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DEPOLYMERIZATION OF CHONDROITIN 6-SULFATE AND DERMATAN SULFATE WITH DIAZOMETHANE IN THE PRESENCE OF A SMALL PROPORTION OF WATER

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ABSTRACT

Chondroitin 6-sulfate (sodium salt), dermatan sulfate (sodium salt), and their methyl esters were depolymerized into mixtures of methylated, even-numbered oligosaccharides having a 4,5-unsaturated uronic acid, nonreducing end-group, respectively, with excess diazomethane in the presence of a small proportion of water. The methyl ester of chondroitin 6-sulfate was more effectively cleaved than the sodium salt, whereas the methyl ester of dermatan sulfate was depolymerized at a rate slightly higher than the sodium salt. About half of the acetamido group in the depolymerized product of the methyl ester of these polysaccharides was *N*-methylated.

INTRODUCTION

In a previous paper[1], we described the depolymerization of heparin by β-elimination with diazomethane to give a mixture of methylated di-, tetra-, and hexa-saccharides having a 4,5-unsaturated uronic acid, nonreducing end-group. It was suggested that the effective cleavage of heparin was due to both the α-L-iduronic acid residue and the sulfoamino group. In order to investigate the cleavage of other glycosaminoglycuronans that contain a D-glucuronic acid residue but no sulfoamino group, chondroitin 6-sulfate, dermatan sulfate, and their methyl esters were treated with diazomethane in the presence of a small proportion of water.

RESULTS AND DISCUSSION

A solution of the sodium salt or the methyl ester of chondroitin 6-sulfate in a small volume of water was treated with an excess solution of diazomethane in

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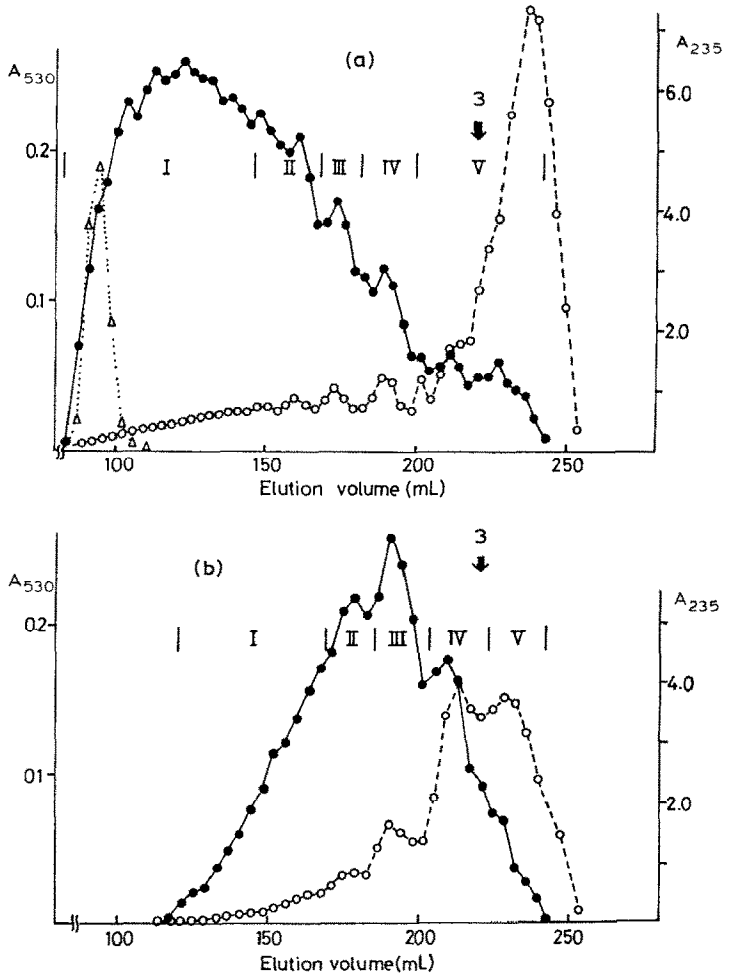


Fig. 1. Diagrams of gel-filtration, on Sephadex G-50 (superfine), of the sodium salt of chondroitin 6-sulfate (absorbance at 530 nm, · · · △ · · ·) and the reaction products of: (a) the sodium salt (absorbance at 530 nm, —●—●—; at 235 nm, ---○---○---), and (b) the methyl ester of chondroitin 6-sulfate (absorbance at 530 nm, —●—●—; at 235 nm, ---○---○---), treated with diazomethane at 25°. The arrow indicates the position of elution of standard **3**. The absorbance at 235 nm is due to the 4,5-unsaturated, nonreducing terminal uronic acid group. The u.v.-absorbing area that emerges after the disaccharides is probably due to both the 4,5-unsaturated uronic acid monosaccharide and u.v.-absorbing material originating from diazomethane during the reaction.

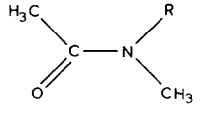
diethyl ether with vigorous stirring for a long period at 25°. Figs. 1a and 1b show the diagrams of the gel-filtration of the reaction products on a column of Sephadex G-50. The methyl ester of chondroitin 6-sulfate was more effectively depolymerized than the sodium salt.

When the reaction product of the sodium salt was again treated with excess diazomethane, it was slightly depolymerized (elution diagram not shown). A

separate experiment on the methyl glycoside of dimeric *N*-acetylchondrosine indicated that it was slightly cleaved by the treatment with excess diazomethane (data not shown). On the other hand, similar treatment cleaved reducing dimeric *N*-acetylchondrosine into methylated disaccharides, in ~35% yield, as previously reported[1]. The oligosaccharide obtained by treatment of chondroitin 6-sulfate with diazomethane showed no reducing activity by the Park–Johnson method[2], suggesting methylation of the reducing group.

When the concentration of the solution was changed from 5% in 0.1M phosphate buffer (pH 8.0) (Figs. 1a and b) to 10 or 1.7%, the degree of the depolymerization was reduced. These results suggested that depolymerization was suppressed by excess of water owing to rapid decomposition of the reagent, or by insufficient water owing to incomplete reaction in the highly viscous solution. The addition of a radical scavenger (Garvinoxyl) to the reaction medium did not affect the degree of depolymerization. Since the sodium salt of chondroitin was depolymerized to the same extent as that of chondroitin 6-sulfate, the presence of sulfate groups did not influence the depolymerization.

Each fraction pooled as indicated in Fig. 1b was collected and rechromatographed on a column of Sephadex G-25. The ^{1}H-n.m.r. spectra of Fractions III and IV indicated that they were tetrasaccharides and disaccharides, respectively, and that about 50% of the acetamido groups was *N*-methylated (Table I). The ^{1}H-n.m.r. spectrum of Fraction IV showed two sharp signals with similar chemical shift (δ 2.94 and 2.90) and a broad peak (δ 3.15–3.03) in the region of the *N*-methyl resonance. These signals are probably due to the two rotational isomers **1** and **2**.



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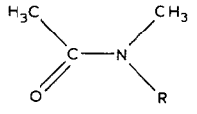

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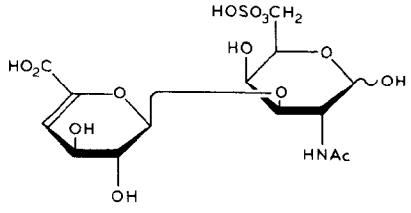

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Four distinct signals observed in the spectrum region of an acetamido group resonance probably correspond to the α- and β-glycosides of **1** and **2**. Since these spectra showed a signal for two H-6 protons on the carbon atom bearing a sulfate group and a weak signal for the methyl ester group, the sulfate group appeared to be stable, whereas a large proportion of the methyl ester group (not quantitatively estimated) was removed during the reaction with diazomethane. These results were also supported by the paper-electrophoretic behavior of Fractions III and IV (Fig. 2). Fraction IV gave a major spot migrating close to 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose 6-sulfate (**3**) and a minor spot that migrated much slower than **3**, whereas the reesterified product (IV′) preponderantly gave the latter one. The electrophoretic behavior of Fraction

TABLE I

^{1}H-N M R DATA (δ) FOR OLIGOSACCHARIDES PREPARED BY TREATMENT OF CHONDROITIN 6-SULFATE METHYL ESTER, DERMATAN SULFATE METHYL ESTER, AND SODIUM DERMATAN SULFATE WITH DIAZOMETHANE

Polysaccharide	*Fraction separated on Sephadex G-50*	*Proton*					
		$\Delta_{4,5}$	$NHCOCH_3$		NCH_3		CO_2CH_3
Chondroitin 6-sulfate (methyl ester)	III	6.02–6.18 (0.95 H) (6.10)[b]	2.08 2.21	(6 H)	2.85 2.90 2.94–3.13	(~3 H)	[a]
	IV	5.98–6.18 (0.98 H) (6 08)[b]	2.07 2.10 2.20 2.22[c]	(3 H)	2.90 2 94 3.03–3.15	(~1.4 H)	[a]
	III'[d]	6.30–6.50 (0.97 H) (6.40)[b]	2.07 2.21	(6 H)	2.84 2.88 2.99–3.10	(~3.3 H)	3.88 (~6.4 H)
	IV'[e]	6.31–6 51 (1.0 H) (6.41)[b]	2.09 2 12 2 23 2.26[c]	(3 H)	2.95 3.08–3 17	(~1.5 H)	3.92 (~6.1 H)
Dermatan sulfate (methyl ester)	III	6.00–6.20 (1.0 H) (6.10)[b]	2.06 2.19 2.22 2 33	(6 H)	2.84 2.98–3.08	(~2 5 H)	
	IV	6.00–6.16 (1.0 H) (6.08)[b]	2.05 2 19 2.23 2.33	(3 H)	2.90 3.02–3.10	(~1.4 H)	
Dermatan sulfate (sodium salt)	IV V	6.02 (0 93 H) 6 06 (1.0 H) J 3 0 Hz (doublet)	2.07 2.07	(6 H) (3 H)			

[a]The spectrum showed broad peaks at δ 3.78–4.03, probably due to the methyl ester group (not quantitatively estimated). [b]Center of the peak. [c]Shoulder [d]Reesterified Fraction III. [e]Reesterified Fraction IV.

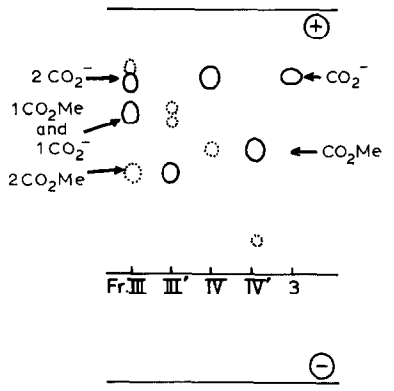

Fig. 2. Electrophoretic patterns of Fractions III and IV (Fig. 1b) and their re-esterified products (Fractions III' and IV'), and standard **3**. The electrophoresis was performed at pH 5.5. Patterns of substitution of the carboxyl group are indicated beside the spots.

III, before and after reesterification, was also consistent with the results of Fraction IV. Di- and tetra-saccharide fractions obtained by the reaction of the sodium salt of chondroitin 6-sulfate with diazomethane gave a broad spot migrating close to **3**, suggesting that the carboxyl group in these oligosaccharides was not esterified during the reaction.

The methyl ester of dermatan sulfate was depolymerized at a rate slightly higher than that of its sodium salt (Figs. 3a and 3b). The glycosyl linkage at C-4 and H-5 of the L-iduronic acid residue of dermatan sulfate are in *trans*-diaxial relationship. Owing to this orientation favorable for β-elimination, the sodium salt and the methyl ester were expected to be affected by treatment with diazomethane. The ^{1}H-n.m.r. data indicated that Fraction III, obtained from the methyl ester, and Fraction IV, obtained from the sodium salt, contained tetrasaccharides, and that Fraction IV, obtained from the methyl ester, and Fraction V, obtained from the sodium salt, contained disaccharides (Table I). About half the acetamido group of the methyl ester was found to be *N*-methylated, whereas those of the sodium salt were not affected. These results suggest that the amide proton was stabilized by the carboxylate anion at the adjacent sugar residue [$^4C_1$(D) conformation of the D-glucuronic acid residue or $^1C_4$(L) conformation of the L-iduronic acid residue] and, thus, unreactive with diazomethane. Hydrogen bonding between the amide proton and the carboxylate oxygen atom is likely to exist in a dimethyl sulfoxide or deuterium oxide solution of some glycosaminoglycuronans[3]. The ^{1}H-n.m.r. spectrum of Fraction V (Fig. 3a) obtained from the sodium salt of dermatan sulfate gave a single, sharp signal for the acetamido group at δ 2.07 and a doublet signal for the olefinic proton at δ 6.06 (Table I). These spectral characteistics are much different from those of the disaccharide (Fraction IV in Fig. 3b) obtained from the methyl ester. The ^{1}H-n.m.r. spectra of these fractions, obtained from both chondroitin 6-sulfate and dermatan sulfate, showed several intense signals due to the methoxyl

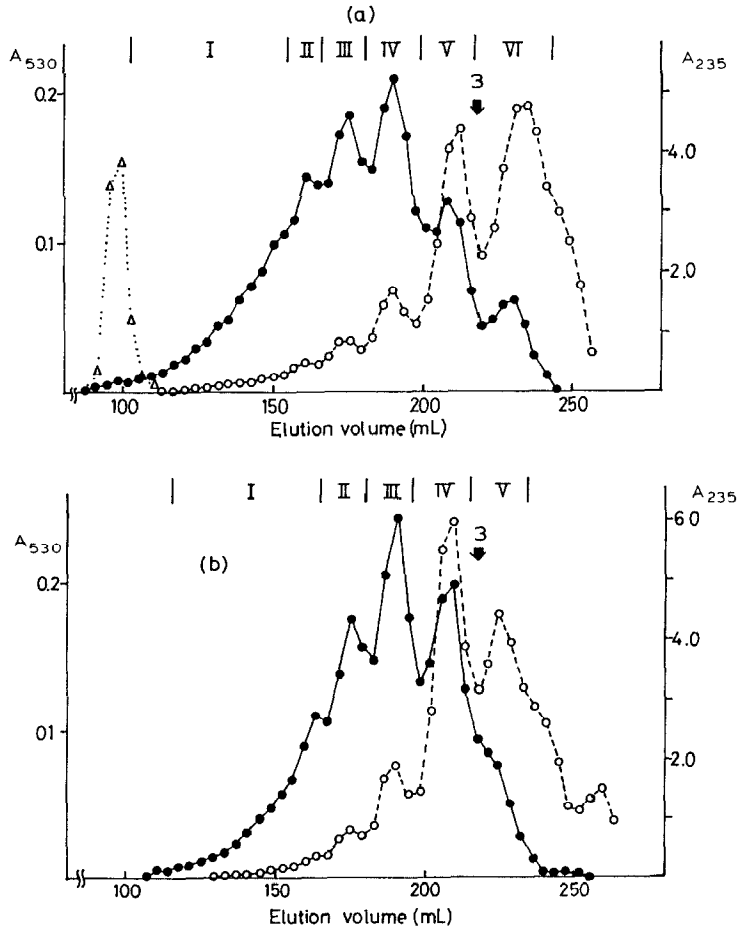

Fig. 3. Diagrams of gel-filtration, on Sephadex G-50 (superfine), of the sodium salt of dermatan sulfate (absorbance at 530 nm, · · · △ · ·) and the reaction products of: (a) the sodium salt (absorbance at 530 nm, —●—●—; at 235 nm, ---○---○---), and (b) the methyl ester of dermatan sulfate (absorbance at 530 nm, —●—●—; at 235 nm, ---○---○---), treated with diazomethane at 25°. The arrow indicates the position of elution of standard **3**. The absorbance at 235 nm is due to the 4,5-unsaturated, nonreducing terminal uronic acid group. The u.v.-absorbing area that emerges after the disaccharides is probably due to both the 4,5-unsaturated uronic acid monosaccharide and u.v.-absorbing material originating from diazomethane during the reaction.

groups at δ 3.3–3.6 (not quantitatively estimated), suggesting that most of the hydroxyl groups in the reaction products had been methylated, as observed previously for heparin[1].

EXPERIMENTAL

Materials and methods. — Chondroitin 6-sulfate (Na salt), pig-skin dermatan sulfate (Na salt), and 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-eno-

pyranosyluronic acid)-D-galactose 6-sulfate (**3**) were obtained from Seikagaku Kogyo Co. (Tokyo). Diazomethane in diethyl ether was prepared from *N*-methyl-*N*-nitroso-4-toluenesulfonamide, obtained from Tokyo Kasei Kogyo Co. (Tokyo). Uronic acid content was determined by the method of Bitter and Muir[4], and reducing value by the method of Park and Johnson[2]. Paper electrophoresis was performed as described previously[5]. ^{1}H-N.m.r. spectra were recorded with a Varian EM-90 spectrometer at 35° (HOD peak, δ 4.71), for solutions in D_2O (99.95%); chemical shifts are expressed downfield from the signal of sodium 4,4-dimethyl-4-silapentane-1-sulfonate; samples were preliminarily treated with D_2O (99.75%).

Preparation of the methyl esters of chondroitin 6-sulfate and dermatan sulfate. — A solution of the Na salt of chondroitin 6-sulfate (300 mg) in water (10 mL) was passed through a column of Dowex 50W-X2 (H^+, 50–100 mesh). The effluent was lyophilized. The residue was suspended in anhydrous methanol (20 mL) and cooled to 4°. To the suspension was added a solution of diazomethane in diethyl ether (~30 mL) freshly prepared from *N*-methyl-*N*-nitroso-4-toluenesulfonamide (5 g), and the suspension was stirred for 45 min at 4°. After evaporation of the solvent under reduced pressure, a suspension of the residue in water (5 mL) was stirred to give an acidic homogeneous solution. The pH of the solution was adjusted to 6.8 with M NaOH and the solution lyophilized to give the methyl ester of chondroitin 6-sulfate as a white powder.

The Na salt of dermatan sulfate (150 mg) was converted into the methyl ester as just described.

Depolymerization and permethylation of chondroitin 6-sulfate methyl ester with diazomethane. — To a solution of chondroitin 6-sulfate methyl ester (50 mg) in 0.1M phosphate buffer (pH 8.0, 1.0 mL) was added a solution of diazomethane in diethyl ether (~60 mL), freshly prepared from *N*-methyl-*N*-nitroso-4-toluenesulfonamide (10 g). The solution was vigorously stirred for 24 h at 25°. After both diethyl ether and water had been evaporated, the residue was dissolved in water (0.5 mL) and the solution was treated again with the diazomethane solution (~60 mL) for the same period. After evaporation of diethyl ether, the residue was extracted successively with methanol (3 × 5 mL) and water (2 × 5 mL). The combined extracts were concentrated to a small volume *in vacuo*, and applied to a Sephadex G-50 (superfine) column (1.5 × 150 cm). The column was eluted with M NaCl. The eluate was collected in 3.2-mL fractions, and each fraction was analyzed for uronic acid content and for the absorbance at 235 nm, and pooled as indicated in Fig. 1b. Each of Fractions I–V (obtained from three experiments on the scale just described) was separately pooled and lyophilized. The residue was desalted on a Sephadex G-15 column (2.5 × 75 cm) and material from the fractions giving a positive carbazole reaction was rechromatographed on a Sephadex G-25 (superfine) column (1.5 × 150 cm) in 0.2M NH_4HCO_3. The fractions homogeneous in molecular size were pooled, lyophilized, and converted into the free acid by passage through a column of Dowex 50W-X2 (H^+, 50–100 mesh). The pH of the acidic eluate was adjusted to 6.4 with 0.1M NaOH. Each of the fractions was finally

purified by passing a solution of the sodium salt in 1:9 (v/v) ethanol–water through a Sephadex G-15 column (1.0 × 60 cm). Yield of each fraction: Fr. I, 22.5 mg; Fr. II, 17.5 mg; Fr. III, 22.5 mg; Fr. IV, 15.6 mg; and Fr. V, 6.2 mg (Total, 84.3 mg).

Chondroitin 6-sulfate (Na salt), dermatan sulfate (Na salt), and its methyl ester were depolymerized by virtually the same method as just described.

Reesterification of depolymerized and permethylated chondroitin 6-sulfate methyl ester (Fractions III and IV in Fig. 1b). — Fraction III or IV (6 mg for each sample) was converted into the acid form by passage through a column of Dowex 50W-X2 (H^+, 50–100 mesh). Each acidic eluate was lyophilized. To the residue dissolved in anhydrous methanol (1.5 mL) was added a solution of diazomethane in diethyl ether (1.5 mL) at 4°. After being stirred for 30 min at 4°, the solution was concentrated *in vacuo*. A solution of the residue in water (1 mL) was stirred for 4 h at room temperature, the pH of the solution being kept at 6.5–7.0 by the occasional addition of 0.1M NaOH, and the solution stirred overnight. It was concentrated to a small volume *in vacuo* and applied to a Sephadex G-15 column (1 × 60 cm), and the column eluted with 1:9 (v/v) ethanol–water. The fractions giving a positive carbazole reaction were combined and lyophilized to give the methyl ester of Fraction III or IV (III′ or IV′).

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