccharides require a minimum of 12–14 saccharide residues (corresponding to 6–7 kDa depending on the level of sulfation) to exhibit HCII-mediated anti-factor IIa activity. It is interesting to note that *in vivo* antithrombotic activity of LMW-DS 105-4 (having an average molecular weight below the 4200 of LMW-DS 105-3) is diminished markedly, as measured by both a modified Wessler stasis model and a rat jugular clamping model and a rat jugular clamping model (Fig. 6).

The disaccharide composition of the LMW-DS children showed some interesting correlation to biological activities. A substantial increase in disaccharides **5** and **7** was seen when going from LMW-DS 105-2 to LMW-DS 105-3 (Table 2). LMW-DS 105–3 was enriched in disaccharides corresponding to →4)IdoAp or GlcAp(1→3)-β-D-GalNpAc4S6S(1→4)-α-L-IdoAp(1→ and →4)-IdoAp2S(1→3)-β-D-GalNpAc4S(1→4)UAp(1→ sequences. These data suggest that enhanced sulfation, particularly in the form of 4,6- or 2,4-disulfation, leads to increased biological activity.

Examination of the oligosaccharide map for LMW-DS 105-3 shows it to have low susceptibility to digestion by chondroitin B lyase (Fig. 5), suggesting a reduced iduronic acid content. Interestingly, the oligosaccharide map for LMW-DS 105-3 shows that while it was highly sensitive to chondroitin ABC lyase (Fig. 5), some residual polymeric material remained undigested. It was a concern that much of its biological activity might be attributable to this undigestible material, if it were a heparin or heparan sulfate contaminant. To examine this possibility, each DS sample was treated with an equi-unit mixture of heparin lyase I and heparin lyase II, which is known to depolymerize heparin primarily to a single trisulfated disaccharide and heparan sulfate to a mixture of two monosulfated disaccharides [39, 45]. Analysis by CE, an ultra-sensitive method capable of detecting a <0.1% (w/w) contamination of heparin in heparan sulfate, showed no contamination of any of the DS samples.

In conclusion, this study demonstrated that a LMW-DS can be prepared from DS by controlled depolymerization using hydrogen peroxide, which retains substantial *in vitro* and *in vivo* activity. Size fractionation of this LMW-DS showed that the highest *in vivo* activity corresponded to a fraction of 4.2 kDa and with an optical rotation of −51°. Disaccharide compositional analysis showed that this LMW-DS fraction was enriched in 2,4- and 4,6-disulfated disaccharide sequences. Further studies are underway to further characterize the *in vivo* properties of this active LMW-DS including its pharmacokinetics and bioavailability.

Acknowledgements—The authors thank the National Institute of Health (Grant GM38060) for supporting this research and Opocrin for supplying the dermatan sulfate samples. The authors also acknowledge Dr. G. Mascellani of Opocrin for his helpful suggestions in the preparation of this manuscript. Mr. Jian Liu is supported by a predoctoral fellowship from the American Heart Association, Iowa Affiliate.

REFERENCES

1. Bossennec V, Petitou M and Perly B, ^{1}H-n.m.r. investigation of naturally occurring and chemically oversulphated dermatan sulphates. Identification of

minor monosaccharide residues. *Biochem J* **267**: 625–630, 1990.
2. Linhardt RJ and Loganathan D, Heparin, heparinoids and heparin oligosaccharides: Structure and biological activities. In: *Biomimetic Polymers* (Ed. Gebellein G), pp. 135–175. Plenum Press, New York, 1990.
3. Linhardt RJ, Heparin: An important drug enters its seventh decade. *Chem Ind* **2**: 45–50, 1991.
4. Bjork I, Olson ST and Shore JD, Molecular mechanisms of the accelerating effect of heparin on the reactions between antithrombin and clotting proteinases. In: *Heparin. Chemical and Biological Properties, Clinical Applications* (Eds. Lane DA and Lindahl U), pp. 229–255. CRC Press, Boca Raton, FL, 1989.
5. Tollefsen DM, Heparin cofactor II. In: *Heparin. Chemical and Biochemical Properties, Clinical Applications* (Eds. Lane DA and Lindahl U), pp. 257–273. CRC Press, Boca Raton, FL, 1989.
6. Marcum JA and Rosenberg RD, The biochemistry, cell biology and pathophysiology of anticoagulantly active heparin-like molecules of the vessel wall. In: *Heparin. Chemical and Biochemical Properties, Clin Applications* (Eds. Lane DA and Lindahl U), pp. 275–294. CRC Press, Boca Raton, FL, 1989.
7. Gervin AS, Complication of heparin therapy. *Surg Gynecol Obstet* **140**: 789–796, 1975.
8. Walenga JM and Fareed J (Eds.), Development of non-heparin glycosaminoglycans as therapeutic agents. *Semin Thromb Hemost* **17** (Suppl 1): 1–135, 1991.
9. Tollefsen DM, Petska CA and Monafo WJ, Activation of heparin cofactor II by dermatan sulfate. *J Biol Chem* **258**: 6713–6716, 1983.
10. Hoppensteadt D, Racanelli A, Walenga JM and Fareed J, Comparative antithrombotic and hemorrhagic effects of dermatan sulfate, heparan sulfate, and heparin. *Semin Thromb Hemost* **15**: 378–385, 1989.
11. Ferro PR, Provasoli A, Ragazzi M, Casu B, Torri G, Bossennec V, Perly B, Sinay P, Petitou M and Choay J, Conformations of the pentasaccharide corresponding to the binding site of heparin for antithrombin III. *Carbohydr Res* **195**: 157–167, 1990.
12. Casu B, Structure, shape and function of glycosaminoglycans. In: *New Trends in Haemostasis* (Eds. Harenberg J, Heene D, Stehle G and Schettle G), pp. 2–11. Springer, Heidelberg, 1990.
13. Linhardt RJ, Chemical and enzymatic methods for the depolymerization and modification of heparin. In: *Carbohydrates—Synthetic Methods and Applications in Medicinal Chemistry* (Eds. Ogura H, Hasegawa A and Sumani T), pp. 385–401. VCH, Weinheim, 1992.
14. Breddin HK, Fareed J and Bender N, Low-molecular-weight heparins pharmacology, *in vitro* and *ex vivo* effects and clinical results. *Haemostasis* **18** (Suppl 3): 1–2, 1988.
15. Volpi N, Mascellani G, Bianchini P and Liverani L, Analytical techniques for studying structures of dermatan sulfate and low molecular weight dermatan sulfate. *Farmaco* **47**: 841–853, 1992.
16. Van Ryn-McKenna J, Ofosu FA, Gray E, Hirsh J and Buchanan MR, Effects of dermatan sulfate and heparin on inhibition of thrombus growth *in vivo*. *Ann NY Acad Sci* **556**: 304–312, 1989.
17. Thomas DP, Merton RE and Barrowcliffe TW, Relative efficacy of heparin and related glycosaminoglycans as antithrombotic drugs. *Ann NY Acad Sci* **556**: 313–322, 1989.
18. Al Hakim A and Linhardt RJ, Capillary electrophoresis for the analysis of chondroitin sulfate-derived disaccharides. *Anal Biochem* **195**: 68–73, 1991.
19. Linhardt RJ, Wang HM, Loganathan D, Lamb DJ and Mallis LM, Analysis of glycosaminoglycan-derived oligosaccharides using fast-atom-bombardment mass-spectrometry. *Carbohydr Res* **225**: 137–145, 1992.
20. Lamb DJ, Wang HM, Mallis LM and Linhardt RJ, Negative ion-fast-atom bombardment tandem mass spectrometry to determine sulfate and linkage position in glycosaminoglycan-derived disaccharides. *J Am Soc Mass Spectrum* **3**: 797–803, 1992.
21. Lohse DL and Linhardt RJ, Purification and characterization of heparin lysases from *Flavobacterium heparinum*. *J Biol Chem* **267**: 24347–24355, 1992.
22. Ahsan A, Jeske W, Mardiguian J and Fareed J, Feasibility study of HMC as a GPC calibrator for heparins and low molecular weight heparins. *J Pharm Sci*, in press.
23. Linhardt RJ, Loganathan D, Al-Hakim A, Wang HM, Walenga JM, Hoppensteadt D and Fareed J, Oligosaccharide mapping of low molecular weight heparin: Structure and activity differences. *J Med Chem* **33**: 1639–1645, 1990.
24. Rice KG, Rottink MK and Linhardt RJ, Fractionation of heparin-derived oligosaccharides by gradient polyacrylamide-gel electrophoresis. *Biochem J* **244**: 515–522, 1987.
25. Linhardt RJ, Al-Hakim A, Liu SY, Kim YS and Fareed J, Molecular profile and mapping of dermatan sulfates from different origins. *Semin Thromb Hemost* **17** (Suppl 1): 15–22, 1991.
26. Ampofo SA, Wang HM and Linhardt RJ, Disaccharide compositional analysis of heparin and heparan sulfate using capillary electrophoresis. *Anal Biochem* **199**: 249–255, 1991.
27. Walenga JM, Fareed J, Hoppensteadt D and Emanuele RM, *In vitro* evaluation of heparin fractions: Old vs. new methods. *Crit Rev Clin Lab Sci* **22**: 361–389, 1986.
28. Walenga JM, Bara L, Samama MM and Fareed J, Amidolytic antifactor Xa assays in the laboratory evaluation of heparin and low molecular weight fractions. *Semin Thromb Hemost* **11**: 100–107, 1985.
29. Kim YS and Linhardt RJ, Structural features of heparin and their effect on heparin cofactor II mediated inhibition of thrombin. *Thromb Res* **53**: 55–71, 1989.
30. Dupouy D, Sie P, Dol F and Boneu B, A simple method to measure dermatan sulfate at sub-microgram concentrations in plasma. *Thromb Haemost* **60**: 236–239, 1988.
31. Fareed J, Walenga JM, Kumar A and Rock AL, A modified stasis thrombosis model to study the antithrombotic actions of heparin and its fractions. *Semin Thromb Hemost* **11**: 155–175, 1985.
32. Raake W and Elling H, Rat jugular vein hemostasis—A new model for testing antithrombotic agents. *Thromb Res* **53**: 73–77, 1989.
33. Edens RE, Al-Hakim A, Weiler JM, Rethwisch DG, Fareed J and Linhardt RJ, Gradient polyacrylamide gel electrophoresis for determination of molecular weights of heparin preparations and low-molecular-weight heparin derivatives. *J Pharm Sci* **81**: 83–827, 1992.
34. Biller J, Massey EW, Marler JR, Adams HP, Davis JN, Bruno A, Henriksen RA, Linhardt RJ, Goldstein OB, Alberts M, Kisker CT, Toffol GJ, Greenberg CS, Banwart KJ, Bertels C, Beck DW, Walker M and Magnani HN, A dose escalation study of ORG 10172 (low molecular weight heparinoid) in stroke. *Neurology* **39**: 262–265, 1989.
35. Gordon DC, Linhardt R and Adams HP, Low-molecular-weight heparins and heparinoids and their use in acute progressing ischemic stroke. *Clin Neuropharmacol* **13**: 522–543, 1990.
36. Linhardt RJ, Al-Hakim A, Liu J, Hoppensteadt D, Mascellani G, Bianchini P and Fareed J, Structural features of dermatan sulfates and their relationship to anticoagulant and antithrombotic activities. *Biochem Pharmacol* **42**: 1609–1619, 1991.
37. Maimone M and Tollefsen DM, Structure of a dermatan

sulfate hexasaccharide that binds to heparin cofactor II with high affinity. *J Biol Chem* **265**: 18263–18271, 1990.
38. Mathews MB and Cifonelli JA, Acid mucopolysaccharide reference standards data sheet. NIAMD Contract N. NO1-AM-5-2205, April 1977.
39. Linhardt RJ, Turnbull JE, Wang HM, Loganathan D and Gallagher JT, Examination of the substrate specificity of heparin and heparan sulfate lyases. *Biochemistry* **29**: 2611–2617, 1990.
40. Whinna HC, Choi HU, Rosenberg LC and Church FC, Interaction of heparin cofactor II with biglycan and decorin. *J Biol Chem* **268**: 3920–3924, 1993.
41. Linhardt RJ, Polysaccharide lyases for glycosaminoglycan analysis. In: *Current Protocols in Molecular Biology* (Ed. Varki A). Wiley Interscience, New York, in press.
42. Gu K, Liu J, Pervin A and Linhardt RJ, Comparison of the activity of two chondroitin AC lyases on dermatan sulfate. *Carbohydr Res* **244**: 369–377, 1993.
43. Linhardt RJ, Cooney CL and Galliher PM, Polysaccharide lyases. *Appl Biochem Biotechnol* **12**: 135–177, 1986.
44. Tollefsen DM, Peacock ME and Monafo WJ, Molecular-size of dermatan sulfate oligosaccharides required to bind and activate heparin cofactor-II. *J Biol Chem* **261**: 8854–8858, 1986.
45. Linhardt RJ, Rice KG, Kim YS, Lohse DL, Wang HM and Loganathan D, Mapping and quantification of the major oligosaccharide components of heparin. *Biochem J* **254**: 781–787, 1988.