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THE STRUCTURE OF ROOSTER-COMB DERMATAN SULFATE. FRAGMENTATION OF THE POLYSACCHARIDE CHAINS BY CHONDROITINASE AC-II DIGESTION, AND BY PERIODATE OXIDATION, FOLLOWED BY ALKALI CLEAVAGE

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ABSTRACT

The polysaccharide-chain fragments of rooster-comb dermatan sulfates (RC-20 and RC-30) were obtained by chondroitinase AC-II digestion and by periodate oxidation, followed by alkaline cleavage, and their structures analyzed both quantitatively and qualitatively. RC-20 having a lower D-glucuronic acid content (22.6%) is composed preponderantly of large clusters of *N*-acetyldermosine sulfate (M_r ~17 600–41 000) at the nonreducing terminal, whereas RC-30, having a higher D-glucuronic acid content, (41.4%) is poor in this cluster. Both RC-20 and RC-30 have an *N*-acetyldermosine sulfate cluster (M_r 6500–7300) within the polysaccharide chains. Most *N*-acetylchondrosine sulfate units of RC-20 and RC-30 exist as clusters, the large clusters (M_r ~17 600) being preponderant in RC-30; both RC-20 and RC-30 contain a large proportion of *N*-acetylchondrosine sulfate clusters (M_r 3500 and 9000) that corresponds to the uronic acid content. In RC-30, most *N*-acetyldermosine disulfate units (13.4%) are linked to *N*-acetylchondrosine sulfate units or clusters.

INTRODUCTION

In the preceding communication[1], it was shown that the two fractions, RC-20 and RC-30, are representative, both quantitatively and qualitatively, of rooster-comb dermatan sulfate and that these polysaccharides (M_r ~41 000) show the highest content of D-glucuronic acid ever observed[2–4]. These fractions were desulfated and depolymerized under solvolytic conditions[5] in hot dimethyl sulfoxide containing water to give fractions of di-, tetra-, and hexa-saccharides as the main components. Quantitative and qualitative analyses of these fractions suggested that the polysaccharide chains contain many clusters of *N*-acetylchondrosine or *N*-acetyl-

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dermosine sulfate units, especially the latter ones. Further information on the copolymeric structure of the chains was obtained by digestion with chondroitinase AC-II, and by periodate oxidation followed by alkaline cleavage, and the results are described herein.

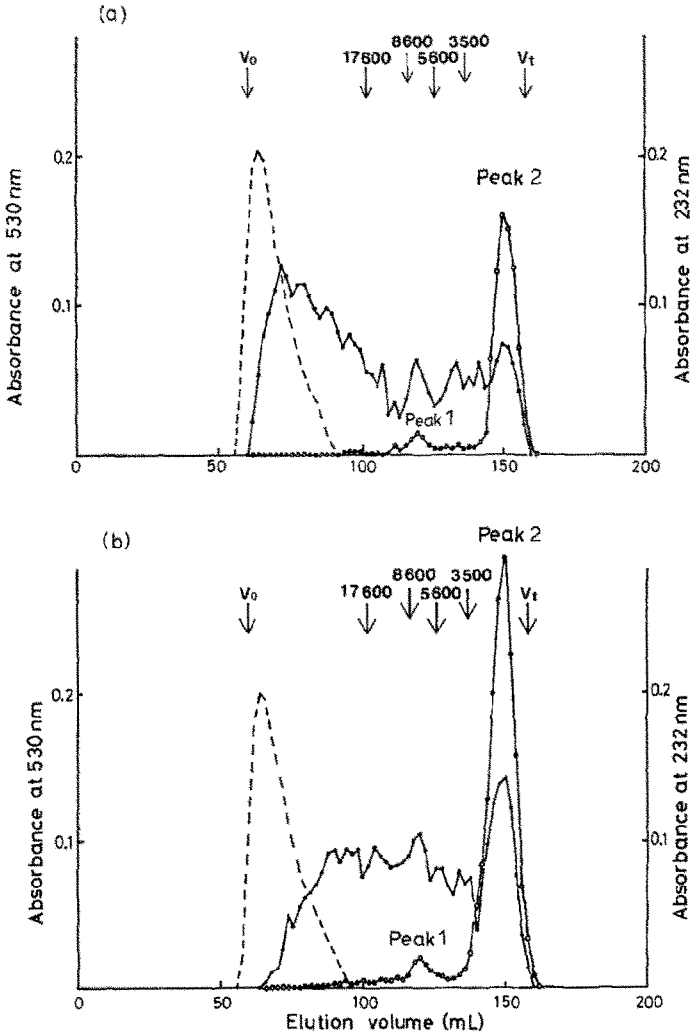

Fig. 1. Separation, on Sephadex G-200, of the products formed by digestion, with chondroitinase AC-II, of rooster-comb dermatan sulfates: (a) Fraction RC-20 and (b) Fraction RC-30. Each column was calibrated with Blue dextran (V_0), depolymerized chondroitin 6-sulfates (M_r 17 600, 8600, 5600, and 3500), and 2M sodium chloride (V_t). (– – –) Elution diagram of original dermatan sulfate (RC-20 or RC-30), (—●—●—●—) A_{530nm}, and (—○—○—○—) A_{232nm}.

EXPERIMENTAL

Materials. — The purified fractions (RC-20 and RC-30) of rooster-comb dermatan sulfate were described previously[1]. A series of standard chondroitin 6-sulfates (sodium salt) having different M_r values (3500, 5600, 8600, and 17 600) were obtained by limited depolymerization of chondroitin 6-sulfate with bovine testis hyaluronidase and by repeated gel-chromatography of digestion products[6]. Chondroitinase AC-II and chondroitinase ABC were products of Seikagaku Kogyo Co. Ltd., Tokyo.

Analytical methods. — Reducing power was determined by the method of Park–Johnson[7]. Other analytical procedures were carried out as previously described[1].

Digestion of rooster-comb dermatan sulfates with chondroitinase AC-II. — To a solution of the polysaccharide (RC-20 or RC-30, 2 mg/0.2 mL of water) and enriched Tris buffer[8], pH 8.0 (0.1 mL), was added chondroitinase AC-II (10 units/0.2 mL of water), and the mixture incubated for 3 h at 37°. After being heated in boiling water for 10 min, the mixture was centrifuged, and the supernatant solution was applied to a Sephadex G-200 column (1.5 × 90 cm) prepared in 0.15M sodium chloride. The column was eluted with the same solvent at a flow rate of 20 mL/h, and each fraction collected (2 mL) was analyzed for both uronic acid content and absorbance at 232 nm.

On a preparative scale, a solution of RC-20 or RC-30 dermatan sulfate (50 mg/3 mL of water) and enriched Tris buffer (pH 8.0, 1 mL) was incubated with chondroitinase AC-II (20 units/1 mL of water) for 16 h at 37°. After treating the incubation mixture as just described, the supernatant solution was chromatographed on a Sephadex G-200 column (2.5 × 86 cm) prepared in 0.15M sodium chloride. Fractions corresponding to the elution area to be isolated (for example, Peak 1 or 2 in Fig. 1b) were pooled, lyophilized, and desalted on a Sephadex G-25 column (2.5 × 80 cm) prepared in 10% ethanol and eluted with the same solvent. The solution was evaporated *in vacuo* at 35°, and lyophilized. The Peak 2 material of RC-20 or RC-30 was analyzed for 4,5-unsaturated disaccharide composition according to the procedure previously described[1]. The Peak 1 material of RC-30 was digested with chondroitinase ABC according to the determination of uronic acid composition of the preceding paper[1] to obtain a value of A_{232} chondroitinase AC-II/A_{232} chondroitinase ABC.

Fragmentation of rooster-comb dermatan sulfates with periodate oxidation, followed by alkali cleavage. — The polysaccharide fractions, RC-20 and RC-30, were oxidized and cleaved by the procedure described by Fransson *et al.*[9,10]. A solution of the polysaccharide (100 mg) in 20mM sodium periodate and 50mM sodium citrate buffer (pH 3.0, 50 mL) was kept in the dark for 24 h at 4°. A 10% solution of mannitol (5 mL) was added, the mixture dialyzed against distilled water (5 L × 5) for 48 h, and the dialyzate lyophilized. The residue was dissolved in water (5 mL), and the pH adjusted to 12 with M sodium hydroxide. The solution was kept

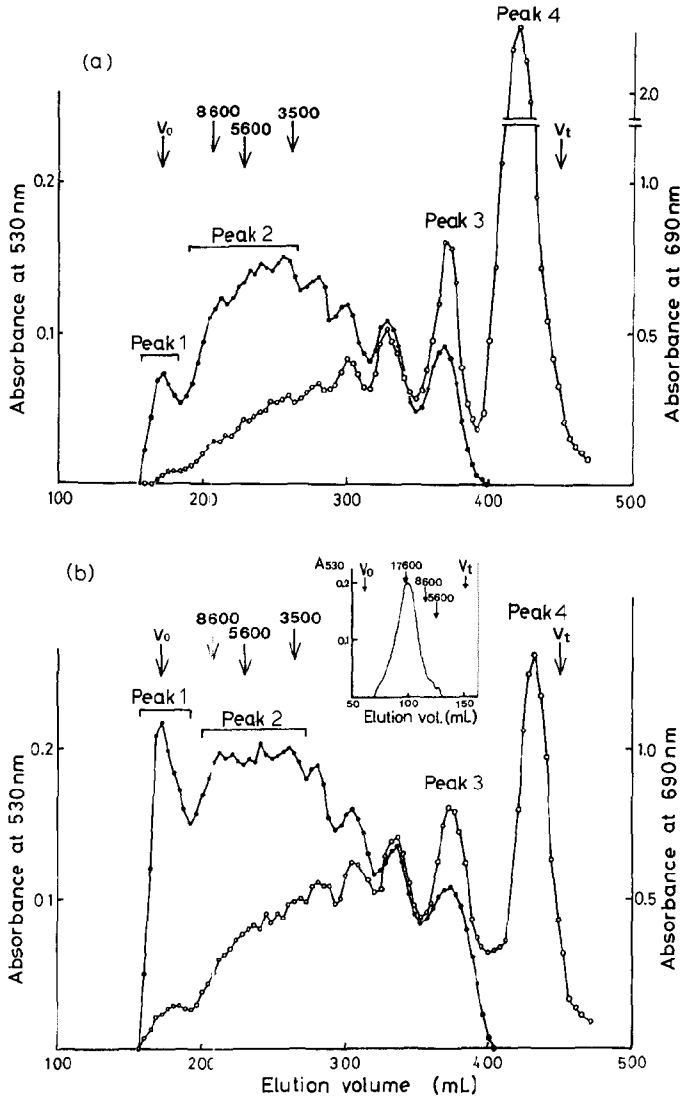

Fig. 2. Separation, on Sephadex G-50, of the products formed by periodate oxidation and alkali degradation of rooster-comb dermatan sulfates: (a) RC-20, and (b) RC-30. Each column was calibrated with Blue dextran (V_0), depolymerized chondroitin 6-sulfates (M_r 8600, 5600, and 3500), and 2M sodium chloride (V_t). The elution diagram, from Sephadex G-200, of Peak 1 material of RC-30 is shown in the inset of Fig. 2b. (—●—●—●—) A_{530nm}, and (—○—○—○—) A_{690nm}.

for 30 min at room temperature, made neutral with M hydrochloric acid, and applied to a Sephadex G-50 column (2.5 × 91 cm) prepared in 0.15M sodium chloride. The column was eluted with the same solvent at a flow rate of 20 mL/h. Each 4 mL-fraction was collected and analyzed for both uronic acid content and reducing power (Figs. 2a and b).

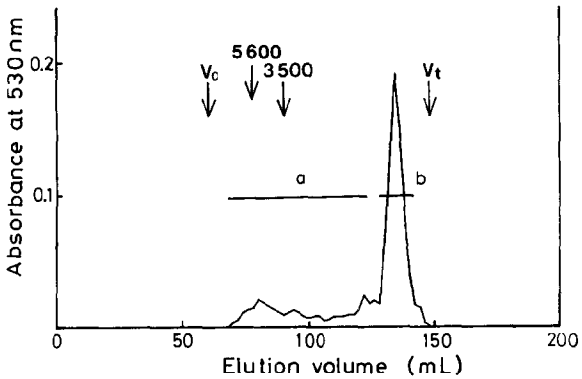

Fig. 3. Separation, on Sephadex G-50, of the digestion products, with chondroitinase AC-II, of the high-mol.-wt. fraction (Peak 1 of Fig. 2b) obtained by periodate oxidation of rooster-comb dermatan sulfate, RC-30. The column was calibrated with Blue dextran (V_0), depolymerized chondroitin 6-sulfates (M_r 5600 and 3500), and 2M sodium chloride (V_t). The horizontal bars indicate (a) higher oligo- and (b) di-saccharide areas.

Fractions constituting the peak near V_0 (Peak 1 in Fig. 2b; elution vol., 156–192 mL) were pooled, lyophilized, and desalted on a Sephadex G-25 column (2.5 × 80 cm) by elution with 10% ethanol, and the desalted solution (pH 6.5) evaporated at 35° to remove ethanol and lyophilized. The residue was dissolved in 0.15M sodium chloride (1 mL; 1 mg/mL) and chromatographed on a Sephadex G-200 column (1.5 × 83 cm), prepared in 0.15M sodium chloride and eluted with the same solvent. The column was calibrated with Blue dextran (V_0), depolymerized chondroitin 6-sulfates (M_r 17 600, 8600, and 5600), and 2M sodium chloride (V_t) (inset of Fig. 2b).

The ability of Peak 1 material to be degraded by chondroitinase AC-II was examined as follows. To a solution of this material (1 mg/0.1 mL of water) and enriched Tris buffer, pH 8.0 (0.05 mL), was added chondroitinase AC-II (5 units/0.1 mL of water), and the mixture incubated for 3 h at 37°. After being heated in boiling water for 10 min, the mixture was centrifuged, and the supernatant solution applied to a Sephadex G-50 column (1.5 × 86 cm) prepared in 0.15M sodium chloride. The column was eluted with the same solvent at a flow rate of 20 mL/h, and each fraction collected (0.5 mL) was analyzed for uronic acid content (Fig. 3).

Fractions corresponding to a broad area at the rear of Peak 1 (Peak 2 in Fig. 2b; elution vol., 200–274 mL) were pooled, and Peak 2 material was isolated as described previously. The materials of Peaks 1 and 2 (each 100 μg) were digested separately with chondroitinase ABC (0.2 unit in 20 μL of water) in enriched Tris buffer, pH 8.0 (30 μL), and with chondroitinase AC-II (0.5 unit in 20 μL of water) in 50mM acetate buffer, pH 6.0 (30 μL) for 3 h at 37°, respectively. After dilution of the reaction mixtures with 10mM hydrochloric acid to a final volume of 1.5 mL, the absorbance at 232 nm was measured, and the ratio between the values obtained from the two enzymic digests was calculated (Table I).

TABLE I

DEGRADATION WITH CHONDROITINASES AC-II AND ABC OF THE PRODUCTS OF PERIODATE OXIDATION OF RC-30[a]

Degradation with chondroitinase	*Peak*[b]	
	1	*2*
AC-II	0.476	0.515
ABC	0.670	0.678
Ratio of AC-II to ABC value	0.71	0.76

[a]Materials from Peaks 1 and 2 (see legend to Fig. 2b). [b]A_{232} for 100 μg of material oxidized.

RESULTS AND DISCUSSION

Fractions RC-20 and RC-30 were digested with chondroitinase AC-II according to the procedure of Saito *et al.*[8]. The gel-filtration diagrams, on Sephadex G-200 (Fig. 1a and 1b), of the digestion products showed a large peak (Peak 2; elution volume for RC-20, 145–160 mL; and for RC-30, 140–160 mL) of unsaturated disaccharide monosulfate, originating from the sequentially linked *N*-acetylchondrosine units, and a group of peaks differing in the degree of polymerization (elution vol. for RC-20, 60–145 mL; and for RC-30, 65–140 mL), which originate from the sequentially linked *N*-acetyldermosine units. The digestion products of RC-20 or RC-30 were fractionated on a preparative scale by Sephadex G-200 gel-filtration to isolate Peak-2 materials. Each of these was then subjected to analytical gel-filtration on Sephadex G-25 and paper chromatography, and was identified as ΔDi-4S (containing a negligible proportion of ΔDi-OS) (data not shown). The ratio of total peak area A_{232}, as measured by the elution diagrams Fig. 1a and 1b for RC-20 to that for RC-30, was found to be 0.56:1. Since the elution diagrams were obtained for the digestion products resulting from treatment of equal amounts of RC-20 and RC-30 with chondroitinase AC-II, the ratio (0.56:1) represents that of the contents of total *N*-acetylchondrosine units of RC-20 and RC-30. This result agrees well with the ratio[1] (0.55:1) of the D-glucuronic acid contents (22.6% for RC-20 and 41.1% for RC-30). Each of the A_{232} area of Peaks 2 of RC-20 and RC-30 (representing the total amounts of the unsaturated disaccharide from the sequentially linked *N*-acetylchondrosine units) corresponds to 87% of the total A_{232} peak areas, indicating a distribution of *N*-acetylchondrosine units very similar in RC-20 and RC-30; this ratio corresponds to the uronic acid composition. As indicated in the preceding paper[1], most of the *N*-acetylchondrosine units exist as clusters, as a cluster of 8 *N*-acetylchondrosine units between *N*-acetyldermosine units, corresponds to a content of unsaturated disaccharides and tetra- or higher oligosaccharides of 87.5 and 12.5%, respectively.

A small, distinct peak (Peak 1), near K_{av} 0.61 (elution vol., 114–126 mL), was observed for both Fractions RC-20 and RC-30 (Fig. 1a and 1b). In order to es-

timate the average M_r of Peak 1 material, Fraction RC-30 was digested with chondroitinase AC-II on a preparative scale, and Peak-1 material, isolated by gel-filtration of the digestion product, was digested with chondroitinase ABC. The ratio of the A_{232} value for the digestion product with chondroitinase AC-II to that with chondroitinase ABC was found to be 0.081–0.072:1. From this value, it was estimated that Peak-1 material contains, on average, 12–14 disaccharide units (11–13 *N*-acetyldermosine units plus 1 unsaturated disaccharide unit), assuming that the value of A_{232} due to the unsaturated uronic acid residue was not influenced by the length of the saccharide sequence linked to the residue*. From the average M_r of the RC-30 disaccharide unit (524), the M_r of Peak-1 material was calculated to be 6500–7300. This value agrees with the M_r range obtained by gel chromatography (Figs. 1a and 1b). Thus, Peak-1 material resulted from a polysaccharide chain having, on average, 11–13 sulfated *N*-acetyldermosine units between *N*-acetylchondrosine units. This structure seems characteristic for rooster-comb dermatan sulfate.

A large proportion of high-mol.-wt. products, in which no unsaturated uronic acid (A_{232}) was detected and which corresponds to 46% of the uronic acid area of the total digestion products, was eluted early (Fig. 1a; elution vol., 60–92 mL). Taking into consideration the sensitivity of the A_{232} measurement, it seems that most of the materials corresponding to this area derived from large *N*-acetyldermosine clusters containing nonreducing, terminal portions of RC-20. The M_r value estimated at >17 600 (>34 sulfated *N*-acetyldermosine units; Fig. 1a) suggests the presence of a few particularly large clusters having a size comparable to that of the original polysaccharide chain[1] of RC-20 (M_r ~41 000). Similarly, a material that may have derived from large *N*-acetyldermosine clusters was also observed for RC-30, but in far smaller proportions and derived probably from contaminating RC-20.

As expected, the proportions of tetra- or higher oligosaccharides (up to M_r ~17 600) carrying an unsaturated uronic acid residue (a relatively small proportion, as estimated from the A_{530} to A_{232} ratios) or not carrying the residue (relatively abundant) were small for RC-20 (40% of total uronic acid content) and large for RC-30 (59%) (see Fig. 1a, elution vol. 94–144 mL; and Fig. 1b, elution vol. 90–140 mL). These different proportions reflect the contents of D-glucuronic acid and are also related to the different chain structures at the nonreducing, terminal end.

Periodate oxidation of RC-20 and RC-30, followed by alkali degradation[9,10], gave fragments that were chromatographed on Sephadex G-50 (Figs. 2a, 2b). A substantial proportion of the fragments from RC-20 was included in the gel, and the smallest product arising from this degradation of the sequentially linked *N*-acetyldermosine monosulfate units [GalNAc-O-C(CHO)=CH-CO_2H] gave a large peak (Peak 4, Fig. 2a; elution vol. 400–460 mL). Peak 3 (Fig. 2a; elution vol. 350–390 mL) contained (GalNAc→UA)→GalNAc-O-C(CHO)=CH-CO_2H (UA:

*The method for measurement of the GlcA-to-IdoA ratio of dermatan sulfate using digestion with chondroitinases AC-II and ABC is based on this assumption.

GlcA or IdoA sulfate), which originated from D-glucosyluronic acid→GalNAc 4-sulfate or L-idosyluronic acid sulfate→GalNAc 4-sulfate units between monosulfated *N*-acetyldermosine units. The total amount of uronic acid in the peak areas of Fig. 2a corresponds to 33% of the uronic acid present in the starting fraction RC-20. This value is approximately equal to the sum[1] of D-glucuronic acid (23%) and ΔDi-diS (7%)[1]. A similar agreement between these corresponding values was observed for RC-30. Thus the disaccharide giving ΔDi-diS contains L-iduronic acid sulfate.

In RC-30, the formation of GalNAc-O-C(CHO)=CH-CO_2H (Peak 4, Fig. 2b) was less significant than in RC-20, but the proportion of high-molecular-weight products (Peaks 1 and 2; elution vol., 156–272 mL) was large, and the total uronic acid content of the Peak-1–3 area corresponded to 56% of the uronic acid present in starting RC-30 (in contrast to 33% for RC-20). In particular, Peak 1 of RC-30 (Fig. 2b), which contains the large-size fragments excluded from the Sephadex G-50 gel, was much greater than Peak 1 of RC-20 (Fig. 2a). The different elution diagrams for RC-30 and RC-20 results from the higher content in D-glucuronic acid (41%) and higher content in disaccharide disulfate (13%) in RC-30 than in RC-20 (23 and 7%, respectively), which suggests that RC-30 is rich in large-size clusters of sulfated (or nonsulfated) *N*-acetylchondrosine and *N*-acetyldermosine disulfate units.

The structure of the large-size clusters of RC-30 was elucidated by digesting, with chondroitinases AC-II and ABC, the fractions of higher M_r (Fig. 2b, Peak 1 and following broad Peak-2-fraction of M_r >3500) and by examining, on Sephadex G-50, the product of digestion of Peak 1 material with chondroitinase AC-II (see Table I and Fig. 3). The polysaccharide chain of these higher-molecular-weight products is constituted preponderantly of *N*-acetylchondrosine monosulfate units with several *N*-acetyldermosine disulfate units distributed within the chain.

For Peak 4 material of RC-20, the proportion of uronic acid, oxidized to give GalNAc-O-C(CHO)=CH-CO_2H, relative to the total uronic acid of the starting material is 67% (100 − 33%), which is approximately equal to the proportion of nonsulfated IdoA, 70% [total IdoA (77) − ΔDi-diS (7)]. For RC-30, these values are 44 (100 − 56) and 45% (59 − 14), nearly equal. Thus, it is highly likely that most *N*-acetyldermosine disulfate units that give ΔDi-diS are distributed within the *N*-acetylchondrosine sulfate clusters rather than within the *N*-acetyldermosine monosulfate clusters.

In summary, in agreement with our previous results[1], both RC-20 and RC-30 fractions have many *N*-acetylchondrosine or *N*-acetyldermosine clusters of various sizes, but they differ in the proportions of the uronic acids and in the content of disaccharide disulfate units. In addition, the present work, has shown: (a) RC-20, but not RC-30, contains many *N*-acetyldermosine sulfate clusters of a large size (M_r ~17 600 or more) at the nonreducing, terminal end. (b) Both fractions have an *N*-acetyldermosine sulfate cluster of M_r 6500–7300 within the chain. This seems characteristic of rooster-comb dermatan sulfate. (c) RC-30 contains more D-

glucuronic acid than RC-20. In both fractions, most *N*-acetylchondrosine sulfate units form clusters. Several of these have a large size (M_r ~17 600, ~34 disaccharide sulfate units) in RC-30, but not in RC-20. In both fractions, many of the *N*-acetylchondrosine sulfate clusters have a M_r of ~3500–9000. (d) In RC-30, several *N*-acetyldermosine disulfate units (13%) may be linked to *N*-acetylchondrosine sulfate units or clusters, suggesting that, in both fractions, most *N*-acetylchondrosine sulfate clusters having a large molecular size exist as a copolymer with *N*-acetyldermosine disulfate units.

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