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THE STRUCTURE OF ROOSTER-COMB DERMATAN SULFATE. CHARACTERIZATION AND QUANTITATIVE DETERMINATION OF COPOLYMERIC, ISOMERIC TETRA- AND HEXA-SACCHARIDES

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ABSTRACT

Rooster-comb dermatan sulfates RC-20 and RC-30 were subjected to depolymerization–desulfation in hot dimethyl sulfoxide containing 10% of water to give almost quantitatively nonsulfated, even-numbered oligosaccharides having 2-acetamido-2-deoxy-D-galactose residues at the reducing terminals. They were fractionated on an anion-exchange resin with a linear gradient of lithium chloride into even-numbered oligosaccharide fractions from di- to dodeca-saccharide. Each fraction of di-, tetra-, and hexa-saccharide was isolated and further fractionated on an anion-exchange resin with a linear gradient of formic acid. All the oligosaccharide isomers that could theoretically exist (two disaccharide, four tetrasaccharide, and eight hexasaccharide fractions) were separated, and the sequences in disaccharide units were determined. The recoveries of the total amount of the di-, tetra-, and hexa-saccharide isomers isolated based on the starting RC-20 and RC-30 were 63.1 and 50.4%, respectively. The *N*-acetyldermosine-to-*N*-acetylchondrosine ratio in the disaccharide fractions from RC-20 and RC-30 had a value approximately equal to the IdoA-to-GlcA ratio. The yield of the hybrid oligomer containing *N*-acetylchondrosine and *N*-acetyldermosine was lower than expected, suggesting that the polysaccharide-chain structure of the dermatan sulfates (RC-20 and RC-30) of rooster comb contains many saccharide-chain blocks in which *N*-acetylchondrosine or *N*-acetyldermosine units are bonded sequentially.

INTRODUCTION

The copolymeric structure of pig-skin dermatan sulfate has been studied by fragmentation of the chain by chemical reactions, such as periodate oxidation followed by alkaline cleavage, combined with chondroitinases or testicular hyaluronidase[1]. Inoue and Nagasawa[2] have reported the formation of *N*-acetyl-

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chondrosine and *N*-acetyldermosine in excellent yields by solvolytic depolymerization, followed by desulfation of dermatan sulfate in dimethyl sulfoxide–water, and the formation of a series of oligomeric *N*-acetylchondrosine (di- to octa-decasaccharide) by a similar treatment of chondroitin 6-sulfate. The study of the copolymeric structure of rooster-comb dermatan sulfate by the previously described method[2] is reported herein.

EXPERIMENTAL

Materials. — Chondroitin 4- and 6-sulfates, and pig-skin dermatan sulfate (all reagent grade), were obtained from Seikagaku Kogyo Co. Ltd., bovine liver β-D-glucuronidase and D-glucurono-6,3-lactone from Sigma Chemical Co. (St. Louis, MO 63178), and chondroitinase AC-II from *Arthrobacter aurescens,* chondroitinase ABC from *Proteus vulgaris* (Chondroitin sulfate lyases, EC 4.2.99.6), chondro-4- and -6-sulfatases from *Proteus vulgaris,* and 4,5-unsaturated disaccharide standards (ΔDi, ΔDi-4S, and ΔDi-6S)* from Seikagaku Kogyo Co. Ltd. Standard *N*-acetylchondrosine and its oligomers (dimer and trimer), and standard *N*-acetyldermosine were obtained by solvolysis of chondroitin 6-sulfate or dermatan sulfate (pyridinium salt) in hot 10% water–dimethyl sulfoxide, followed by separation of the products on AG 1-X2 (Cl^-) or AG 1-X4 (HCO_2^-) anion-exchange resin (Bio-Rad Laboratories, Richmond, CA 94804). Standard tri- (GalNAc→GlcA→GalNAc) and penta-saccharide (GalNAc→GlcA→GalNAc→GlcA→GalNAc) were prepared by β-D-glucuronidase digestion of *N*-acetylchondrosine dimer and trimer, followed by separation of the products on AG 1-X4 (Cl^-) anion-exchange resin, respectively.

Analytical procedures. — Uronic acid was determined by the method of Bitter and Muir, modified by increasing the borate concentration to 0.2M, and using D-glucurono-6,3-lactone and 1,2-*O*-isopropylidene-L-iduronolactone as standards[3]; by the method of Dische[4]; and by the orcinol method using a 20-min boiling time[5]. 2-Amino-2-deoxyhexose was determined by the method of Antonopoulos[6] after hydrolysis with 3M hydrochloric acid for 15 h at 100°, 2-acetamido-2-deoxyhexose reducing end-group by the method of Reissig *et al.*[7], and sulfate groups by the method of Dodgson and Price[8]. Reduction of the oligosaccharides with sodium borohydride was carried out as described by Linker *et al.*[9]. 2-Amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-galactose content was determined with a Jeol 5AH automatic amino acid analyzer, after hydrolysis of the sample (3 mg) with 3M hydrochloric acid (1 mL) in evacuated sealed tubes for 16 h at 100°. Amino acid analysis was performed with the amino acid analyzer after hydrolysis[10] of the sam-

*Abbreviations: ΔDi, 2-acetamido-2-deoxy-3-*O*-(α-L-*threo*-4-enopyranosyluronic acid)-D-galactose; ΔDi-4S, 2-acetamido-2-deoxy-3-*O*-(α-L-*threo*-4-enopyranosyluronic acid)-4-*O*-sulfo-D-galactose; ΔDi-6S, 2-acetamido-2-deoxy-3-*O*-(α-L-*threo*-4-enopyranosyluronic acid)-6-*O*-sulfo-D-galactose; ΔDi-diS, 2-acetamido-2-deoxy-3-*O*-[α-L-*threo*-2-(or 3-)-*O*-sulfo-4-enopyranosyluronic acid]-4-*O*-sulfo-D-galactose; ChoNAc, *N*-acetylchondrosine; and DerNAc, *N*-acetyldermosine.

ple (10 mg) with 6M hydrochloric acid (1 mL) in an evacuated, sealed tube for 20 h at 100°.

Cellulose acetate membrane electrophoresis[11] was carried out on Separax strips (Fuji Photo Film Co., Tokyo, Japan) for a solution in 0.3M calcium acetate, pH 7.25, with a current of 1 mA/cm for 3 h. The strip was stained with a 0.5% Alcian Blue solution in 3% acetic acid. T.l.c. was performed on precoated cellulose plates (Merck, Darmstadt, West Germany) developed ascendingly with 15:10:3:12 (v/v) 1-butanol–pyridine–acetic acid–water. Papers or thin-layer cellulose plates were stained with the *p*-aminohippuric acid reagent[12]. Descending paper chromatography was performed on Toyo Roshi No. 51A paper in a buffer solution, pH 5.2, of 2:3:1 (v/v) 1-butanol–acetic acid–M ammonium hydroxide for 10–15 h at room temperature. After detection of the spots under u.v. irradiation, extraction and quantitative determination of the spots were carried out according to the method of Saito *et al.*[13]. The weight-average molecular mass (M_r) was determined by viscometry using the equation $[\eta] = 3.1 \cdot 10^{-4} M_r^{0.74}$ proposed for dermatan sulfate[14].

Determination of uronic acid composition in dermatan sulfates. — To a mixture of the sample (100 μg/20 μL of water) were added enriched Tris buffer*, pH 8.0 (10 μL), and chondroitinase AC-II (0.5 unit/20 μL of water), and the mixture was incubated for 5 h at 37°. To another sample of the material (100 μg/20 μL of water) were added enriched Tris buffer(10 μL) and chondroitinase ABC (0.2 unit in 20 μL of water), and the mixture was incubated for 5 h at 37°. The absorbance at 232 nm was measured for each incubation mixture to obtain a ratio of A_{232} chondroitinase AC-II:A_{232} chondroitinase ABC that gave[13] the content of the D-glucuronic in the total uronic acid content (%).

Analysis of location of sulfate groups in dermatan sulfates. — To a mixture of sample solution (100 μg/20 μL of water) and enriched Tris buffer, pH 8.0 (10 μL), was added either chondroitinase ABC (0.2 unit/20 μL of water) alone, or chondroitinase ABC and chondro-4-sulfatase (0.2 unit/20 μL of water), or chondroitinase ABC and chondro-6-sulfatase (0.2 unit/20 μL of water), or chondroitinase ABC and chondro-4- and -6-sulfatases (each 0.2 unit/20 μL of water). The mixtures were incubated for 6 h at 37°, and the digestion products in each incubation mixture were characterized and quantitatively determined by paper chromatography as described by Suzuki *et al.*[15].

Analysis of the sequence of disaccharide units of oligosaccharides. — To a mixture of sample (100 μg/20 μL of water) and enriched Tris buffer, pH 8.0 (10 μL), was added either chondroitinase ABC (0.2 unit/20 μL of water) of chondroitinase AC-II (0.2 unit/20 μL of water), and the mixtures were incubated for 5 h at 37°. To another solution of sample and 50mM acetate buffer, pH 5.0 (10 μL), was added β-D-glucuronidase (200 units/20 μL of water), and the mixture was incu-

*Enriched Tris buffer[13] contains 0.3M sodium acetate, 0.25M sodium chloride, 0.25M Tris · hydrochloride (pH 8.0), and bovine serum albumin (0.5 mg/mL).

bated for 5 h at 37°. The products of digestion were characterized by paper chromatography in 15:10:3:12 (v/v) 1-butanol–pyridine–acetic acid–water with 4,5-unsaturated disaccharide (ΔDi), *N*-acetylchondrosine, *N*-acetyldermosine, D-glucuronic acid, and trisaccharide (GalNAc→GlcA→GalNAc) as standards.

Preparation of dermatan sulfate fraction from rooster comb[16]. — Rooster comb (1 kg, wet wt.) was boiled in 0.5% benzalkonium chloride (2 L) for 30 min, minced, and then digested with Pronase (5 g; Kaken Kagaku Co., Tokyo, Japan) in a solution containing calcium acetate (100 mg) and a few drops of toluene (2 L, pH 7.0–8.0) for 12 h at 50°. After filtration, the filter cake obtained was extracted with an equal volume of M sodium chloride under stirring for 3 h at room temperature. The final concentration of sodium chloride was adjusted to 0.65M, and the extract was applied to a column (3 × 20 cm) of Diaion HPA-10 anion-exchange resin, equilibrated in 0.5M sodium chloride. The column was washed successively with 0.65M sodium chloride (0.5 L) and 1.1M sodium chloride (0.3 L), and the fractions eluted with 1.8M sodium chloride were collected, and concentrated *in vacuo* to a small volume. The solution was dialyzed against running tap-water overnight, evaporated *in vacuo* to a 10-mL volume, and 5M sodium hydroxide was added to a final concentration of 0.5M. The alkaline solution was kept for 90 min at 37°, and then made neutral by addition of acetic acid under cooling. Ethanol (2 vol.) was added and the precipitate washed successively with 70% ethanol, ethanol, and ether, and dried *in vacuo* in the presence of phosphorus pentaoxide (crude rooster-comb dermatan sulfate, sodium salt, 650 mg).

Purification of rooster-comb dermatan sulfate. — A solution of crude rooster-

TABLE I

PROPERTIES AND COMPOSITION OF ROOSTER-COMB DERMATAN SULFATES

Properties and composition	*Dermatan sulfate*	
	RC-20	*RC-30*
Yield[a] (g)	2.80	1.11
(%)	61.7	24.4
M_r	41 000	40 800
Ratio of D-glucuronic acid to total uronic acid (%)	22.6	41.4
Ratio of carbazole to orcinol color	0.35	0.47
S (mol)[b]	0.98	1.20
Ratio of 2-amino-2-deoxy-D-glucose to total 2-amino-2-deoxyhexose (%)	[c]	0.2
Composition of chondroitinase ABC digestion products (%)		
ΔDi	4.45	1.67
ΔDi-4S	88.40	84.90
ΔDi-diS	7.14	13.40

[a]Amount of preparation purified from 5 g of sodium dermatan sulfate. [b]Mole ratio relative to disaccharide unit. [c] No measurable amount of 2-amino-2-deoxy-D-glucose was detected.

comb dermatan sulfate (5 g, sodium salt) in water (125 mL) was kept overnight at room temperature. A small amount of the insoluble material formed was centrifuged off (10 000 r.p.m. for 15 min at 10°). The supernatant solution was treated by the procedure of Meyer *et al.*[17] as follows. It was mixed with 10% calcium acetate in M acetic acid (125 mL), and ethanol was added to a final concentration of 20% with stirring in an ice-bath. The mixture was kept for 20 h at 4°, and then centrifuged off. The supernatant solution was further fractionated with ethanol at successive concentrations of 30, 40, and 80% by the same procedure. The precipitates were washed successively with 90% ethanol, ethanol, and ether, and dried *in vacuo* in the presence of phosphorus pentaoxide. Of these fractions, the 20%-and 30%-ethanol precipitates [yields of 2.80 g (61.7%) and 1.11 g (24.4%), respectively] were shown, by electrophoresis on a cellulose acetate membrane, to be composed of dermatan sulfates only, and the 40%- and 80%-ethanol precipitates [yields of 0.34 g (7.5%) and 0.29 g (6.4%), respectively] to contain a considerable proportion of chondroitin 4-sulfate. The 20%- and 30%-ethanol precipitates (designated as RC-20 and RC-30, respectively) were subjected to chemical and enzymic analyses (Table I).

Desulfation and depolymerization of dermatan sulfates with dimethyl sulfoxide containing 10% of water. — A solution of the calcium salt of dermatan sulfate (RC-20 or RC-30, each 1.2 g) in water (35 mL) was passed through a column of Dowex 50W-X8 (H^+, 50–100 mesh) at 0–4°, and the effluent was made neutral by the addition of pyridine and lyophilized to give the pyridinium salt as a white powder. Desulfative depolymerization of the pyridinium salt (500 mg) was carried out in dimethyl sulfoxide containing 10% of water, by the procedure previously reported[2], for 14 h at 100° for RC-20, and for 20 h at 95° for RC-30. Nonsulfated tetra- and hexa-saccharides were the main products.

Fractionation of a mixture of nonsulfated oligosaccharides. — Each reaction mixture obtained by desulfative depolymerization of RC-20 and RC-30 was evaporated *in vacuo* at 55°, the residue dissolved in water (5 mL), and the solution applied to a column (3 × 35 cm) of AG 1-X4 (Cl^-, 200–400 mesh) anion-exchange resin, which was eluted at room temperature with a linear gradient of 0–0.2 (1.3 L) and 0.2–0.3M lithium chloride (1.3 L) at a flow rate of 50 mL/h. The eluate was collected in 7-mL fractions, and each fraction was analyzed for uronic acid content (Fig. 1a,b). The material from each peak was pooled, lyophilized, and desalted on a column (2.6 × 90 cm) of Sephadex G-15 by elution with 10% ethanol. The solution was concentrated *in vacuo* to a small volume, and lyophilized. Of these isolated oligosaccharide fractions, di-, tetra-, and hexa-saccharide fractions were separately rechromatographed on the column (3 × 35 cm) of AG 1-X4 (Cl^-) with linear gradients of 0–0.2, 0.1–0.3, and 0.2–0.3M lithium chloride, respectively, and each of the rechromatographed oligosaccharide fractions was desalted and lyophilized to give the lithium salt as a white powder. This was dissolved in a minimum volume of methanol, and the solution was poured into 1:1 (v/v) acetone–ether with stirring to give a white precipitate. The precipitate was collected by cen-

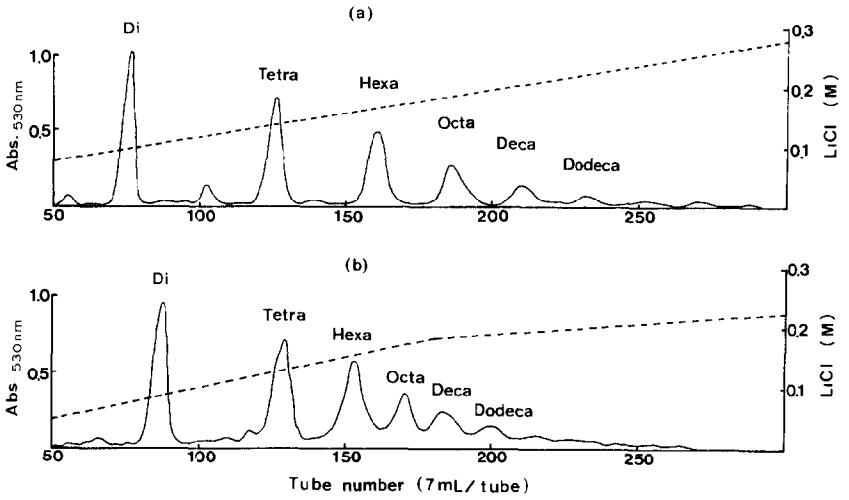

Fig. 1. Separation, on AG 1-X4 (Cl^-) anion-exchange resin, of even-numbered oligosaccharides obtained by desulfative depolymerization of rooster-comb dermatan sulfates, RC-20 (a) and RC-30 (b): Carbazole reaction (———), and concentation of lithium chloride (----). The peak areas, based on uronic acid determination, of di- to deca-saccharides were: for (a) 22.2, 20.9, 21.1, 9.5, and 6.3; and for (b) 18.8, 17.3, 14.9, 7.9, and 6.0%, respectively.

trifugation and dried in air, then *in vacuo* in the presence of phosphorus pentaoxide. Analytical data of the isolated oligosaccharide fractions are summarized in Table II.

Separation of di-, tetra-, and hexa-saccharide fractions into isomeric oligosac-

TABLE II

CHARACTERIZATION OF DI-, TETRA-, AND HEXA-SACCHARIDE FRACTIONS OBTAINED FROM ROOSTER-COMB DERMATAN SULFATE RC-20

Sample	*T.l.c.* R_{ChoNAc}	*Content in 2-amino-2-deoxy-D-galactose*		*Molar ratio of contents in 2-amino-2-deoxy-D-galactose before and after reduction*	
		Before reduction μmol/mg (%)	*After reduction* μmol/mg (%)	*Found*	*Calc.*
Standards					
N-Acetylchondrosine	1.00	2.41 (43.3)	0.06 (1.0)	1:0.02	1:0
N-Acetyldermosine	1.13	2.28 (40.9)	0.05 (1.0)	1:0.02	1:0
N-Acetylchondrosine dimer	0.58				
N-Acetyldermosine dimer	0.73				
Fractions					
Disaccharide	0.97, 1.09	2.26 (40.5)	0.06 (1.1)	1:0.03	1:0
Tetrasaccharide	0.60, 0.72	2.28 (40.8)	1.15 (20.6)	1:0.50	1:0.50
Hexasaccharide	0.38	2.28 (40.9)	1.47 (26.3)	1:0.64	1:0.67

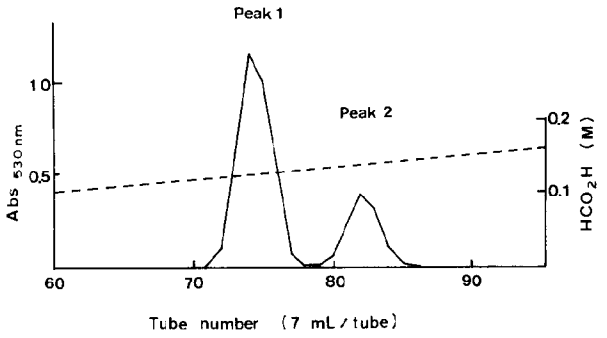

Fig. 2. Separation, on AG 1-X4 (HCO_2^-) anion-exchange resin, of the disaccharide fraction obtained by desulfative depolymerization of rooster-comb dermatan sulfate RC-20: Carbazole reaction (———); and concentration of formic acid (----); Peak 1 (*N*-acetyldermosine) 74.3%, and Peak 2 (*N*-acetylchondrosine) 25.7%. Chromatography, on the same column, of the disaccharide fraction obtained from dermatan sulfate RC-30 gave two peaks with 55.2 and 44.8% peak areas.

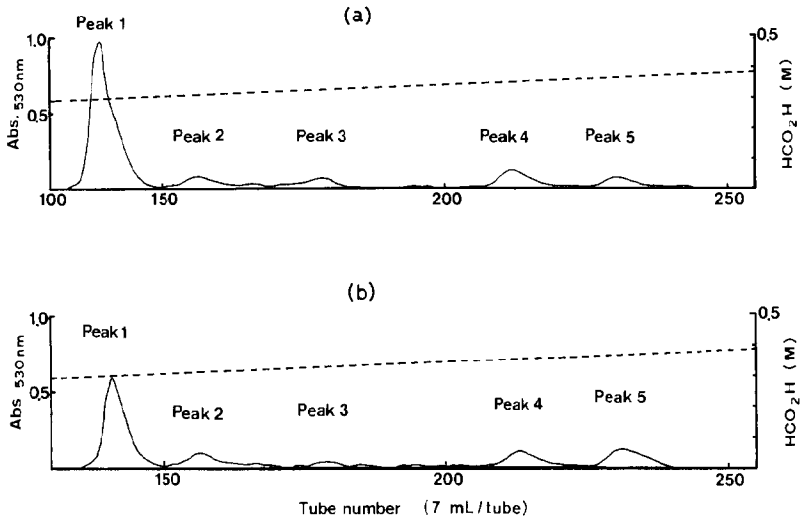

Fig. 3. Separation, on AG 1-X4 (HCO_2^-) anion-exchange resin, of tetrasaccharide fractions obtained by desulfative depolymerization of rooster-comb dermatan sulfates RC-20 (a) and RC-30 (b). The peak areas (%), based on uronic acid determination, of Peaks 1–5 were: for (a) 69.6, 8.3, 5.4, 10.9, and 5.8; and for (b) 56.5, 9.9, 4.7, 12.0, and 16.8, respectively.

charides. — The purified di-, tetra-, and hexa-saccharide fractions were separately applied onto a column (1 × 85 cm) of AG 1-X4 (HCO_2^-, 200–400 mesh) kept at 37°, and eluted with linear gradients of 0–0.2M formic acid (1 L) for the disaccharide fraction, 0.1–0.3 and 0.3–0.4M formic acid (each 1 L) for the tetrasaccharide fraction, and 0.3–0.5, 0.5–0.7, and 0.7–0.8M formic acid (each 1 L) for the hexasaccharide fraction, at a flow rate of 40 mL/h. Each of the effluents was analyzed for uronic acid (Figs. 2–4). Isolation of each peak material was carried out as follows.

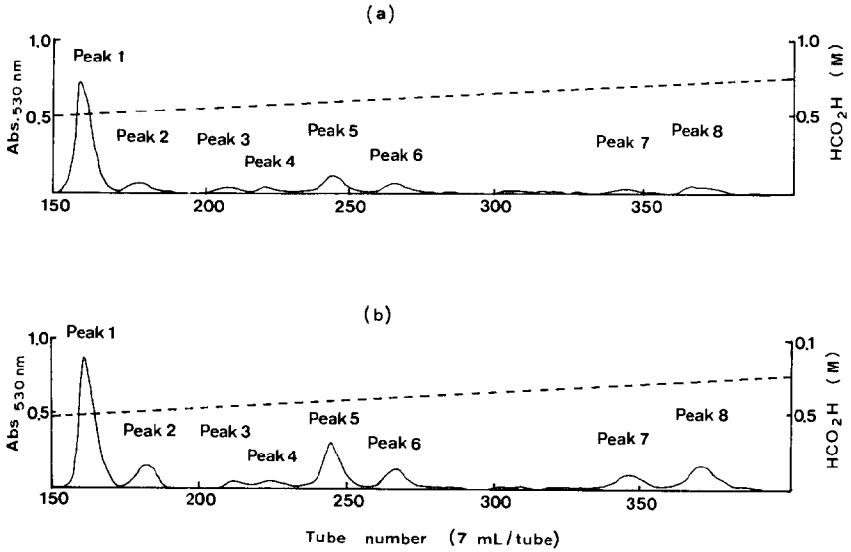

Fig. 4. Separation, on AG 1-X4 (HCO_2^-) anion-exchange resin, of hexasaccharide fractions obtained by desulfative depolymerization of rooster-comb dermatan sulfates RC-20 (a) and RC-30 (b). The peak areas (%), based on uronic acid determination, of Peaks 1–8 were: for (a) 62.9, 5.2, 3.0, 2.7, 10.7, 5.5, 3.2, and 6.9; and for (b) 47.0, 7.2, 1.9, 2.3, 17.3, 7.2, 5.4, and 11.7, respectively.

The pooled fractions corresponding to each peak were made neutral by addition of pyridine, and lyophilized. The residue was dissolved in water, and the solution passed through a column of Dowex 50W-X4 (Li^+, 50–100 mesh). The effluent was lyophilized, the residue dissolved in a minimum volume of methanol, and the solution poured into 1:1 (v/v) acetone–ether with stirring to give a white precipitate, which was dried in air, and then *in vacuo* in the presence of phosphorus pentaoxide. The sequence of disaccharides in each oligosaccharide was analyzed by the enzymic procedure described in the preceding section.

RESULTS AND DISCUSSION

Examination of crude dermatan sulfate, prepared from rooster comb, by electrophoresis on cellulose acetate membrane (data not shown) revealed that it contained chondroitin 4-sulfate, which was purified according to the procedure of Meyer *et al.*[17] to give RC-20 and RC-30 (see Table I). Both fractions had a M_r of ~41 000, which is larger than that of pig-skin dermatan sulfate[18] (M_r 25 000) or those of the whale-intestinal dermatan sulfates[19] (M_r 11 300 and 13 000). The contents of D-glucuronic acid of RC-20 and RC-30 were clearly higher than that of the dermatan sulfates from other origins[19–21]. The uronic acid compositions of RC-20 and RC-30 were markedly different. The sulfate contents showed a similar trend (Table I). The contents of serine (data not shown) corresponded to 1 mol/20 mol and 1 mol/12 mol of dermatan sulfate in RC-20 and RC-30, respectively. This indi-

cates that the peptide component represents only 5–8% of the whole dermatan sulfate molecule. The location of the sulfate ester residues was determined by digestion with chondroitinase ABC and chondrosulfatases, followed by analysis by paper chromatography. Digestion with chondroitinase ABC of RC-20 and RC-30 gave three unsaturated disaccharides, ΔDi, ΔDi-4S, and ΔDi-diS (see Table I). Digestion with chondrosulfatases (data not shown) suggested that ΔDi-diS had the second sulfate ester linked to C-2 or -3 of the uronic acid residue. The different yields of the three unsaturated disaccharides from RC-20 and RC-30 reflect the different sulfate contents in RC-20 and RC-30. The position of the sulfate group on the 2-acetamido-2-deoxy-D-galactose unit of these polysaccharides was exclusively at C-4, as observed for the dermatan sulfates from other origins previously reported.

A limited depolymerization, followed by desulfation, with dimethyl sulfoxide containing 10% of water was performed in order to separate and characterize the hybrid tetra- and hexa-saccharides containing both *N*-acetylchondrosine unit(s) and *N*-acetyldermosine unit(s). The best conditions to obtain the oligosaccharides were 100° and 14 h for RC-20, and 95° and 20 h for RC-30. In the reaction of chondroitin 4- and 6-sulfate with aqueous dimethyl sulfoxide, rapid initial cleavage of sulfate groups occurred, and the 2-acetamido-2-deoxy-β-D-galactosyl linkage of the resulting desulfated polysaccharides was preferentially hydrolyzed without side reactions. Consequently, a series of nonsulfated, even-numbered oligosaccharides having 2-acetamido-2-deoxy-D-galactose residue at the reducing end was obtained with fairly good yields. In the case of the dermatan sulfate containing L-iduronic acid in highest content, such as pig-skin dermatan sulfate, it was observed that the L-idopyranosyluronic linkage in the polysaccharide was partially hydrolyzed[2]; thus the reaction conditions just described were selected. The desulfated, depolymerized products of RC-20 and RC-30 were separated into di- to dodeca-saccharide fractions, respectively (Figs. 1a,b). The respective fractions corresponding to the di-, tetra- and hexa-saccharide peaks were collected, each of which was isolated as lithium salt and analyzed. The molar ratio of 2-amino-2-deoxy-D-galactose content, before and after reduction of each oligosaccharide with sodium borohydride, coincided well with the value calculated for each even-numbered oligosaccharide (Table II). Thus, the main peaks in Figs. 1a,b are even-numbered oligosaccharides (di- to dodeca-saccharide) having a 2-acetamido-2-deoxy-D-galactose residue at the reducing end.

T.l.c. of the materials of the two small peaks (tube numbers 50–60 and 98–110, Fig. 1a) showed R_F values (data not shown) that agreed with those of GalNAc→GlcA→GalNAc and GalNAc→GlcA→GalNAc→GlcA→GalNAc. These compounds were presumably formed by the loss of the L-idopyranosyluronic acid group at the nonreducing end of tetra- or hexa-saccharides.

The t.l.c. data of the di- and tetra-saccharide fractions in Table II indicated that each of them was a mixture containing several isomers. That the hexasaccharide fraction was also a mixture of several isomers giving only one spot was also

most probable. The disaccharide fraction of rooster-comb dermatan sulfates was completely separated into two isomeric disaccharides, *N*-acetyldermosine and *N*-acetylchondrosine, according to the procedure of Fransson *et al.*[22] with some modification (Peaks 1 and 2, Fig. 2). The *N*-acetyldermosine to *N*-acetylchondrosine ratios of RC-20 and RC-30 were ~3:1 and 11:9, respectively. These ratios were nearly identical with those of the respective uronic acid content shown in Table I. This indicated that the hydrolysis of the 2-acetamido-2-deoxy-β-D-galactosyl linkages proceeded uniformly irrespective of the nature of the uronic acid component.

Fransson and Rodén[20] isolated a hybrid tetrasaccharide, GlcA→GalNAc(SO_4)→IdoA→GalNAc(SO_4), by degradation of pig-skin dermatan sulfate with testicular hyaluronidase. Suzuki *et al.*[23] reported the isolation of ΔGlcA→GalNAc→IdoA→GalNAc(4-SO_4) and ΔGlcA→GalNAc(6-SO_4)→IdoA→GalNAc(4-SO_4) from the chondroitinase AC digestion product of a dermatan sulfate–chondroitin sulfate copolymer in fibrous cartilage of the human-knee joint. The tetrasaccharide fractions shown in Figs. 1a,b may have been considered to be a mixture of four isomeric tetrasaccharides including an isomer having the same sequence as the hybrid tetrasaccharide isolated by Fransson and Rodén[20], and by Suzuki *et al.*[23]. Unexpectedly, chromatography of the tetrasaccharide fractions on anion-exchange resin gave five peaks (Figs. 3a,b). The material of each peak was isolated as lithium salt and was analyzed for their disaccharide-unit sequences by digestion with chondroitinases, or bovine liver β-D-glucuronidase, or both. The materials of the four peaks, except that of Peak 3, were identified as the four isomeric tetrasaccharides that could theoretically exist (Table III). As expected from the uronic acid composition of RC-20 and RC-30, the amount of *N*-acetyldermosine dimer was the greatest among those of the four isomeric tetrasaccharides. Comparison of the order of elution of these isomers with the sequence of disaccharide units suggested that the *N*-acetylchondrosine dimer was the most negative in terms of ionic be-

TABLE III

CHARACTERIZATION OF ISOMERIC TETRASACCHARIDES OF RC-20 SEPARATED ON AG 1-X4 (HCO_2^-) ANION-EXCHANGE RESIN

Material of Peak[a]	*Digestion product*			*Sequence*
	Chondroitinase ABC	*Chondroitinase AC*	*β-D-Glucuronidase*	
1	ΔDi, DerNAc	Resistant	Resistant	IdoA→GalNAc→IdoA→GalNAc (*N*-acetyldermosine dimer)
2	ΔDi, ChoNAc	Resistant	Partially decomp. (GlcA, trisaccharide)	GlcA→GalNAc→IdoA→GalNAc
3	Resistant	Resistant	Resistant	Unidentified
4	ΔDi, DerNAc	ΔDi, DerNAc	Resistant	IdoA→GalNAc→GlcA→GalNAc
5	ΔDi, ChoNAc	ΔDi, ChoNAc	GlcA, trisaccharide	GlcA→GalNAc→GlcA→GalNAc (*N*-acetylchondrosine dimer)

[a]Materials correspond to Peaks 1–5 of Fig. 3a.

havior, and the *N*-acetyldermosine dimer the least negative. The relation between the sequence of disaccharide units of the hybrid isomers (Peaks 2 and 4) and the order of elution also suggested this.

The material of Peak 2 (GlcA→GalNAc→IdoA→GalNAc) was partially (~50%) hydrolyzed into D-glucuronic acid and trisaccharide by the use of a large excess (ten times as much as the usual proportion) of bovine liver β-D-glucuronidase. This is probably due to the inhibitory effect of the tetrasaccharide *per se* or the trisaccharide formed. The material of Peak 3 was resistant to all enzymes (see Table III). The chromatographic behaviors of this material on anion-exchange resin and cellulose plate closely resembled those of the materials of Peaks 1, 2, 4 and 5 (data not shown). Considering the data of Table I and the experimental results not shown herein, it was difficult to suspect participation of a third uronic acid component other than D-glucuronic and L-iduronic acid. However, transglycosidation may have partially occurred in the course of desulfative depolymerization of the polysaccharides to form a product having a structure resistant to chondroitinase ABC (see Scheme 1) although this is not highly likely. Since the amount of the Peak 3 material obtained was not sufficient for structural elucidation, no further examination was attempted. The formation of Peak-3 material was ultimately disregarded in the present work, because its amount was only ~1% of the original polysaccharides (based on uronic acid content).

UroA-(1→3)-GalNAc-(1→4)-UroA-(1→3)-GalNAc ⇒
UroA-(1→3)-GalNAc-(1→2 or 3)-UroA-(1→3)-GalNAc

Scheme 1

The eight peaks, which presumably correspond to the theoretically possible eight isomers constituting the hexasaccharide fraction, were separated completely (Figs. 4a,b). The chromatogram shown in Fig. 3 revealed that, among these isomers, (a) the *N*-acetyldermosine trimer was the most rapidly eluted, (b) the *N*-acetylchondrosine trimer was eluted last, and (c) the residual six hybrid isomers were eluted inbetween these trimers. Peaks 1 and 8 were expected to be *N*-acetyldermosine and *N*-acetylchondrosine trimer, respectively, and Peak 5 to be one of the six hybrid isomers, the yield of which was comparatively higher. Each of these was isolated as lithium salt, and analyzed for disaccharide-unit sequence by digestion with chondroitinases AC and ABC. Table IV shows the results obtained for the three isomers separated from the hexasaccharide fraction of RC-20 (the same results were also obtained for Peaks 1, 5, and 8 of the hexasaccharide fraction of RC-30).

In addition to the relationship between ionic negativity and the sequence of disaccharide units in the four tetrasaccharide isomers as previously described, the relationship between ionic negativity and the sequence of disaccharide unit in the hexasaccharide isomers 1, 5 and 8 (as shown in Figs. 4a,b and Table IV) suggests

TABLE IV

CHARACTERIZATION OF ISOMERIC HEXASACCHARIDES OF RC-20 SEPARATED ON AG 1-X4 (HCO_2^-) ANION-EXCHANGE RESIN

Material of Peak(s)[a]	*Digestion product*		*Sequence*
	Chondroitinase ABC	*Chondroitinase AC*	
1	ΔDi, DerNAc	Resistant	DerNAc trimer
2, 3, and 4	[b]	[b]	ChoNAc→DerNAc→DerNAc or DerNAc→ChoNAc→DerNAc or ChoNAc→ChoNAc→DerNAc
5	ΔDi, DerNAc	ΔDi, tetrasaccharide	DerNAc→DerNAc→ChoNAc
6 and 7	[b]	[b]	ChoNAc→DerNAc→ChoNAc or DerNAc→ChoNAc→ChoNAc
8	ΔDi, ChoNAc	ΔDi, ChoNAc	ChoNAc trimer

[a]Materials correspond to Peaks 1–8 of Fig. 4a. [b]Enzymic experiments were not performed.

that the *N*-acetylchondrosine unit located at the reducing terminal may contribute appreciably to the ionic negativity of the oligosaccharides. On the basis of this relationship, each hybrid isomer of the yet unidentified Peaks 2, 3, 4, 6, and 7 was estimated to have the sequence of disaccharide units as shown in Table IV.

Based on uronic acid determination, the proportions of respective isomers (two single isomers and one hybrid, isomeric mixture) constituting the di-, tetra- and hexa-saccharide fractions are expressed as percentages of the total uronic acid amounts of starting RC-20 and RC-30, respectively (Table V). The recovery of the total amount of these respective isomers was 63% for RC-20 and 50% for RC-30. The uronic acid contents that could not be determined were those of even-num-

TABLE V

RECOVERY[a] (%) OF EACH SINGLE AND HYBRID ISOMER IN DI-, TETRA-, AND HEXA-SACCHARIDE FRACTIONS

Fraction	*Disaccharide unit(s)*	*Dermatan sulfate*	
		RC-20	*RC-30*
Disaccharide	ChoNAc	5.7	8.4
	DerNAc	16.5	10.4
Tetrasaccharide	ChoNAc dimer	1.2	2.9
	Hybrid dimer	4.0	3.8
	DerNAc dimer	14.6	9.8
Hexasaccharide	ChoNAc trimer	1.5	1.8
	Hybrid trimer	6.4	6.2
	DerNAc trimer	13.2	7.1
Total		63(.1)	50(.4)

[a]Calculation based on uronic acid determination.

TABLE VI

COMPARISON BETWEEN EXPERIMENTAL AND CALCULATED COMPOSITIONS (%) OF EACH SINGLE AND HYBRID ISOMER IN DI-, TETRA-, AND HEXA-SACCHARIDE FRACTIONS

Fractions	*Disaccharide unit(s)*	*Dermatan sulfate*			
		RC-20		*RC-30*	
		Obs.[a]	*Calc.*[b]	*Obs.*[a]	*Calc.*[b]
Disaccharide	ChoNAc	25.7	22.6	44.8	41.4
	DerNAc	74.3	77.4	55.2	55.2
Tetrasaccharide	ChoNAc dimer	6.1	5.1	17.6	17.1
	Hybrid dimer	20.3	34.9	23.0	48.6
	DerNAc dimer	73.6	60.0	59.3	34.3
Hexasaccharide	ChoNAc trimer	6.9	1.2	11.7	7.1
	Hybrid trimer	30.0	52.4	41.3	72.8
	DerNAc trimer	62.9	46.4	47.0	20.1

[a]Calculated from the data of Table V. [b]When dermatan sulfate, in which the uronic acid composition has a ratio of D-glucuronic acid to L-iduronic acid = a:b and the sequence of uronic acids is arranged irregularly in the copolymeric chain, is subjected to fragmentation by the reaction described herein to give 100% of disaccharides, the theoretical percentage of two isomers (*N*-acetylchondrosine and *N*-acetyldermosine) formed is identical with the uronic acid compositions given in Table I. When dermatan sulfate is subjected to 100% fragmentation into tetrasaccharide, the theoretical proportions (%) of two single isomers and one hybrid isomer was calculated from Equation (*1*), where

$$(a + b)^2/100 = (a^2 + 2ab + b^2)/100 \qquad (1)$$

a is the percentage of *N*-acetylchondrosine, b that of *N*-acetyldermosine, $a^2/100$ that of *N*-acetylchondrosine dimer, $b^2/100$ that of *N*-acetyldermosine dimer, and $2ab/100$ that of hybrid dimer. When dermatan sulfate is subjected to 100% fragmentation into hexasaccharide, the theoretical quantitative proportions (%) of the two single isomers and one hybrid isomeric mixture was calculated from Equation (*2*), where

$$(a + b)^3/10\,000 = (a^3 + 3a^2b + 3ab^2 + b^3)/10\,000 \qquad (2)$$

a is the percentage of *N*-acetylchondrosine, b that of *N*-acetyldermosine, $a^3/10\,000$ that of *N*-acetylchondrosine trimer, $b^3/10\,000$ that of *N*-acetyldermosine trimer, and $(3a^2b + 3ab^2)/10\,000$ that of hybrid trimer.

bered oligosaccharides (octasaccharide or higher), free uronic acid, and odd-numbered oligosaccharides (small proportion), and the materials lost during processing.

Table VI shows the compositions (%) of isomers (two single isomers and one hybrid, isomeric mixture) for the di-, tetra- and hexa-saccharides from RC-20 and RC-30. These compositions were calculated from the data of Table V. Theoretical compositions of the respective isomers for RC-20 and RC-30 are also shown. These were calculated on the assumption that *N*-acetylchondrosine and *N*-acetyldermosine units would be arranged irregularly to form the copolymeric polysaccharide chain, and that the polysaccharide chain would have been depolymerized to 100%

of disaccharide, or to 100% of tetrasaccharide, or to 100% of hexasaccharide in the present reaction. The data in Table VI would then provide information about the copolymeric chain structure of the dermatan sulfates. With regard to the disaccharide fraction, the percentages of assayed *N*-acetylchondrosine and *N*-acetyldermosine were approximately equal to the theoretical values, for either RC-20 and RC-30. This allows a direct comparison between the assayed values and the calculated values obtained for the isomers of tetra- and hexa-saccharide fractions. As shown in Table VI, both in RC-20 and RC-30, the yields of the hybrid isomers of tetra- and hexa-saccharides were significantly lower than the calculated values. Accordingly, the assayed values of single isomers were without exception higher than the calculated values. Especially the values of the *N*-acetyldermosine dimer, *N*-acetylchondrosine trimer, and *N*-acetyldermosine trimer were very high. These results clearly indicate that the polysaccharide-chain structure of the dermatan sulfates (RC-20 and RC-30) contains many saccharide-chain blocks in which *N*-acetylchondrosine or *N*-acetyldermosine units are linked sequentially.

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