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DETERMINATION OF THE DISTRIBUTION OF D-GLUCURONIC ACID UNITS WITHIN THE CHAIN OF PIG-SKIN DERMATAN SULFATE NEAR THE LINKAGE REGION

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ABSTRACT

A method for analyzing the distribution of D-glucuronic acid units within the chain and near the linkage region of dermatan sulfate has been developed. The method consists of (a) a chemical modification of the reducing terminal residue in the polysaccharide by reductive amination with excess 1,2-diaminoethane in the presence of sodium cyanoborohydride, (b) desulfative fragmentation of the polysaccharide, labeled with 2-aminoethylamino (AEA) groups, in hot dimethyl sulfoxide containing 10% of water followed by 2,4-dinitrophenylation of the 2-aminoethylamino group, (c) separation of the 2-(2,4-dinitrophenylamino)ethylamino labeled dermatan fragments from nonlabeled fragments on Octyl-Sepharose CL-4B gel, and (d) determination of the uronic acid composition of the labeled fragments having various chain-length. A preparation of pig-skin dermatan sulfate (M_r 21 000, ratio of GlcA to total uronic acid, 93:500) showed an average distribution pattern of D-glucuronic acid residues near the linkage region of one *N*-acetylchondrosine unit in the disaccharide sequence 1–5(6) linked to the Xyl→Gal→Gal→GlcA residue, a cluster of 6–8 *N*-acetyldermosine units in the sequence 6(7)–12(13), and four separate *N*-acetylchondrosine units between the sequence adjacent to the *N*-acetyldermosine cluster and the sequence 23 or higher.

INTRODUCTION

The copolymeric structure of pig-skin dermatan sulfate has been successfully studied by fragmentation of the polysaccharide chain by chemical reaction, such as periodate oxidation followed by alkaline cleavage, in combination with chondroitinases or testicular hyaluronidase[1]. Recently, we have reported studies of the structural characteristics of rooster-comb dermatan sulfates by desulfation and depolymerization of the polysaccharide under solvolytic conditions, and fragmenta-

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tion by digestion with chondroitinase AC-II, together with the periodate oxidation procedure[2,3]

We have now devised a method of chemical analysis for the primary structure of complex glycosaminoglycuronans which consists of several procedures based on our previous work[4,5]. A preparation of pig-skin dermatan sulfate (M_r 21 000, GlcA-to-total uronic acid ratio, 93:500) was analyzed by this method for the D-glucuronic acid distribution within the chain near the linkage region, and the results obtained are reported herein.

EXPERIMENTAL

Materials. — A preparation of pig-skin dermatan sulfate, prepared by the procedure similar to the method described previously[2], was donated by Seikagaku Kogyo Co. Ltd. (Tokyo). The pig-skin dermatan sulfate was fractionated with ethanol as calcium salt according to the method of Meyer *et al.*[6]. The 20%-ethanol fraction was purified further by gel filtration on Sepharose 6B. The preparation thus obtained was subjected to chemical and enzymic analysis (Table I).

Standard chondroitins (sodium salt) having different M_r values (2700, 5000, 8000, and 11 000) were described previously[7]. Chondroitinase AC-II from *Arthrobacter aurescens* and chondroitinase ABC from *Proteus vulgaris* were obtained from Seikagaku Kogyo Co. Ltd. Sodium cyanoborohydride was purchased from Aldrich Chem. Co. Inc. (Milwaukee, WI) and was used without further purification.

Analytical methods. — Enzymic determination of uronic acid composition in dermatan samples was carried out as previously described[2,8]. The uronic acid content was determined by a modified method of Bitter and Muir[9]. Analytical gel-chromatography on Sephadex G-25, G-75, or G-100 was carried out by the procedure described previously[7]. Each column was calibrated with Blue dextran (V_0), chondroitin standards (M_r 2700, 5000, 8000, and 11 000), and 2M sodium chloride (V_t).

Chemical determination of uronic acid composition in pig-skin dermatan sulfate and its 2-aminoethylamino derivative. — (a) Hydrolysis of the polysaccharide materials into a mixture of constitutional mono- and di-saccharides with dimethyl sulfoxide containing 10% of water. A solution of the sample (~6 mg/0.4 mL of water) was passed through a column of Dowex 50W-X2 (H^+, 50–100 mesh) at 0–4°. The eluent and washings were combined, made neutral (pH 6.0) by the addition of pyridine, and lyophilized to give the pyridinium salt as a white powder. A solution of the pyridinium salt (~6 mg) in dimethyl sulfoxide containing 10% of water (1.5 mL) was heated in a Pyrex test tube (0.7 × 10 cm) with a Teflon-lined screw-cap under stirring with a Teflon stirrer (0.5 cm in diameter) for 30 h at 108 ±1°. After being cooled in an ice bath, the content of the test tube was diluted with an equal volume of water and transferred into a distillation flask (20 mL in volume) and made neutral (pH 6.0) with 0.1M NaOH. The solution was evaporated to dryness at 50–55° under reduced pressure.

(b) Separation, on Sephadex G-25 gel and AG 1-X4 anion-exchange resin, of the hydrolyzate into N*-acetyldermosine,* N*-acetylchondrosine,* L*-iduronic acid, and* D*-glucuronic acid, and determination of the ratio of* D*-glucuronic acid to total uronic acid.* The hydrolyzate just described was dissolved in 0.1M NH_4HCO_3 (0.5 mL) and loaded onto a column (1.5 × 85.5 cm) of Sephadex G-25 prepared in the same solvent. The column was eluted at 20–25° with the same solvent at a flow rate of 34 mL/h. Each fraction (2 mL) was analyzed for uronic acid. The pooled fractions corresponding to the disaccharide and monosaccharide peaks of the elution diagram were combined and lyophilized. The residue was dissolved in water (0.5 mL) and loaded onto a column (1.0 × 85.5 cm) of AG 1-X4 (HCO_3^-, 200–400 mesh) prepared in water. The column was eluted at 40° with 0.2M formic acid at a flow rate of 24 mL/h. Each fraction (3.9 mL) was analyzed for uronic acid. The sum of the peak areas corresponding to *N*-acetylchondrosine and D-glucuronic acid in each elution diagram provided an estimated value of the content of D-glucuronic acid, and the sum of the peak areas corresponding to *N*-acetyldermosine and L-iduronic acid provided that of L-iduronic acid.

Determination of sugar component at the reducing terminal of pig-skin dermatan sulfate and its 2-aminoethylamino (AEA) derivative. — The determination was performed according to the procedure described by Yamaguchi[10]. A solution of the sample (~10 mg) in 0.3M NaOH (2.5 mL) containing $NaBH_4$ (50 mg) was kept at 50° for 5 h. After decomposition of excess $NaBH_4$ by the addition of 2M acetic acid (1.75 mL), the mixture was made neutral with 0.1M NaOH. It was dialyzed against distilled water (3 × 10 L) for 18 h at 20°, and then lyophilized. Both the reduced starting dermatan sulfate (8.86 mg) and the reduced AEA-dermatan sulfate (~8.88 mg) gave a negative Somogyi–Nelson test[11]. A solution of the reduced sample (6 mg) in 0.5M H_2SO_4 (2 mL) was heated at 100° for 6 h, and then cooled. An internal standard solution (0.1 mL) containing 2-deoxy-D-glucitol (50 μg) was added, and the solution was made neutral with M $Ba(OH)_2$ and then with $BaCO_3$. After centrifugation, the supernatant was concentrated to a small volume, which was loaded onto a column (0.6 × 3.5 cm) of Dowex 50W-X8 (H^+, 200–400 mesh) cation-exchange resin. The column was eluted with water (4 mL) and the eluate was evaporated to dryness. The residue was dissolved in methanol (1 mL), the solution kept at 50° for 15 min and then evaporated to dryness. This operation (dissolution in methanol and evaporation to dryness) was repeated twice. The residue was dissolved in water (1 mL) and was immediately loaded onto a column (0.6 × 3.5 cm) of Dowex 1-X8 (HO^-, 200–400 mesh) anion-exchange resin. The column was eluted with water (6 mL). The eluate containing alditols was concentrated to a small volume and then loaded again onto a column (0.6 × 3.5 cm) of Dowex 50W-X8 (H^+) cation-exchange resin as described above. The eluate was evaporated to dryness in a glass tube (1.0 × 6 cm) with a Teflon-lined screw-cap, and the residue dissolved in pyridine (0.1 mL) and acetic anhydride (0.1 mL). The solution was heated at 100° for 2 h. The acetylated alditols thus obtained were analyzed by g.l.c. on a glass column (0.4 × 200 cm) of 3% ECNSS-M on Gas

Chrom Q (100–200 mesh) at 200° (column temperature), under N_2 as the carrier gas (60 mL/min), by use of a Shimadzu GC-4BM gas chromatograph equipped with a hydrogen-flame-ionization detector.

The amount of sugar component at the reducing terminal of the samples was calculated from the amount of the acetylated alditols determined by g.l.c. The starting dermatan sulfate (Na salt) contained 30.6 μmol of D-xylose and 1.9 μmol of D-galactose per g, and the AEA-dermatan sulfate (Na salt) 8.9 μmol of D-xylose and 0.6 μmol of D-galactose per g. The ratio (D-xylose of starting dermatan sulfate − D-xylose of AEA-dermatan sulfate)/(D-xylose of starting dermatan sulfate) indicates the degree of 2-aminoethylamination of the sugar component (D-xylose) at the reducing terminal of AEA-dermatan sulfate. The ratio of 0.71 calculated from the data just given and the value of 0.778 observed by the trinitrophenylation method are close, especially when experimental errors in the determination of reducing sugar components are taken into consideration.

As indicated earlier, a small proportion of D-galactose (1.9 and 0.6 μmol/g of starting dermatan sulfate and AEA-dermatan sulfate, respectively) was unexpectedly observed as another sugar component located at the reducing end. This sugar resulted probably from the procedures, such as Pronase digestion or alkaline treatment, used for preparing the dermatan sulfate chains from the parent proteoglycan. The ratio of 0.68, calculated from the proportion of D-galactose present in both samples, was also approximately close to the value obtained by the trinitrophenylation method.

Reductive amination of the reducing terminal residue of pig-skin dermatan sulfate with 1,2-diaminoethane in the presence of sodium cyanoborohydride. — The starting dermatan sulfate (Table I, 300 mg) was dissolved in a 12% 1,2-diaminoethane solution in 0.2M K_2HPO_4 (pH 9.0) (6 mL) and the solution was treated for 17 days at 40° in the presence of sodium cyanoborohydride (370 mg). The mixture was dialyzed against distilled water (5 × 10 L) for 48 h at 25°, and the dialyzate concentrated (~10 mL) *in vacuo* at 45°. The solution was passed through a column (1.5 × 25 cm) of Dowex 50W-X2 (H^+, 50–100 mesh) cation-exchange resin at 4°, and the eluent made neutral with M NaOH and lyophilized to give the sodium salt of 2-aminoethylaminodermatan (AEA-dermatan) sulfate (280 mg).

The AEA content of the AEA-dermatan sulfate was determined according to the following procedure. A solution of the sample (sodium salt, ~8 mg) in 0.5M phosphate buffer (pH 8.0, 1 mL) was treated with 0.2% 2,4,6-trinitrobenzenesulfonic acid in water (1 mL) for 15 h at 40°. The mixture was deposited on a Sephadex G-25 column (1.5 × 6 cm) prepared in 10% ethanol. The column was eluted with 10% ethanol and the yellow eluent emerging near the void volume was collected, evaporated, and lyophilized. A part of the residue (~1 mg) was dissolved in 4% $NaHCO_3$ and the absorbancy at 348 nm measured. 2-Aminoethylamination of the AEA-dermatan sulfate was estimated by use of the ε value (1.54×10^4 at 3348 nm) of standard TNP-glycine, and expressed as the number of TNP groups/mol of the starting dermatan sulfate (M_r 21 000). The degree of 2-aminoethyl-

amination of the AEA-dermatan sulfate obtained was 0.778.

Desulfative fragmentation of AEA-dermatan sulfate with dimethyl sulfoxide containing 10% of water. — A solution of AEA-dermatan sulfate (sodium salt, 100 mg) in water (5 mL) was passed through a column (1.5 × 10 cm) of Dowex 50W-X2 (H^+, 50–100 mesh) cation-exchange resin at 4°, and the eluent was made neutral by addition of pyridine and lyophilized to give the pyridinium salt of AEA-dermatan sulfate (100 mg). Both complete desulfation and limited depolymerization of the pyridinium salt thus obtained (100 mg) were carried out in dimethyl sulfoxide containing 10% of water (25 mL) for 4 h at 90° by the procedure previously reported[4], and a mixture of nonsulfated dermatan fragments with and without the AEA group was obtained as sodium salt (82 mg). The M_r range of the product was estimated to be 1600–10 000 by analytical gel-filtration on a Sephadex G-100 column calibrated with standard chondroitins (Fig. 1).

2,4-Dinitrophenylation of AEA-dermatan fragments. — A solution of AEA-dermatan fragments (72 mg) in 0.5M carbonate buffer (pH 9.0, 10 mL) was treated with 20% 2,4-dinitrofluorobenzene in methanol (10 mL) at 20–25°, for 15 h in the dark, to convert the AEA group into a 2,4-dinitrophenyl-AEA (DNP-AEA) group. The mixture was evaporated *in vacuo* to a small volume (~2 mL), and deposited onto a Sephadex G-25 column (1.5 × 20 cm) prepared in 10% ethanol. The column was eluted with 10% ethanol and the yellow eluent emerging near the void volume was collected, evaporated, and lyophilized to afford the DNP-AEA product as sodium salt (64 mg). A part of the DNP-AEA product was subjected to analytical gel-filtration on Sephadex G-100 (Fig. 1).

Separation of the DNP-AEA-labeled dermatan fragments from unlabeled fragments on Octyl-Sepharose CL-4B. — A solution of the DNP-AEA product (60 mg) in 4M $(NH_4)_2SO_4$ in 50mM phosphate buffer (pH 6.0, 10 mL) was deposited on a column (1.5 × 25 cm) of Octyl-Sepharose CL-4B prepared in the same solution. The column was eluted successively with 4M $(NH_4)_2SO_4$ in 50mM phosphate buffer (pH 6.0, 300 mL) and water (200 mL) at 25°. The flow rate was 34 mL/h and 10-mL fractions were collected. An aliquot (25 μL) of each fraction was analyzed for uronic acid (A_{530}) and DNP-AEA group (A_{365}) (Fig. 2). The pooled fractions eluted with 4M $(NH_4)_2SO_4$ in 50mM phosphate buffer (pH 6.0) and with water were collected, respectively, and each was dialyzed against distilled water (3 × 10 L) for 24 h at 25° and lyophilized. The yields of DNP-AEA-labeled dermatan fragments and unlabeled fragments were 26 and 24 mg, respectively, and the K_{av} values, determined by analytical gel-filtration on Sephadex G-100, 0.53 and 0.55, respectively.

Fractionation of DNP-AEA-dermatan fragments by gel-filtration on Sephadex G-100, and determination of uronic acid composition and average molecular weight of the fractions separated. — A solution of DNP-AEA-labeled dermatan fragments (25 mg) in 0.1M NH_4HCO_3 (4 mL) was deposited on a Sephadex G-100 column (2.6 × 84 cm) prepared in 0.1M NH_4HCO_3. The column was eluted at 25° with the same solvent at a flow rate of 42 mL/h. The eluent was collected in 5.3-mL fractions and each fraction analyzed for uronic acid and DNP-AEA group contents; the ratio of

DNP-AEA group content (A_{365}) to uronic acid content (A_{530}) was calculated and plotted (Fig. 3). The elution diagram based on A_{365} determination was divided into ten fractions (Fractions 1–10) of equal peak-area, and each of the fractions was lyophilized. Average M_r values of the DNP-AEA-dermatan fragments thus obtained were estimated by separate analytical gel-filtration on Sephadex G-100 for Fractions 1–4, G-75 for Fractions 5–9, and G-25 for Fraction 10, respectively, using the chondroitin standards. The ratios of D-glucuronic acid content to total uronic acid content in the fractions (except Fraction 10) were determined by the enzymic procedure. The uronic acid composition of Fraction 10 could not be determined because of its minute amount and contamination with inorganic salts. The data obtained are summarized in Table III.

RESULTS AND DISCUSSION

The pig-skin dermatan sulfate prepared by a known procedure, including Pronase digestion (pH 7.0–8.0, 12 h, 50°) and alkali treatment[2] (0.5M sodium hydroxide, 90 min, 37°), was further purified by a series of conventional fractionation procedures. As shown in Table I, the purified material gave an M_r value of 21 000 by the reducing-power measurement[11], and this value corresponded well to its elution data on Sepharose 6B gel (elution diagram not shown), indicating that the dermatan sulfate chains had been completely released from the parent proteoglycan.

A preliminary examination of the reaction conditions for 2-aminoethylamination of the dermatan sulfate was carried out with chondroitin 6-sulfate as a model substance. From the reaction temperatures (20–60°), pH conditions (6.0–9.0), and reaction periods (1–17 days) tested, an appropriate set of conditions (40°, pH 9.0,

TABLE I

ANALYTICAL DATA OF PIG-SKIN DERMATAN SULFATE SODIUM SALT

M_r	21 000[a]
K_{av} on Sepharose 6B	0.47
Ratio of D-glucuronic acid to total uronic acid (%)	18.6
S (mol)[b]	1.08
Composition of chondroitinase ABC digestion products (%)[c]	
ΔDi	0.8
ΔDi-4S	85.7
ΔDi-6S	5.3
ΔDi-diS	8.0

[a]Determined by reducing-power measurement using the Somogyi–Nelson method[11]. [b]Ratio relative to disaccharide unit. [c]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)-D-galactose 4-sulfate; ΔDi-6S, 2-acetamido-2-deoxy-3-*O*-(α-L-*threo*-4-enopyranosyluronic acid)-D-galactose 6-sulfate; ΔDi-diS, 2-acetamido-2-deoxy-3-*O*-[α-L-*threo*-2-(or 3-)-4-enopyranosyluronic acid sulfate]-D-galactose 4-sulfate.

TABLE II

RATIOS OF D-GLUCURONIC ACID CONTENT TO TOTAL URONIC ACID CONTENT IN PIG-SKIN DERMATAN SULFATE AND ITS DERIVATIVES, ASSAYED BY BOTH ENZYMIC AND CHEMICAL METHODS

Sample	*Ratio of D-glucuronic acid to total uronic acid (%)*	
	Enzymic method	*Chemical method*
Starting pig-skin dermatan sulfate	18.6	19.1
AEA-dermatan sulfate[a]	19.1	21.6
DNP-AEA-dermatan fragments[a]	20.1	[b]

[a]Abbreviations: AEA and DNP-AEA, 2-aminoethylamino- and 2,4-dinitrophenylaminoethylamino-, respectively. [b]Not determined.

17 days) was selected for the preparation of the derivative of pig-skin dermatan sulfate. The polysaccharide was treated with a large excess of 1,2-diaminoethane at pH 9.0 for 17 days in the presence of sodium cyanoborohydride[12,13] and the derivative was isolated as the sodium salt. The degree of 2-aminoethylamination was found to be 77.8% by the trinitrophenylation method. In order to confirm that the reducing terminal residue of the dermatan sulfate had undergone exclusively 2-aminoethylamination under the reaction conditions used, the polysaccharide and its derivative were analyzed for the sugar component at the reducing end by sodium borohydride reduction and hydrolysis. After separation, the unlabeled D-xylose residue at the reducing terminal of each sample was recovered as D-xylitol, determined by g.l.c.*. The degree of 2-aminoethylamination of the AEA derivative, calculated from the amount of recovered D-xylose, was 71%, which corresponded approximately to the value (78%) measured by the trinitrophenylation method. From these results, it was estimated that the sugar component at the reducing terminal of the polysaccharide almost exclusively underwent 2-aminoethylamination.

The results of the enzymic assay for uronic acid composition, which is one of the procedures essential for the present study, has been demonstrated by us (unpublished) to be influenced by various factors specific for enzymic reactions, such as variance in the structure of substrates (the existence of various dermatan sulfate–chondroitin sulfate copolymers) or in the enzyme source (chondroitinase AC-I from *Flavobacterium* and AC-II from *Arthrobacter*). Accordingly, the data for uronic acid composition in the starting polysaccharides, especially the synthetically modified samples, were supported by another method. As shown in Table II, the determinations of the uronic acid composition of the starting dermatan sulfate and its AEA derivative gave approximately the same results by both enzymic and chemical methods. Furthermore, the values of the uronic acid composition of the DNP-AEA-dermatan fragments determined by the enzymic method were similar

*G.l.c. analysis of the starting dermatan sulfate and its AEA derivative indicated the presence of D-galactose besides the D-xylose residue as the reducing residues (1.9 and 0.6 μmol/g, respectively).

TABLE III

MOLECULAR WEIGHT AND URONIC ACID COMPOSITION OF DNP-AEA-DERMATAN FRAGMENTS HAVING DIFFERENT CHAIN-LENGTHS

Fraction	M_r	*D.p.*$_{av}$	D-*Glucuronic acid/ Total uronic acid (%)*
1	10000 (9120)[a]	23[b]	19.3 (4.44)[c]
2	8000 (7120)	18	19.7 (3.55)
3	6600 (5720)	14	13.8 (1.93)
4	4500 (3620)	9	12.1 (1.09)
5	3600 (2720)	7	13.5 (0.95)
6	2800 (1920)	5	19.6 (0.98)
7	2400 (1520)	4	21.7 (0.87)
8	1900 (1020)	3	22.3 (0.67)
9	1600 (720)	2	26.5 (0.53)
10	~900	0	

[a]In parentheses, less 880 [molecular weight of GlcA-Gal-Gal-Xylityl-$NHCH_2CH_2NH$-$C_6H_3(NO_2)_2$]. [b]Number of repeating disaccharide unit. [c]In parentheses, number of D-glucuronic acid residues, calculated from the ratio D-glucuronic acid/total uronic acid (%) and d.p.$_{av}$.

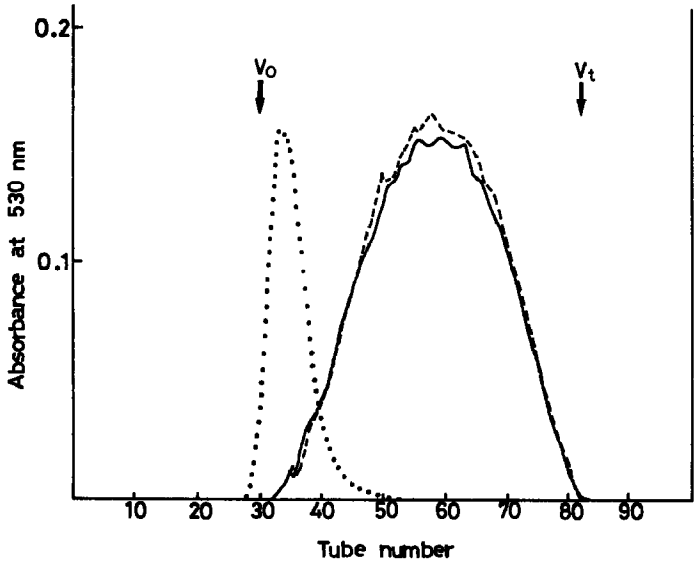


Fig. 1. Elution diagrams of pig-skin dermatan sulfate and its derivatives by gel-filtration on Sephadex G-100. The pig-skin dermatan sulfate (0.6 mg), AEA-dermatan fragments (1.4 mg), and DNP-AEA-dermatan fragments (1.5 mg) were separately deposited on a Sephadex G-100 column (1.5 × 95 cm) prepared in 0.2M NaCl. The column was eluted at 25° with the same solvent at a flow rate of 29 mL/h. The eluent was collected in 2.0-mL fractions, and each fraction was analyzed for uronic acid: Pig-skin dermatan sulfate (● ● ● ●); AEA-dermatan fragments (——); and DNP-AEA-dermatan fragments (-----).

to those of the starting dermatan sulfate and its AEA derivative. The values (not shown) for the smaller DNP-AEA-dermatan fragments (Fractions 1–9 in Table III) were in good correlation with those estimated by the carbazole–orcinol ratio.

As separation of DNP-AEA-labeled from unlabeled compounds as dermatan sulfate was not perfect, separation of both compounds as dermatan fragment was investigated. Thus, the pyridinium salt of AEA-dermatan sulfate, which consists of 78% of AEA-labeled and 22% of unlabeled dermatan sulfate, was completely desulfated and partially depolymerized in dimethyl sulfoxide containing 10% of water[4] to give a mixture of nonsulfated dermatan fragments, with and without the AEA group, having a mol. wt. range of 1600–10 000 (Fig. 1). The dermatan fragments having the AEA group consist solely of compounds containing the linkage region. Those without the AEA group are expected to consist mainly of the fragments originating from the peripheral portion of both polysaccharides with and without the AEA group, in addition to the fragments containing the linkage region originating from the polysaccharide without the AEA group (22%).

The mixture of dermatan fragments with and without the AEA group was treated with 2,4-dinitrofluorobenzene to convert the AEA group into the 2,4-dinitrophenyl-AEA (DNP-AEA) group. As shown in Fig. 1, the elution diagram of the DNP-AEA compound was nearly identical with that of the AEA-dermatan fragments, indicating that dinitrophenylation of the AEA group had not affected the gel-filtration properties of the dermatan fragments. Furthermore, the uronic acid composition of the DNP-AEA compound (a mixture of DNP-AEA-labeled

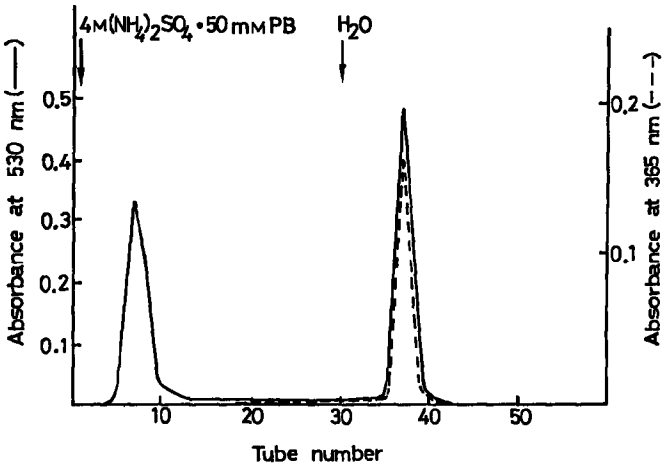


Fig. 2. Separation of DNP-AEA-dermatan fragments from non-labeled fragment on Octyl-Sepharose CL-4B. The DNP-AEA product (60 mg) was deposited on an Octyl-Sepharose CL-4B column (1.5 × 25 cm) prepared in 4M $(NH_4)_2SO_4$ in 50mM phosphate buffer (pH 6.0). The column was eluted at 25° successively with the same solvent (0.3 L) and water (0.2 L) at a flow rate of 34 mL/h. The eluent was collected in 10-mL fractions and each fraction analyzed for uronic acid (A_{530}) and DNP-AEA group (A_{365}).

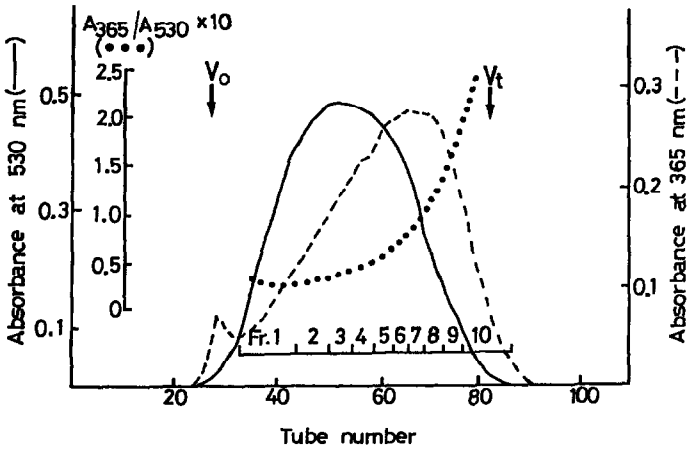


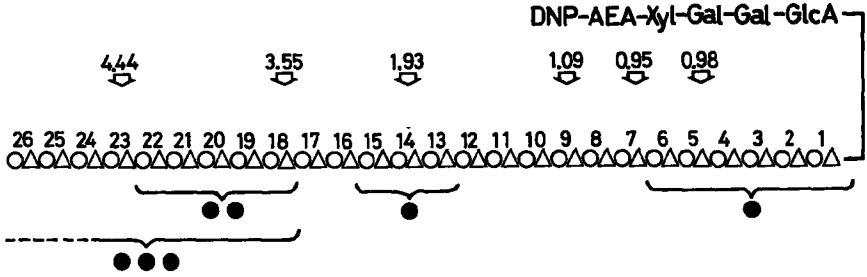
Fig. 3. Gel-filtration of DNP-AEA-dermatan fragments on Sephadex G-100. The DNP-AEA-dermatan fragments (25 mg) were deposited on a Sephadex G-100 column (2.6 × 84 cm) prepared in 0.1M NH_4HCO_3. The column was eluted at 25° with the same solvent at a flow rate of 42 mL/h. The eluent was collected in 5.3-mL fractions, and each fraction was analyzed for uronic acid and DNP-AEA group contents. The ratio of DNP-AEA group content (A_{365}) to uronic acid content (A_{530}) was calculated. The elution diagram (------) based on A_{365} determination was divided into ten fractions (Fr. 1–10) of equal peak area.

and -unlabeled dermatan fragments), determined by the enzymic method was approximately identical with that of the starting dermatan sulfate and its AEA derivative within the limits of experimental errors (Table II). This suggests that the enzymic method of determination is not affected by the presence of DNP-AEA groups in dermatan fragments.

We have reported[5] previously the separation of fluorescein-labeled species from -unlabeled heparins with Octyl-Sepharose CL-4B, and this procedure was successfully employed to separate the DNP-AEA-containing dermatan fragments from the compounds without a DNP-AEA group (Fig. 2). The DNP-AEA-dermatan fragment was further subjected to gel-filtration on Sephadex G-100 and the eluted products were divided into ten equal fractions (Fig. 3). The ratio of D-glucuronic acid to total uronic acid (%) of these fractions (Fraction 1–9, Table III) was determined by the enzymic method, and the values obtained were in good correlation with those estimated by the carbazole–orcinol ratio (data not shown). The average M_r value of each fraction was estimated by analytical gel-filtration.

On the basis of these data (Table III), the average distribution of the D-glucuronic acid residue within the polysaccharide chain near the linkage region was assigned as follows. The D-glucuronic acid content of the fractions having a $d.p._{av}$ 2–5 suggests a location of the first *N*-acetylchondrosine unit in the area of the disaccharide sequence 1–5 or 6. The change in D-glucuronic acid content for the fractions having $d.p._{av}$ 5, 7, 9, and 14 suggests a limited presence of D-glucuronic acid residues in the sequence 6 or 7–12 or 13. Furthermore, it is likely that a cluster

of 6–8 *N*-acetyldermosine units is present in this area. The mode of distribution of the *N*-acetylchondrosine units within the further peripheral area of the polysaccharide chain (the sequence 13 or 14–23 or higher) seems to be as follows. The data of the fractions having $d.p._{av}$ 9, 14, and 18 suggest that the 2nd *N*-acetylchondrosine unit and two separate or adjacent *N*-acetylchondrosine units are located near the sequences 14 and 20, respectively. The data of the fraction having $d.p._{av}$ 23 also suggest the presence of a fifth *N*-acetylchondrosine unit in the area of the sequence 23 or higher. These conclusions on the average distribution of the D-glucuronic acid residues within the polysaccharide chain near the linkage region are illustrated in Scheme 1. The dermatan fragments having $d.p._{av}$ 23 correspond to 56% of the whole polysaccharide chain of the starting dermatan sulfate.



Scheme 1. Distribution of D-glucuronic acid residue within the polysaccharide chain near the linkage region of pig-skin dermatan sulfate. The numerical value above the arrow sign indicates the number of D-glucuronic acid residues: (△) GalNAc, (○) IdoA, and (●) GlcA.

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