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Note

Preparation and biological activity of *N*-sulfonated chondroitin and dermatan sulfate derivatives

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Chondroitin and dermatan sulfates are families of structurally complex, sulfated, linear polysaccharides called glycosaminoglycans (GAGs) with alternating uronic acid and *N*-acetylated D-galactosamine residues [1] (Scheme 1). Chondroitin sulfate A (**1A**) consists of unsulfated D-glucuronic acid $1 \rightarrow 3$ linked to *N*-acetylated, 4-sulfated D-galactosamine, which in turn is attached to the next D-glucuronic acid residue by a $1 \rightarrow 4$ linkage. Chondroitin sulfate C (**1B**) has a 6-sulfate group on the *N*-acetylated D-galactosamine in place of the 4-sulfate found in chondroitin sulfate A. Dermatan sulfate, also called chondroitin sulfate B (**2**), is similar to chondroitin sulfate A but instead of D-glucuronic acid it consists mainly of L-iduronic acid. Both dermatan and chondroitin sulfates contain, on the average, one sulfate group per disaccharide unit.

Chondroitin sulfates are localized on cell surfaces and in the extracellular matrix and are important in cell–cell communication [2,3]. They are also involved in the comple-

Abbreviations: NMR: nuclear magnetic resonance; GAG: glycosaminoglycan; HIV: human immunodeficiency virus; MWCO: molecular weight cut-off; CE: capillary electrophoresis; PAGE: polyacrylamide gel electrophoresis; HPLC: high-performance liquid chromatography

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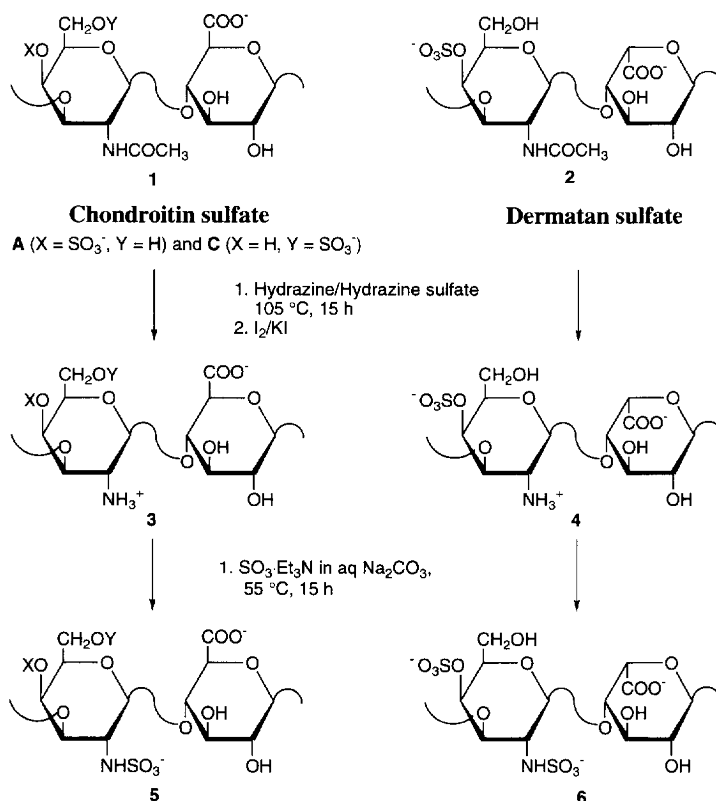

Scheme 1.

ment cascade and the coagulation cascade [4]. Chondroitin sulfates lack clinically significant anticoagulant activity, presumably in part because of their low level of sulfation; however, dermatan sulfate exhibits venous antithrombotic activity and has been studied clinically [1]. Dermatan sulfate's antithrombotic activity results from its acceleration of heparin cofactor II mediated inhibition of thrombin [1,5,6].

Heparin is a related, highly sulfated GAG comprised primarily of alternating L-iduronic acid 2-sulfate $1 \rightarrow 4$ linked to *N*-sulfonated, 6-sulfated D-glucosamine [7]. Because of its high level of sulfonation (an average of 2.7 sulfates per disaccharide unit) and its sequence microheterogeneity, heparin is able to bind a wide range of proteins and regulate a number of important biological functions. Heparin, clinically used as an anticoagulant, is being studied for a wide variety of additional therapeutic applications [7]. Recently, other polysaccharides and modified polysaccharides have been examined as potential heparin analogues in drug development [7]. Curdlan, a linear polysaccharide with a β-$(1 \rightarrow 3)$ glucan backbone, has been chemically sulfonated [8]. The resulting curdlan sulfate has a high level of sulfonation, and has inhibitory activity against HIV strains but has a low anticoagulant activity. Chemical sulfonation of pentosan and dextran also affords highly sulfonated polysaccharides, and these have enhanced an-

tithrombotic activities [9,10]. Oversulfated chondroitin sulfates with two to three sulfate groups per disaccharide unit have been shown to exhibit enhanced antithrombotic activity [11]. However, these oversulfated chondroitin sulfates are chemically *O*-sulfo compounds and retain *N*-acetyl groups, making them quite different from the *N*-sulfo GAG, heparin.

Replacing the *N*-acetyl groups of the D-galactosamine in chondroitin and dermatan sulfates with *N*-sulfo groups should result in GAGs that have structures which more closely resemble heparin. While the level of *O*-sulfonation in these derivatives will be lower than that of heparin, it will be comparable to the level of sulfonation found in heparan sulfate, the GAG that is endogenously responsible for many of the activities associated with pharmaceutical heparin [12].

Another area of increasing scientific interest is the preparation of GAGs having unsubstituted amino groups. GAGs from endothelial cells with unsubstituted amino groups are presumed to be involved in L-selectin binding [13,14]. This note describes the *N*-deacetylation of chondroitin sulfate and dermatan sulfate to obtain GAG chains with unsubstituted hexosamine amino groups without concomitant reduction in GAG chain molecular weight caused through the cleavage of glycosidic linkages. These chondroitin sulfate and dermatan sulfate derivatives were then *N*-sulfonated to give oversulfonated GAGs.

1. Results and discussion

The structure of commercial chondroitin sulfate (**1**) and dermatan sulfate (**2**) (Scheme 1) were first confirmed by ^{1}H NMR spectroscopy (Fig. 1a and Fig. 2a). The chondroitin sulfate was determined to be a mixture of chondroitin 4-sulfate (A) and chondroitin 6-sulfate (C). Disaccharide compositional analysis, performed by HPLC and ^{1}H NMR showed unsulfated, 4-sulfated, and 6-sulfated disaccharides present in 3.7, 14.2, and 82.1 mol%, respectively [15]. Dermatan sulfate (**2**) disaccharide analysis showed unsulfated, 4-sulfated, and 6-sulfated disaccharides present in 1.8, 96.0, and 2.2 mol%, respectively. ^{1}H NMR analysis demonstrated an L-iduronic acid:D-glucuronic acid ratio of 90.4:9.6%. Chondroitin sulfate (**1**) and dermatan sulfate (**2**) were *N*-deacetylated by treatment with anhydrous hydrazine in the presence of hydrazine sulfate. The resulting *N*-deacetylated GAGs contain uronides formed as a result of the coupling of the uronic acid carboxy groups with hydrazine. These undesired uronides are presumably afforded through the formation of uronic acid lactones, under anhydrous conditions, that are subsequently opened with hydrazine [16]. These uronides were broken down by oxidation using iodine. The *N*-deacetylated GAGs (**3** and **4**) were characterized by ^{1}H NMR spectroscopy (Figs. 1b and 2b, and Table 1). The *N*-acetyl signal at 2.04 ppm had completely disappeared, and its disappearance was accompanied by a new signal at 3.4 ppm in chondroitin sulfate and 3.0 ppm in dermatan sulfate corresponding to the H-2 of D-galactosamine residues. The *N*-deacetylated GAGs were then *N*-sulfonated using the sulfur trioxide–triethylamine complex. The resulting *N*-sulfo GAG derivatives **5** and **6** gave ^{1}H NMR spectra consistent with structure showing a downfield shift in the H-2 signals of the newly sulfonated D-galactosamine residues. The H-1 of the D-galacto-

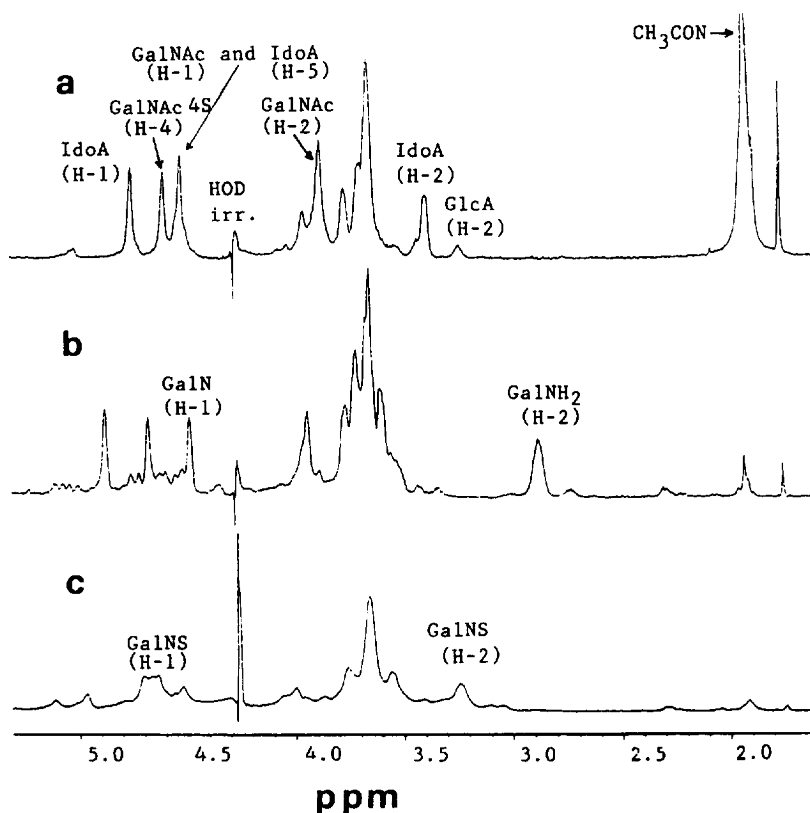

Fig. 1. ^{1}H NMR spectrum of (a) dermatan sulfate, (b) *N*-deacetylated dermatan sulfate, and (c) *N*-deacetylated, *N*-sulfonated dermatan sulfate.

samine and *N*-sulfo D-galactosamine residues also underwent a downfield shift with respect to the *N*-acetyl/D-galactosamine residues as a result of the derivatization (Table 1). Integration of the signals corresponding to the D-galactosamine residues of the *N*-deacetylated GAGs showed the same percentage of 4- and 6-sulfation as found in the parent dermatan sulfate and chondroitin sulfate polymers, confirming the expected stability of *O*-sulfo groups to hydrazinolysis and workup. Polyacrylamide gel electrophoresis (PAGE) analysis of each GAG and GAG derivative (**1** or **2** and **5** or **6**) showed no apparent differences in their molecular size. In addition, both the GAG derivatives with unsubstituted and *N*-sulfonated amino groups were susceptible to nitrous acid depolymerization [17] (as determined by PAGE [18]), while chondroitin and dermatan sulfate were not. Treatment of the modified GAGs with chondroitin ABC lyase afforded no products as detected by ultrasensitive capillary electrophoresis [18], confirming that the derivatives were modified. Furthermore, the modified GAGs were also resistant to heparin lyases I, II, and III.

Modified **5** and **6** are polymers containing repeating units of uronic acid and D-galactosamine *N*-sulfonate. Thus, their sequence does not correspond to any GAG,

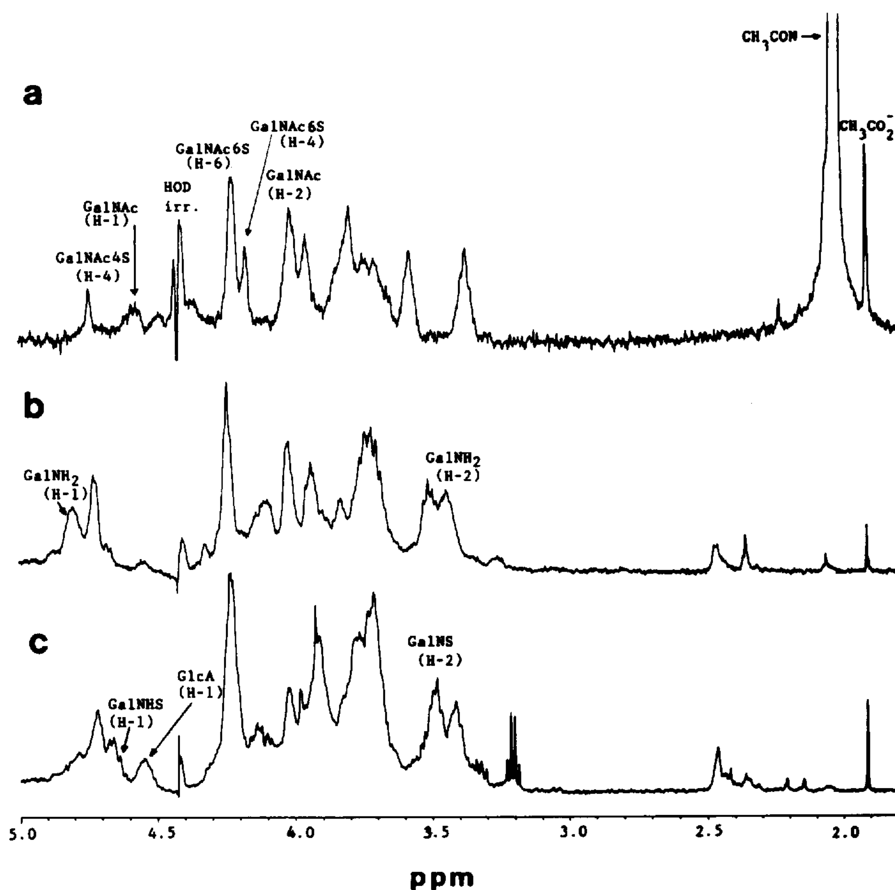

Fig. 2. ^{1}H NMR spectrum of (a) chondroitin sulfate, (b) *N*-deacetylated chondroitin sulfate, and (c) *N*-deacetylated, *N*-sulfonated chondroitin sulfate.

Table 1
Chemical shifts of structural-reporter groups in chondroitin sulfate, dermatan sulfate, and their derivatives

Sample	D-Galactosamine residue (shift in ppm)	
	H-1	H-2
Chondroitin sulfate (**1**)	4.57	4.02
N-Deacetylated chondroitin sulfate (**3**)	4.82	3.40
N-Deacetylated, *N*-sulfonated chondroitin sulfate (**5**)	4.80	3.50
Dermatan sulfate (**2**)	4.67	4.03
N-Deacetylated dermatan sulfate (**4**)	4.72	2.85
N-Deacetylated, *N*-sulfonated dermatan sulfate (**6**)	4.80	3.23

Table 2
Biological activity of chondroitin and dermatan sulfates and their derivatives

Sample	Anti-IIa activity (U/mg)	Anti-Xa activity (U/mg)	Complement inhibitory activity (% inhib. at 40 μg)
Chondroitin sulfate (**1**)	< 6	< 6	24
N-Deacetylated Chondroitin sulfate (**3**)	< 6	< 6	36
N-Deacetylated, *N*-sulfonated chondroitin sulfate (**5**)	< 6	< 6	50
Dermatan sulfate (**2**)	23	< 6	66
N-Deacetylated dermatan sulfate (**4**)	14	< 6	29
N-Deacetylated, *N*-sulfonated dermatan sulfate (**6**)	17	< 6	61

found in nature. These modified GAGs do not closely resemble the *N*-acetylated natural chondroitin and dermatan sulfates, nor do they closely resemble the more highly sulfated D-glucosamine containing heparin and heparan sulfates. Hence, they do not satisfy the substrate specificities of microbial polysaccharide lyases that normally act upon these GAGs [19]. Mammalian heparinases and chondroitinases will probably also be unable to depolymerize these GAGs [20]. This resistance to metabolic enzymes may afford prolonged half-life possibly resulting in increased toxicity of these agents if they are used as therapeutic agents.

A second distinguishing property of these GAGs is their low level of structural heterogeneity. Unlike heparin and heparan sulfate, chondroitin sulfates consist primarily of one repeating disaccharide unit. Their modification by *N*-deacetylation and *N*-sulfonation also results in homocopolymers if the modification is complete. Thus, these polymers may be useful in the study of protein–polysaccharide interactions [21,22]. These modified GAGs can be depolymerized using nitrous acid to give homogenous oligosaccharides that may be useful as building blocks in the synthesis of new oligosaccharides [23] or in the study of protein–oligosaccharide interactions [21,22].

The anticoagulant activity of the modified GAGs was determined using chromogenic, amidolytic assays. Modified chondroitin sulfates **3** and **5** show no measurable anticoagulant activity (Table 2). This was as expected since chondroitin sulfate showed no anticoagulant activity by these assays. While the modified dermatan sulfates **4** and **6** had anti-IIa-amidolytic activity comparable with the parent glycosaminoglycan **2**, none of these samples showed measurable anti-Xa-amidolytic activity. The *N*-sulfonated derivative **6** prepared from dermatan sulfate **2** resembles heparin more closely than the other derivatives because it consists of alternating iduronic acid and *N*-sulfonated hexosamine units. Thus, while its amidolytic activity is low it may have antithrombotic properties similar to that of heparin [24]. The importance of *N*-sulfonation on mimicking the ability of heparin to inhibit complement activation [4] was next examined. Each sample was assayed for ability to inhibit complement activation using a cellular based, classical pathway assay [25]. The results presented in Table 2 show dermatan sulfate is clearly more inhibitory than chondroitin sulfate A as previously reported [25]. *N*-Deacetylation

markedly reduces activity while *N*-resulfonation results in the recovery of activity in the case of dermatan sulfate and the enhancement of activity in the case of chondroitin sulfate. It appears that both *N*-substitution and chirality at the C-5 of the uronic acid residue affect complement inhibitory activity. Heparin (the positive control) shows substantially higher activity than the chondroitin and dermatan sulfates and their derivatives exhibiting 92% inhibition at a concentration of 10 μg/100 μL buffer. These modified GAGs may also have anti-HIV activity similar to curdlan sulfate [8]. Since the *N*-deacetylated chondroitin and dermatan sulfates **3** and **4** contain unsubstituted amino groups, they are potential L-selectin binding ligands [13,14]. Thus, these derivatives may be useful as therapeutic agents for the prevention of abnormal leukocyte migration into tissues. More studies are required to fully establish the activities of these new GAG derivatives.

2. Experimental

Materials.—Chondroitin sulfate (shark cartilage), dermatan sulfate (porcine skin), heparin lyase I (heparinase I, EC 4.2.2.7), heparin lyase II (no EC number), and heparin lyase III (heparitinase, EC 4.2.2.8), were obtained from Seikagaku America (Rockville, MD). Chondroitin ABC lyase was from IBEX (Montreal, Quebec). Anhydrous hydrazine, was purchased from Pierce (Rockford, IL). ^2H_2O (99.9 and 99.96%); 3-(trimethylsilyl)propionic acid, sodium salt (TSP); and acetic anhydride were from Aldrich (Milwaukee, WI). Spectra/Por dialysis tubing [1000 molecular weight cut-off (MWCO)] was from Spectrum (Houston, TX). Microfilters (0.25 μm) were from Millipore (Bedford, MA). Dowex-1 strongly basic anion-exchange resin was from Sigma (St. Louis, MO). HV syringe filters (0.45 μm) were from Nihon (Tokyo, Japan). All other reagents were from either Fisher (Pittsburgh, PA) or Aldrich (St. Louis, MO). Human thrombin (Factor IIa) and bovine Factor Xa were supplied by Haemachem (St. Louis, MO). The Virtis 100 SRC freeze driers were from the Virtis (Gardiner, NY). The ZIP Zone Chamber, Super Z Application System and the auxiliaries were supplied by Helena (Beaumont, TX). Guinea pig serum was from Rockland (Gilbertville, PA). Fourier-transform ^{1}H NMR analysis spectra were recorded on a Varian Unity 500 MHz spectrometer controlled by a SUN SPARC station 2 workstation using software supplied by the manufacturers. Probe temperature was 333 K. Chemical shifts are reported relative to TSP at 0.00 ppm.

Methods.—N-*deacetylation of chondroitin and dermatan sulfates.* — Chondroitin sulfate or dermatan sulfate (20 mg, 0.8 μM), was dissolved in 1 mL of anhydrous hydrazine following a modified labelling procedure for heparin [26]. Hydrazine sulfate (15 mg, 116 μM) was added, and the reaction mixture was heated in a sealed tube at 105 °C for 15 h. The resulting product mixture was diluted with 1 mL of 16% NaCl, and 9 mL of MeOH was added to precipitate the chondroitin sulfate or dermatan sulfate. The precipitate was recovered by centrifugation, dissolved in H_2O, and dialyzed. The retentate was lyophilized and dissolved in 10 mL of 0.25 M $NaHCO_3$. A solution of 0.2 M iodine in 0.4 M KI was added dropwise with continuous stirring until the solution

turned yellow. Then 25% v/v hydrazine was added until the solution turned colourless. The resulting mixture was dialyzed, and lyophilized to obtain the *N*-deacetylated GAG with a 75% yield.

N-*sulfonation of* N-*deacetylated chondroitin and dermatan sulfates.* — The *N*-deacetylated chondroitin or dermatan sulfate (10 mg) was dissolved in H_2O (1 mL). Sodium carbonate (36 mg) was added, and the solution was heated to 55 °C. Sulfur trioxide–triethylamine complex (3 × 15 mg) was added to the solution at 0, 5, and 10 h intervals, and the reaction was maintained at 55 °C for 15 h. The sample was then cooled, diluted with 1 mL of 16% NaCl, and 9 mL of MeOH precipitate was recovered by centrifugation, dissolved in H_2O, and dialyzed. The retentate was lyophilized to give the *N*-deacetylated, *N*-sulfonated chondroitin and dermatan sulfates in 80% yield.

NMR sample preparation. — Lyophilized samples were prepared for NMR by exchange with ^2H_2O (once with 0.5 mL of 99.9 at-% ^2H_2O containing 0.03% w/v TSP, followed by repeated lyophilization from 0.5 mL of 99.9 at-% ^2H_2O) [27]. After the final lyophilization, the sample was dissolved in 99.96 at-% ^2H_2O and filtered through a 0.45 μM syringe filter into a clean and dry NMR tube. Chemical shifts are reported relative to TSP at 0.00 ppm.

Susceptibility to polysaccharide lyases. — Polysaccharide samples (500 μg/10 μL) in the appropriate lyase buffers were added to the lyase aliquots containing lyases in (10 mU/10 μL) in lyase buffer [18,19]. The heparin lyases were used in 50 mM sodium phosphate buffer at pH 7.1 to 7.6 and chondroitin ABC lyase was used in TRIS-acetate buffer at pH 8.0 [18,19]. The solutions were mixed and maintained at the optimum temperature of the lyase for 12 h. At the end of 12 h, the solutions were heated at 100 °C for 1 min to deactivate the lyase. The samples were then lyophilized, dissolved in 10 μL of H_2O, and analyzed by capillary electrophoresis (CE) and polyacrylamide gel electrophoresis (PAGE) [18].

Depolymerization using nitrous acid. — Nitrous acid depolymerization was carried out as described by Shively and Conrad [17]. Briefly, 0.1 mL of the stock solution (100 mg/mL) of *N*-deacetylated, *N*-sulfonated GAGs was treated with freshly prepared 0.2 mL of pH 1.5 nitrous acid (100 mg of sodium nitrite was dissolved in 1.0 mL of N hydrochloric acid). After 10 min at room temperature the reaction was quenched by the addition of 60 μL of 2 M sodium bicarbonate, and then freeze dried. The dried sample was dissolved in 0.5 mL of H_2O and subjected to copper(II) sulfate CE. In case of *N*-deacetylated GAGs, nitrous acid (100 mg/mL) dissolved in hydrochloric acid adjusted to pH 4.0 with NaOH was used for depolymerization, and the sample was further treated as described above.

Capillary electrophoresis. — Separation and analysis were carried out using a fused silica (externally coated except where the tube passed through the detector) capillary tube (75 mm i.d., 375 mm 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 distilled water, and then filled with the operating buffer (i.e., 10 mM sodium borate, 50 mM boric acid, pH 8.8; or 10 mM sodium borate, 50 mM SDS, pH 8.5) and inserted into the instrument [28]. An ACI Model I computer by Dionex performed the retention time analysis and integration of the electropherogram. In case of samples depolymerized using nitrous acid, 5 mM copper(II) sulfate adjusted to pH 4.5 with 0.1 M sulfuric acid

was used as the running buffer, and the UV absorbance was monitored at 240 nm [29]. When using copper(II) sulfate as an electrolyte, the cathode platinum wire was plated by copper. This plating of the cathode resulted in severe baseline noise on using other buffer systems with UV detection. Thus, it was important to clean the cathode after the use of copper sulfate and prior to the use of a second buffer system. The cathode was cleaned by washing it with concentrated nitric acid, followed by water.

Polyacrylamide gel electrophoresis. — Gradient PAGE was performed using a 14 × 28 cm vertical slab gel prepared with a 12–22% total acrylamide gradient as previously described [30]. GAGs and oligosaccharides were visualized by Alcian blue staining.

Anti-Xa and anti-IIa activities. — Normal human plasma was diluted 4-fold in water. Anti-Xa activity was determined by incubating 50 μL of chondroitin sulfate, dermatan sulfate and their derivatives in diluted normal human plasma with 50 μL of bovine Factor Xa (60 PRP ICTH/2.5 ml) at 37 °C for 120 s. Then, 50 μL (2 μmol/mL) of Chromogenic XA substrate (4-methyl-(2*R*)-amino[methoxy(ethoxycarbamate)]pentanoyl-Gly-Arg-*p*-nitroanilide hydrochloride), was added, and residual Factor Xa was measured at 405 nm. Anti-IIa activity was determined by incubating 50 μL of GAG or derivatized GAG sample in diluted normal human plasma with 50 μL of human thrombin (12 NIH units/mL) at 37 °C for 30 s. Then, 50 μL (2.5 μmol/mL) of Chromogenic TH (ethylmalonyl-Pro-Arg-*p*-nitroanilide hydrochloride) was added, and the amidolytic thrombin activity was measured at 405 nm. Measurements were performed on an ACL 300 Plus from Instrumentation (Lexington, MA) and calculated in comparison with USP Heparin Reference Standard (K-3) supplied by U.S. Pharmacopeial Convention (Rockville, MD).

Complement inhibitory activity. — Chondroitin and dermatan sulfate and their derivatives were tested for capacity to regulate the classical pathway of complement as previously described [25]. Briefly, the GAGs and GAG derivatives were prepared at various concentrations (from 0.15 to 40 μg) in 100 μL of dextran–gelatin veronal-buffered saline containing calcium and magnesium (DGVB + +) and added to tubes on ice. Then, guinea pig C2, pre-titered to produce an average of one hemolytic event per cell (one Z of lysis) was added to each tube in 100 μL of DGVB + +. Lastly 1×10^7 of EAC 1, 4b (sheep erythrocytes that contained surface C1 and C4) in 100 μL of DGVB + + were added to each tube and the tubes were immediately incubated in a shaking water bath at 30 °C for 7 min (the t_{max}). Then, 0.3 mL of guinea pig complement diluted in gelatin-veronal-buffered saline that contained 40 mM ethylenediamine was added to each tube as a source of terminal complement pathway components, and incubation was continued for 60 min at 37 °C. Finally, 1.5 mL of saline was added to each tube (except the 100% lysis tubes which received water), the tubes were shaken and centrifuged, and lysis was assessed by determining hemoglobin release at 414 nm. Tubes that contained no GAG or GAG derivatives were designated as non-inhibited controls and were constituted to have about one hemolytic event per cell (one Z of lysis). Reagent blank and 100% lysis tubes did not receive GAG or C2. Inhibition was calculated based upon the lysis of cellular intermediates (Z) in the test sample as compared with the non-inhibited control tubes. Data are reported for the 40 μg tubes, which produced from 24 to 66% inhibition of lysis.

Acknowledgements

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References

[1] R.J. Linhardt and R.E. Hileman, *Gen. Pharmacol.*, 26 (1995) 443–451.
[2] T.E. Hardingham and A.J. Fosang, *FASEB J.*, 6 (1992) 861–870.
[3] D.M. Templeton, *Curr. Rev. Clin. Lab. Sci.*, 29 (1992) 141–184.
[4] R.E. Edens, J.M. Weiler, and R.J. Linhardt, *Complement Profiles*, 1 (1993) 96–120.
[5] R.J. Linhardt, A. Al-Hakim, J. Liu, D. Hoppensteadt, G. Mascellani, P. Bianchini, and J. Fareed, *Biochem. Pharmacol.*, 42 (1991) 1609–1619.
[6] R.J. Linhardt, A. Al-Hakim, S.Y. Liu, Y.S. Kim, and J. Fareed, *Sem. Thromb. Hemostas.*, 17 (1991) 15–22.
[7] R.J. Linhardt and T. Toida, in Z.B. Witzak and K.A. Nieforth (Eds.), *Carbohydrates as Drugs*, Marcell Dekker, New York, 1996.
[8] T. Yoshida, Y. Yasuda, T. Mimura, Y. Kaneko, H. Nakashima, N. Yamamoto, and T. Uryu, *Carbohydr. Res.*, 276 (1995) 425–436.
[9] M.F. Scully, V. Ellis, and V.V. Kakkar, *Thromb. Res.*, 41 (1986) 489–499.
[10] Y. Kazama, T. Koide, and N. Sakuragawa, *Thromb. Res.*, 54 (1989) 499–504.
[11] R.M. Maaroufi, J. Tapon-Bretaudiere, J. Mardiguian, C. Sternberg, M.D. Dauztenberg, and A.M. Fischer, *Thromb. Res.*, 59 (1990) 749–758.
[12] J.T. Gallagher, J.E. Turnbull, and M. Lyon, *Int. J. Biochem.*, 24 (1992) 553–560.
[13] K. Norgard-Sumnicht and A. Varki, *J. Biol. Chem.*, 270 (1995) 1–13.
[14] J. Van der Born, K. Gunnarsson, M.A.H. Bakker, L. Kjellen, M.K. Gullberg, M. Maccarana, J.H.M. Berden, and U. Lindahl, *J. Biol. Chem.*, 270 (1996) 31303–31309.
[15] T. Toida, H. Toyoda, and T. Imanari, *Anal. Sci.*, 9 (1993) 53–58.
[16] R.J. Linhardt, in H. Ogura, A. Hasegawa, and T. Suami (Eds.), *Carbohydrates – Synthetic Methods and Applications in Medicinal Chemistry*, VCH Publishers, Weinheim, 1992, pp 385–401.
[17] J.E. Shively and H.E. Conrad, *Biochemistry*, 15 (1976) 3943–3950.
[18] K. Gu, J. Liu, A. Pervin, and R.J. Linhardt, *Carbohydr. Res.*, 244 (1993) 369–377.
[19] R.J. Linhardt, in A. Varki (Ed.), *Current Protocols in Molecular Biology*, Vol. 2, Wiley Interscience, New York, 1992, pp 17.13.17–17.13.32.
[20] J.J. Hopwood, in D.A. Lane and U. Lindahl (Eds.), *Heparin, Chemical and Biological Properties, Clinical Applications*, CRC Press, Baca Raton, 1989.
[21] E.E.O. Caldwell, V.D. Nadkarni, J.R. Fromm, R.J. Linhardt, and J.M. Weiler, *Int. J. Biochem. Cell Biol.*, 28 (1996) 203–216.
[22] J.R. Fromm, R.E. Hileman, E.E.O. Caldwell, J.M. Weiler, and R.J. Linhardt, *Arch. Biochem. Biophys.*, 323 (1995) 279–287.
[23] R.J. Linhardt, R.J. Kerns, and I.R. Vlahov, in M. Yalpani (Ed.), *Biochemical Functions and Biotechnology of Natural and Artificial Polymers*, ATM Press, in press.
[24] R.M. Maaroufi, J. Tapon-Bretaudiere, J. Mardiguian, C. Sternberg, M.D. Dauztenberg, and A.M. Fischer, *Thromb. Res.*, 59 (1990) 749–758.
[25] J.M. Weiler and R.J. Linhardt, *Immunopharmacology*, 17 (1989) 65–72.
[26] E. Coyne, in R.L. Lundblad, W.V. Brown, K.G. Mann, and H.R. Roberts (Eds.), *Chemistry and Biology of Heparin*, Elsevier North-Holland, New York, 1981, pp 9–17.
[27] V.D. Nadkarni, A. Pervin, and R.J. Linhardt, *Anal. Biochem.*, 222 (1994) 57–63.
[28] A. Pervin, C. Gallo, K. Jandik, X.-J. Han, and R.J. Linhardt, *Glycobiology*, 5 (1995) 83–95.
[29] T. Toida and R.J. Linhardt, *Electrophoresis*, 17 (1996) 341–346.
[30] R.E. Edens, A. Al-Hakim, J.M. Weiler, D.G. Rethwisch, J. Fareed, and R.J. Linhardt, *J. Pharm. Sci.*, 81 (1992) 823–827.