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DEPOLYMERIZATION OF GLYCOSAMINOGLYCURONANS INTO DI- AND HIGHER MOLECULAR-WEIGHT OLIGO-SACCHARIDES: IMPROVED PREPARATION OF *N*-ACETYLDERMOSINE AND OLIGOMERIC *N*-ACETYLCHONDROSINES

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ABSTRACT

Nonsulfated di- to octadeca-saccharides having 2-acetamido-2-deoxy-D-galactose at the reducing end were prepared, in 81% yield, by treatment of chondroitin 6-sulfate (pyridinium salt) with dimethyl sulfoxide containing 10% of water for 14 h at 90°. *N*-Acetylchondrosine and *N*-acetyldermosine were obtained from dermatan sulfate of rooster comb, in 30% and 38% yields, respectively, by solvolysis with dimethyl sulfoxide, containing 10% of water, for 30 h at 105°. Hyaluronic acid was also depolymerized by the same solvent in the presence of an equimolar amount of pyridinium sulfate or chloride per disaccharide unit to give reducing di- and higher molecular weight oligo-saccharides. The results of solvolytic desulfation and depolymerization are compared with those of the conventional methods by acid hydrolysis.

INTRODUCTION

Chemical methods for the preparation of repeating di- and oligo-saccharides from glycosaminoglycuronans are acid hydrolysis[1–4], and methanolysis[5]. The hydrolysis of chondroitin 4-sulfate with 40mM hydrochloric acid for 4 h at 100° gives a mixture of di-, tetra-, hexa-, and higher molecular-weight oligo-saccharides, which are partially deacetylated and desulfated[1]. Chondrosine is prepared from chondroitin sulfates by hydrolysis with M sulfuric acid for 4 h at 100° in 67% yield[2]. Dermatan sulfate is generally more labile to acid than are the chondroitine sulfates and is hydrolyzed to give nearly equal amounts of deacetylated, desulfated mono- and oligo-saccharides by heating with M hydrochloric acid for 1 h in a boiling-water bath[3].

In a previous paper[6], we reported that solvolysis of glycosaminoglycuronans, such as chondroitin 4- and 6-sulfates, dermatan sulfate, and hyaluronic acid, consisting of β-linked 2-acetamido-2-deoxy-D-hexopyranosyl and D-glucopyranosyl,

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with dimethyl sulfoxide containing 10% of methanol resulted in the cleavage of the sulfate groups and the 2-acetamido-2-deoxy-D-glycopyranosyl bonds to give methyl β-D-glycosides of *N*-acetylated di- and higher molecular weight oligo-saccharides. The present report describes the results of depolymerization of sulfated or non-sulfated glycosaminoglycuronans by heating in dimethyl sulfoxide containing water, and an improved method for the preparation of a series of reducing oligosaccharides from the polysaccharides.

EXPERIMENTAL

Materials. — The glycosaminoglycuronans used in this study have been described previously[6], except sodium dermatan sulfate of pig skin, which was obtained from Seikagaku Kogyo Co., Tokyo. Standard 1,2-isopropylidene-L-iduronolactone was a gift from Prof. T. Kinoshita of this school, and D-glucurono-6,3-lactone was a product of Sigma Chemical Co. (St. Louis, MO 63178). *N*-Acetylchondrosine was prepared by acetylation of chondrosine according to the method of Danishefsky *et al.*[7]. The free acids of chondroitin 6-sulfate and hyaluronic acid were prepared by the method described in our previous paper[6].

Analytical methods. — Uronic acid was determined by the method of Bitter–Muir, modified by increasing the borate concentration to 0.2M, and using D-glucurono-6,3-lactone and 1.2-isopropylidene-L-iduronolactone as standards[8], by the method of Dische[9], and by the orcinol method using a 20-min boiling time[10]. 2-Amino-2-deoxyhexose was determined by the method of Antonopoulos[11] after hydrolysis with 3M hydrochloric acid for 15 h at 100°, 2-acetamido-2-deoxyhexose reducing end-group according to the method of Reissig *et al.*[12], the reducing value by the method of Park and Johnson[13], and sulfate groups by the method of Dodgson and Price[14]. Reduction of the oligosaccharides with sodium borohydride was carried out as described by Linker *et al.*[15]. Paper electrophoresis was performed as described previously[6], and t.l.c. on cellulose plate in (*A*) 2:1:1 (v/v) 1-butanol–acetic acid–water and (*B*) 5:3 (v/v) 1-butyric acid–0.5M ammonium hydroxide; the papers and t.l.c. cellulose plates were stained with the *p*-aminohippuric acid reagent[16]. Analytical gel chromatography on Sephadex G-25 was carried out as described previously[6]. The yields of *N*-acetylchondrosine oligomers obtained from chondroitin 6-sulfate, and of *N*-acetylchondrosine and *N*-acetyldermosine obtained from dermatan sulfate were calculated, based on the observed value of 2-amino-2-deoxy-D-hexose for the pyridinium salt of the respective polysaccharide.

Depolymerization of chondroitin 6-sulfate and dermatan sulfate of rooster comb and of pig skin with dimethyl sulfoxide containing 10% of water. — A solution of the pyridinium salt of chondroitin 6-sulfate or dermatan sulfate (5 mg for each sample) in dimethyl sulfoxide containing 10% of water (2.5 mL) was heated under the various reaction conditions given in Table I. After the reaction was complete, the solution was cooled and diluted with an equal volume of water, and the pH adjusted to 6.8 by the addition of 0.1M sodium hydroxide. The solution was evaporated *in vacuo*

TABLE I

SOLVOLYTIC DEPOLYMERIZATION OF CHONDROITIN 6-SULFATE AND DERMATAN SULFATE WITH DIMETHYL SULFOXIDE CONTAINING 10% OF WATER

Polysaccharide	*Reaction conditions*			*Reaction products (%)*[a]		
	Concentration of polysaccharide (mg/mL)	*Temp. (degrees)*	*Time (h)*	*Monosaccharide*	*Disaccharide*	*Higher molecular-weight oligosaccharide*
Chondroitin 6-sulfate (C_5H_5N salt)	2	105	18	3	51	46
Chondroitin 6-sulfate (C_5H_5N salt)	2	95	18	3	31	67
Chondroitin 6-sulfate (C_5H_5N salt)	4	95	18	2	54	44
Chondroitin 6-sulfate (free acid)	2	105	18	2	72	26
Chondroitin 6-sulfate (Na salt)[b]	2	105	18	2	55	43
Dermatan sulfate (rooster comb, C_5H_5N salt)	2	105	18	7	35	58
Dermatan sulfate (rooster comb, C_5H_5N salt)	2	105	30	15	55	30
Dermatan sulfate (pig skin, C_5H_5N salt)	2	105	30	33	32	35

[a]Proportion of each fraction-size of the reaction products separated on Sephadex G-25 (based on uronic acid determination). [b]Treated with dimethyl sulfoxide containing 10% of 50mM sulfuric acid.

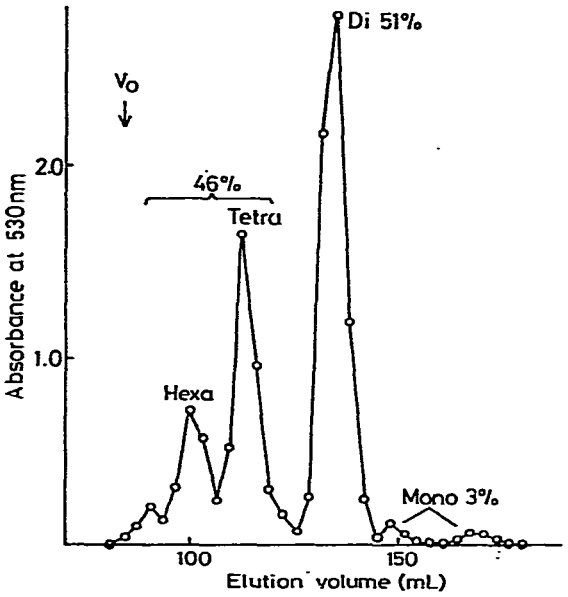

Fig. 1. Gel filtration, on Sephadex G-25, of the reaction products of chondroitin 6-sulfate treated with 1:9 (v/v) water–dimethyl sulfoxide for 18 h at 105°.

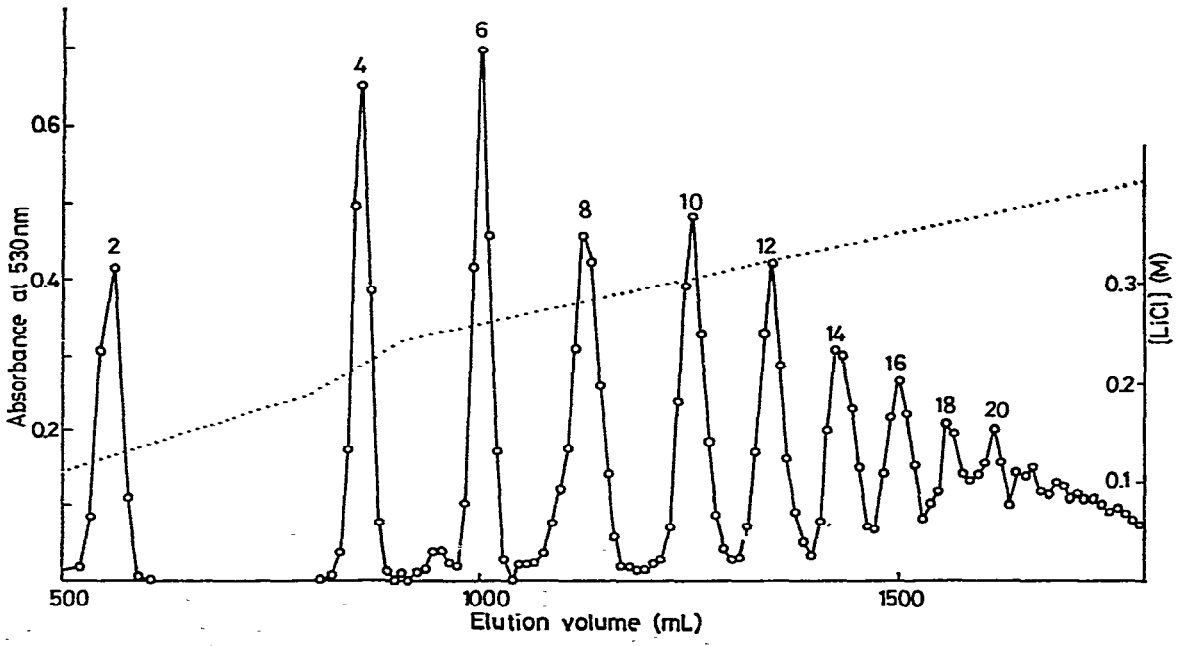

Fig. 2. Anion-exchange chromatography, on AG 1-X2 resin (Cl^-), of the reaction products of chondroitin 6-sulfate treated with 1:9 (v/v) water–dimethyl sulfoxide for 14 h at 90° (——); concentration of lithium chloride (....).

TABLE II

ANALYTICAL DATA OF OLIGOSACCHARIDES (LITHIUM SALTS) PREPARED BY TREATMENT OF CHONDROITIN 6-SULFATE WITH DIMETHYL SULFOXIDE CONTAINING 10% OF WATER FOR 14 H AT 90°

Oligosaccharides (Number of N-acetyl chondrosine units)	*Yield*[a]		$[\alpha]_D^{20°}$[b]	R_{GlA}[c]	R_F[d]	*Uronic acid*[e] (%)	*2-Amino-2-deoxy-hexose* (%)	*Ratio of 2-amino-2-deoxy-hexose to uronic acid residues*			*Ratio of reducing 2-acetamido-2-deoxyhexose to uronic acid residues*[f]		*Reducing value relative to N-acetyl chondrosine*	
	(mg)	*(%)*						*Before reduction*	*After reduction*					
1[g]			−9.7	0.76	0.31	45.8	41.4	0.98	0	(0)[h]	1.00	(1.00)[h]	1.00	(1.00)[h]
2	15.9	6.3	−8.3	0.76	0.31	41.2	36.5	0.96	0.07	(0)	1.04	(1.00)	1.10	(1.00)
4	31.0	12.3	−14.6	0.88	0.12	45.7	40.6	0.97	0.52	(0.50)	0.53	(0.50)	0.82	(0.50)
6	29.0	11.5	−17.9	0.93	0.05	44.5	41.9	1.01	0.65	(0.67)	0.36	(0.33)	0.73	(0.33)
8	33.5	13.3	−15.1	0.97	0.03	46.5	43.4	1.01	0.69	(0.75)	0.25	(0.25)	0.87	(0.25)
10	29.1	11.6	−14.4	0.98	0.02	43.1	39.6	1.00	0.76	(0.80)	0.20	(0.20)	0.53	(0.20)
12	24.7	9.8	−22.8	0.99	0	44.9	42.0	1.01	0.78	(0.83)	0.17	(0.17)	0.46	(0.17)
14	19.2	7.6	−22.1	0.99	0	47.0	44.0	1.01	0.83	(0.86)	0.15	(0.14)	0.37	(0.14)
16	14.0	5.6	−21.1	0.98	0	46.1	42.6	1.00	0.84	(0.88)	0.12	(0.13)	0.34	(0.13)
18	11.8	4.7	−17.3	0.97	0	47.4	43.6	1.00	0.86	(0.89)	0.11	(0.11)	0.29	(0.11)
>20	43.7	17.3	−19.2	0.96	0	43.0	41.6	1.04	0.90	(>0.90)	0.08	(<0.10)	0.23	(<0.10)
Total	251.9[i]	100.0												

[a]Amount of product obtained from 500 mg of the pyridinium salt of chondroitin 6-sulfate. [b]*c* 0.13–0.15, water. [c]Mobility relative to that of D-glucuronic acid. By paper electrophoresis. [d]By t.l.c. on cellulose plate with Solvent *B*. [e]Determined by the modified method of Bitter–Muir[8]. [f]Expressed as a ratio relative to the molar ratio of *N*-acetylhexosamine to uronic acid residues in standard *N*-acetylchondrosine; as the content of 2-acetamido-2-deoxy-D-galactose in *N*-acetylchondrosine found by the Morgan–Elson procedure was 90.7%, which corresponds to 1.93 times the calculated value (55.7%). [g]Standard sample (free acid). [h]In parentheses, calculated value for each oligosaccharide. [i]Corresponds to 81.4% of starting material.

under a nitrogen atmosphere, the residue dissolved in 0.1M sodium chloride (1.0 mL), and the solution passed through a column of Sephadex G-25.

A solution of chondroitin 6-sulfate (free acid, 5 mg) in dimethyl sulfoxide containing 10% of water (2.5 mL) was heated for 18 h at 105°, then cooled, treated as just described, and chromatographed on Sephadex G-25 (Fig. 1).

Depolymerization of sodium chondroitin 6-sulfate with dimethyl sulfoxide containing 10% of 50mM sulfuric acid. — A solution of chondroitin 6-sulfate (sodium salt, 5 mg) in dimethyl sulfoxide containing 10% of 50mM sulfuric acid (2.5 mL) was heated for 18 h at 105°, then cooled, treated as just described, and chromatographed on Sephadex G-25.

Preparation of N-*acetylchondrosine and its oligomers from chondroitin 6-sulfate (pyridinium salt) with dimethyl sulfoxide containing 10% of water.* — A solution of the pyridinium salt of chondroitin 6-sulfate (500 mg) in dimethyl sulfoxide containing 10% of water (125 mL) was heated for 14 h at 90°, cooled, diluted with an equal volume of water, and made neutral with 0.5M sodium hydroxide. The solution was evaporated *in vacuo* under a nitrogen atmosphere and the residue dissolved in water (5 mL). The reaction mixture was applied to a column (2 × 92 cm) of AG 1-X2 (Cl^-, 200–400 mesh) anion-exchange resin, and eluted at room temperature with successively linear gradients of 0–0.2M lithium chloride (total volume 0.8 L) and 0.2–0.4M lithium chloride (total volume 1.2 L) at a flow rate of 51 mL/h (Fig. 2). The eluate was collected in fractions of 9.4 mL, and each fraction was analyzed for uronic acid content. The material from each peak was pooled, lyophilized, and desalted on a column (2.5 × 75 cm) of Sephadex G-15. The desalted solution was lyophilized, and the residue was dissolved in a minimum volume of methanol. The solution was poured into 1:1 (v/v) acetone–ether to give a white powder. Analytical data are summarized in Table II.

Preparation of N-*acetylchondrosine and* N-*acetyldermosine from dermatan sulfate of rooster comb (pyridinium salt) with dimethyl sulfoxide containing 10% of water.* — A solution of the pyridinium salt of dermatan sulfate of rooster comb (200 mg) in dimethyl sulfoxide containing 10% of water (50 mL) was heated for 30 h at 105°, cooled, diluted with an equal volume of water, and made neutral with 0.5M sodium hydroxide. The solution was evaporated *in vacuo* under a nitrogen atmosphere, and the residue dissolved in water (5 mL). An aliquot (0.1 mL) of this solution, diluted with 0.1M sodium chloride (1 mL), was chromatographed on Sephadex G-25 (inset of Fig. 3). That remaining was applied to a column (1.6 × 90 cm) of AG 1-X4 (HCO_2^-, 200–400 mesh) anion-exchange resin and eluted stepwise with 0.3M and M formic acid at 37° (Fig. 3). The eluate was collected in fractions of 16 mL, and each fraction was analyzed for uronic acid content and pooled as indicated in Fig. 3. The pooled fractions corresponding to Peaks A and B were lyophilized, and 0.80 mg of each product, in 0.5M sulfuric acid (0.2 mL), was heated for 2 h in a boiling-water bath. Saturated barium hydroxide solution was added to the hydrolyzates. Barium sulfate was filtered off, and the filtrate was concentrated and examined by t.l.c. on a cellulose plate. Peak A was characterized as *N*-acetyl-

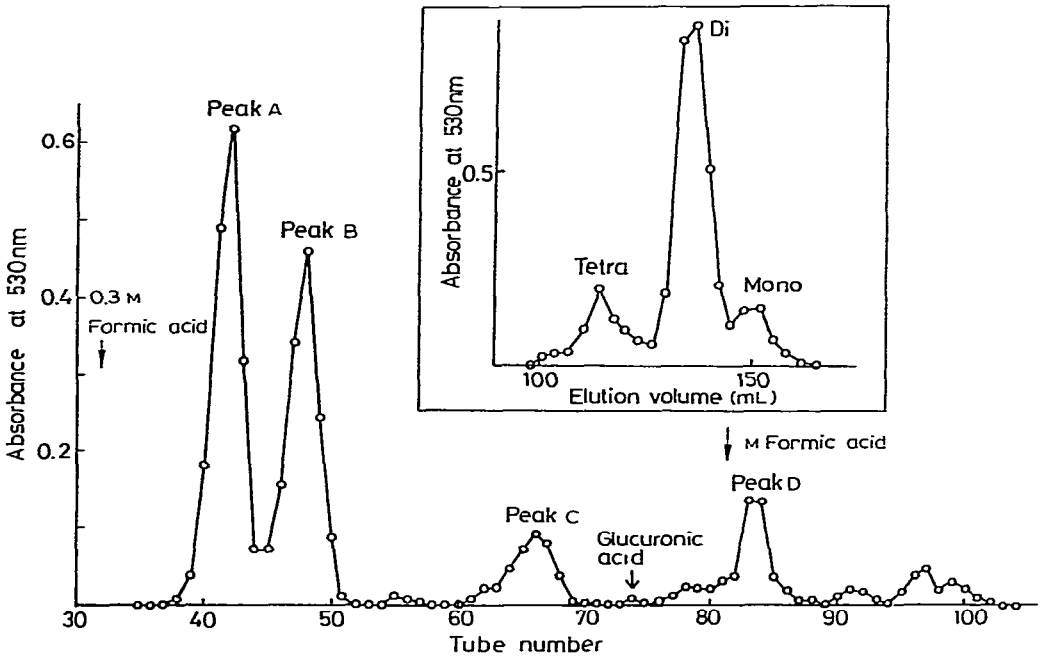

Fig. 3. Anion-exchange chromatography, on AG 1-X4 resin (HCO_2^-), of the reaction products of dermatan sulfate of rooster comb treated with 1:9 (v/v) water–dimethyl sulfoxide for 30 h at 105°. Peak A, *N*-acetyldermosine; Peak B, *N*-acetylchondrosine; Peak C, L-iduronic acid; and Peak D, tetrasaccharide fraction. Gel filtration, on Sephadex G-25, of the same reaction products is shown in the inset.

dermosine, and Peak B as *N*-acetylchondrosine, in a yield of 49 mg (38%) and 38 mg (30%), respectively. The remaining lyophilized products were dissolved in water and passed through a column of Amberlite IRC-50 (Ba^{2+}) cation-exchange resin. The eluate and washings were concentrated *in vacuo* to a small volume and centrifuged. The supernatant solution was introduced dropwise into absolute ethanol. The centrifugation and precipitation were repeated twice to give the barium salts of *N*-acetylchondrosine (35 mg, 28%) and *N*-acetyldermosine (52 mg, 35%). The analytical data are summarized in Table III.

Depolymerization of pig-skin dermatan sulfate (pyridinium salt) with dimethyl sulfoxide containing water or pyridine–water. — Dermatan sulfate (pyridinium salt, 10 mg for each sample) was weighed into three test tubes. To each was added dimethyl sulfoxide (4.5 mL) and, after mixing, water (0.5 mL), 0.3% pyridine in water (0.5 mL) or 1% pyridine in water (0.5 mL), to form a homogeneous solution. All test tubes were heated for 30 h at 105°, and the content of each test tube was cooled and treated as described in the preceding section. A portion of each reaction mixture was subjected to analytical gel chromatography on Sephadex G-25 (Figs. 4a, b, and c).

TABLE III

ANALYTICAL DATA OF *N*-ACETYLCHONDROSINE AND *N*-ACETYLDERMOSINE (BARIUM SALTS) PREPARED BY TREATMENT OF DERMATAN SULFATE OF ROOSTER COMB WITH DIMETHYL SULFOXIDE CONTAINING 10% OF WATER FOR 30 H AT 105°

Disaccharide	*Yield*[a] *(mg)*	$[\alpha]_D^{27°}$ [b]	*Uronic*[c] *acid (%)*	*Hexosamine (%)*	*Ratio of 2-amino-2-deoxyhexose to uronic acid residues*		*Ratio of 2-acetamido-2-deoxyhexose to uronic acid residues*[d]	*Ratio of carbazole to orcinol values*
					Before reduction	*After reduction*		
N-Acetylchondrosine[e]	38 (30%)	−9.7	45.8	41.4	0.98	0	1.00	2.52 (2.40)[f]
N-Acetylchondrosine		−9.6	37.9	34.9	1.00	0	0.97	2.36
N-Acetyldermosine	49 (38%)	−11.8	38.2	34.5	0.98	0	0.96	0.19 (0.24)[f]

[a]Amount of each free acid obtained from 200 mg of the pyridinium salt of dermatan sulfate of rooster comb. [b]*c* 0.90, water. [c]Determined by the method of Bitter–Muir[8], modified by increasing the borate concentration to 0.2M, with D-glucurono-6,3-lactone as standard for *N*-acetylchondrosine, and 1,2-isopropylidene-L-iduronolactone as standard for *N*-acetyldermosine, respectively. [d]Expressed as a ratio to the value of authentic *N*-acetylchondrosine. [e]Standard (free acid). [f]In parentheses, ratio of free acid reported by Fransson *et al.*[4].

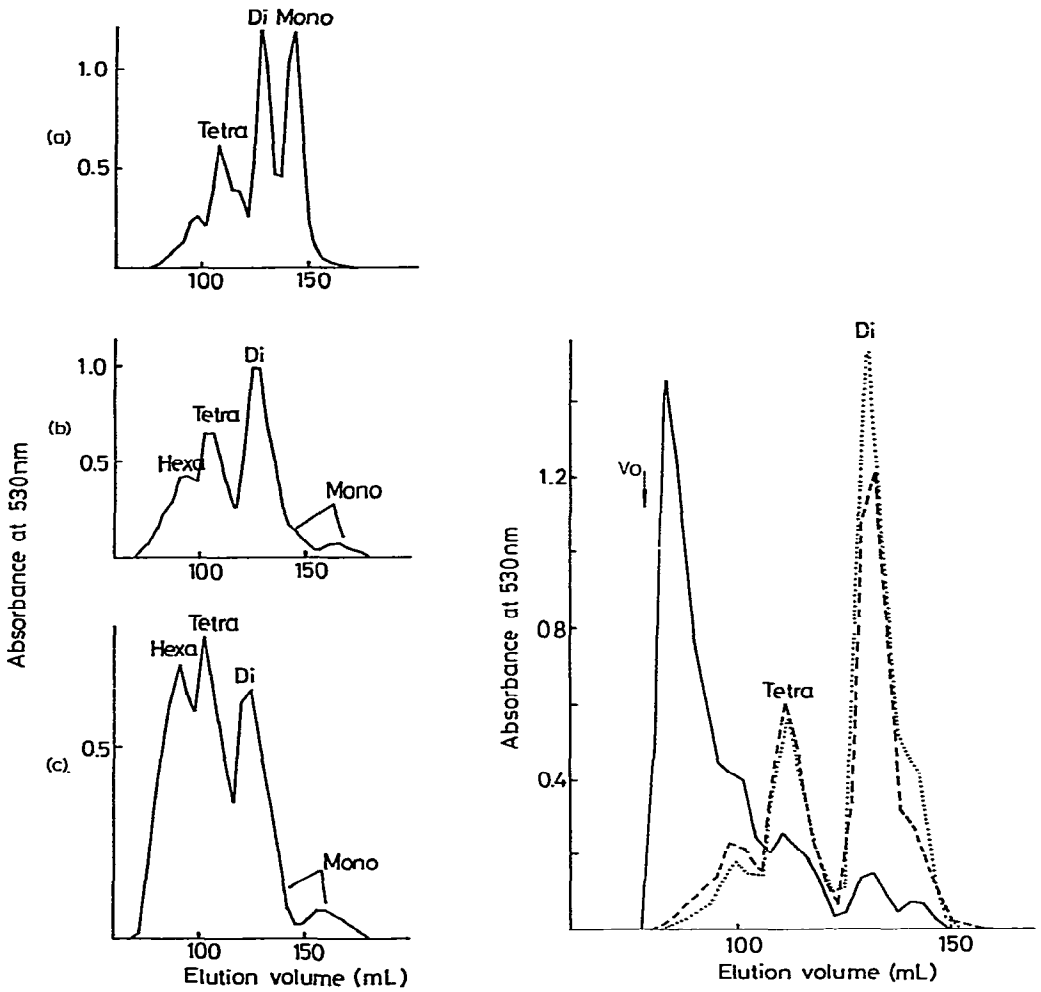

Fig. 4. Gel filtration, on Sephadex G-25, of the reaction products of pig-skin dermatan sulfate treated for 30 h at 105° with: (a) 1:9 (v/v) water–dimethyl sulfoxide; (b) 1:9 (v/v) 0.3% pyridine in water–dimethyl sulfoxide; and (c) 1:9 (v/v) 1% pyridine in water–dimethyl sulfoxide.

Fig. 5. Gel filtration, on Sephadex G-25, of the reaction products of hyaluronic acid treated with 1:9 (v/v) water–dimethyl sulfoxide for 30 h at 105°, in the presence of pyridinium sulfate (– – – –), or pyridinium chloride (.....), and in the absence of any additives (——).

Depolymerization of hyaluronic acid (free acid) with dimethyl sulfoxide containing 10% of water in the presence or absence of acid substances. — A solution of the free acid of hyaluronic acid (4 mg for each experiment) in dimethyl sulfoxide containing 10% of water or of water containing 50mM pyridinium sulfate or pyridinium chloride (each 2 mL) was heated for 30 h at 105°, cooled, treated as described previously, and chromatographed on Sephadex G-25 (Fig. 5).

Depolymerization of N*-desulfated,* N*-acetylated heparin (pyridinium salt) with*

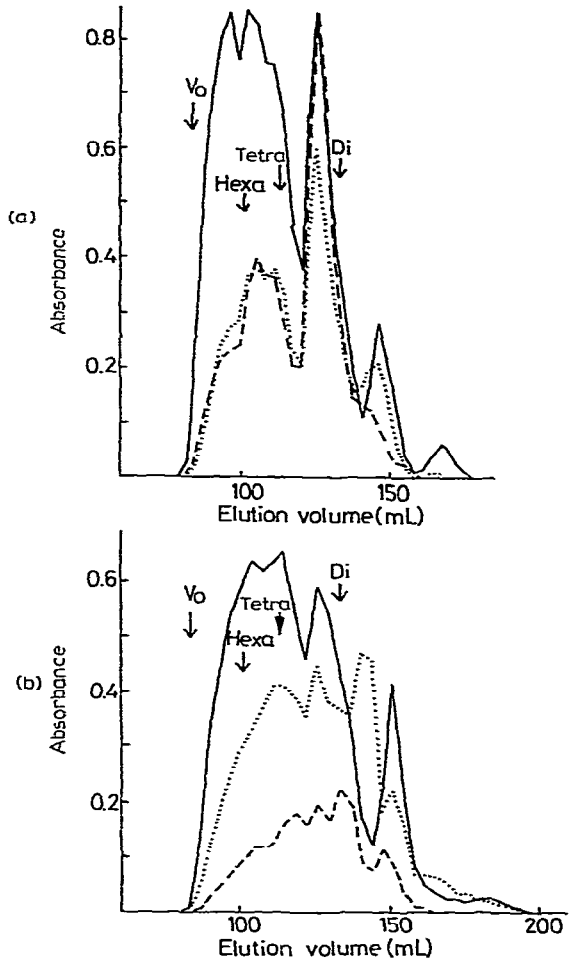

Fig. 6. Gel filtration, on Sephadex G-25, of the reaction products of (a) chondroitin 6-sulfate and (b) dermatan sulfate of rooster comb, both treated with water for 18 h at 105°: (——) carbazole reaction (absorbance at 530 nm), (– – – –) Morgan–Elson reaction (absorbance at 585 nm), and (.....) Park–Johnson method (absorbance at 690 nm). The arrows indicate the position of elution of each nonsulfated oligosaccharide.

dimethyl sulfoxide containing 10% of water. — The pyridinium salt of *N*-desulfated, *N*-acetylated heparin (2.0 mg) was dissolved in dimethyl sulfoxide containing 10% of water (1 mL). The solution was heated for 30 h at 105°, cooled, treated as described previously, and chromatographed on Sephadex G-25.

Depolymerization of dermatan sulfate of rooster comb (pyridinium salt) in hot water. — A solution of the pyridinium salt of dermatan sulfate (43.08 mg) in water (20 mL) was heated for 18 h at 105°, cooled, diluted with an equal volume of water, and made neutral with 0.5M sodium hydroxide. The solution was evaporated *in vacuo* under a nitrogen atmosphere, and the residue dissolved in water (4 mL). A portion

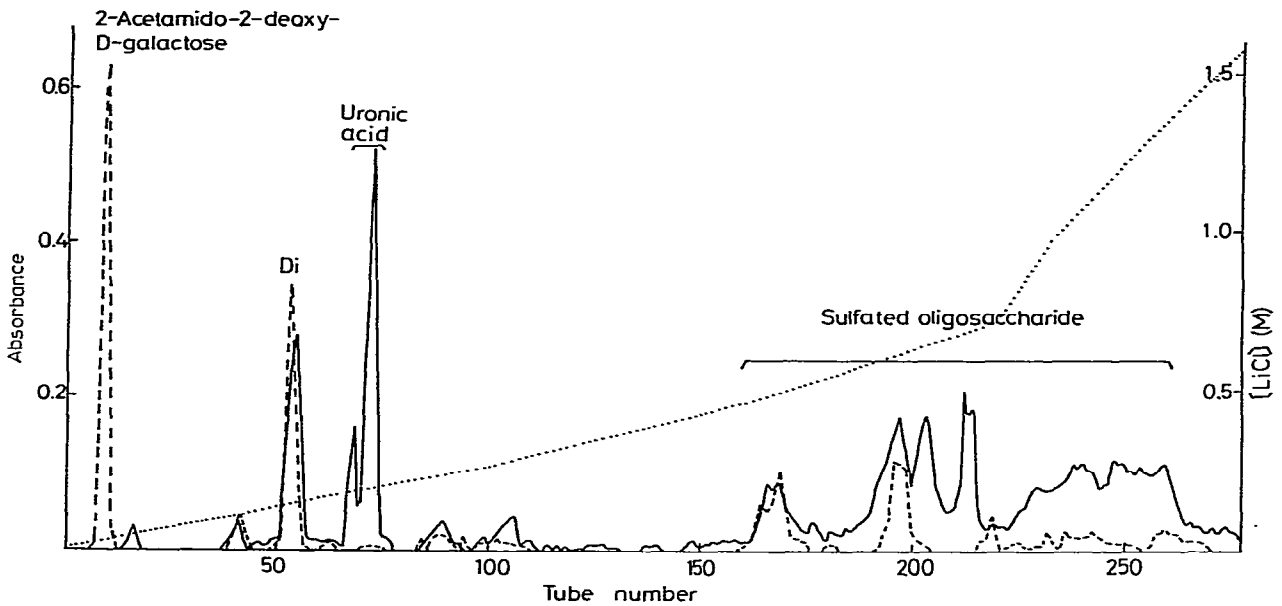

Fig. 7. Anion-exchange chromatography, on AG 1-X4 resin (Cl^-), of the reaction products of dermatan sulfate of rooster comb treated with water for 18 h at 105°: (——) carbazole reaction (absorbance at 530 nm), (– – – –) Morgan–Elson reaction (absorbance at 585 nm), and (.....) concentration of lithium chloride.

(1 mL) of the solution was subjected to analytical gel chromatography on Sephadex G-25 (Fig. 6b). Another portion (2 mL) of the solution was applied to a column (1 × 90 cm) of AG 1-X4 (Cl^-, 200–400 mesh) and eluted at room temperature with a linear gradient of 0–2.0M lithium chloride (total volume 1.5 L) at a flow rate of 48 mL/h. The eluate was collected in fractions of 5.0 mL, and each fraction was analyzed for uronic acid content (Fig. 7). To the remaining reaction mixture (1 mL), 10% barium acetate solution was added. The precipitated barium sulfate was centrifuged off, and the supernatant was passed through a column of Dowex 50W-X2 (H^+, 50–100 mesh) cation-exchange resin, and then washed with water. The eluate and washings were lyophilized, and the residue was dried in the presence of phosphorus pentoxide in a vacuum desiccator to give a brownish film (8.0 mg), which was dissolved in M hydrochloric acid (1 mL) and hydrolyzed for 5 h at 105°. The inorganic sulfate liberated was determined by the turbidimetric method[14].

Depolymerization of chondroitin 6-sulfate (pyridinium salt) in hot water. — A solution of the pyridinium salt of chondroitin 6-sulfate (20.23 mg) in water (10 mL) was heated for 18 h at 105°, cooled, and treated as just described. A portion of an aqueous solution of the product was chromatographed on Sephadex G-25 (Fig. 6a), and the sulfur content of the product was determined as described previously.

RESULTS AND DISCUSSION

Depolymerization of the pyridinium salts of chondroitin 6-sulfate, dermatan sulfate, hyaluronic acid, and N-*desulfated,* N-*acetylated heparin with dimethyl sulfoxide containing water.* — Treatment of the pyridinium salt of chondroitin 6-sulfate with dimethyl sulfoxide containing 10% of water for 18 h at 105° gave a mixture of the oligosaccharides with an even number of residues, as shown in Fig. 1. The pH* at the end of the reaction was 3.52. The amount of uronic acid recovered from the reaction products was almost the same as the amount in the starting material used. The disaccharide fractionated on anion-exchange resin was found to be identical with *N*-acetylchondrosine by t.l.c. The free acid of chondroitin 6-sulfate was depolymerized at a rate higher than that of the pyridinium salt under the same conditions (Table I). The pH at the end of the reaction of the free acid was 3.16. The sodium salt of chondroitin 6-sulfate was also depolymerized with dimethyl sulfoxide containing 10% of 50mM sulfuric acid at nearly the same rate as that of the pyridinium salt in dimethyl sulfoxide containing 10% of water (Table I). When the reaction was over, the pH of the reaction mixture was 3.32. The presence of pyridine, therefore, does not seem to be essential for the solvolytic depolymerization in dimethyl sulfoxide containing water, as observed for the depolymerization with dimethyl sulfoxide containing methanol[6]. On the other hand, the pH of the reaction medium was found to affect the rate of the solvolysis, as will be mentioned later for the depolymerization of pig-skin dermatan sulfate. The results of the solvolytic depolymerization of chondroitin 6-sulfate and dermatan sulfates under various conditions (Table I) indicate that the degree of depolymerization of the polysaccharides depends on the reaction temperature, reaction time, the concentration of the reactants, and the pH of the reaction medium. A comparison of the results in Table I with those reported previously[6] indicates that the rate of depolymerization in dimethyl sulfoxide containing water is lower than that in dimethyl sulfoxide containing methanol under the same reaction conditions (18 h, 95°).

In order to obtain a series of *N*-acetylchondrosine oligomers by solvolysis, the pyridinium salt of chondroitin 6-sulfate was heated in dimethyl sulfoxide containing 10% of water for 14 h at 90°. The reaction products were fractionated on AG 1-X2 (Cl^-) anion-exchange resin into separate oligosaccharide fractions consisting of di- to octadeca-saccharide (Fig. 2). Each oligosaccharide was isolated as the lithium salt and was characterized (Table II). The overall yield of *N*-acetylchondrosine oligomers was 81.4%. The molar ratios of hexosamine to uronic acid after reduction with sodium borohydride, and of reducing-*N*-acetylhexosamine to total uronic acid are in fair agreement with the values calculated for each even-numbered oligosaccharide. In contrast, their reducing values relative to *N*-acetylchondrosine were higher than expected, possibly due to partial degradation of the oligosaccharides under the alkaline condition of the Park–Johnson method as reported by Cifonelli[1].

*The pH was measured after an equal volume of water had been added to the reaction mixture.

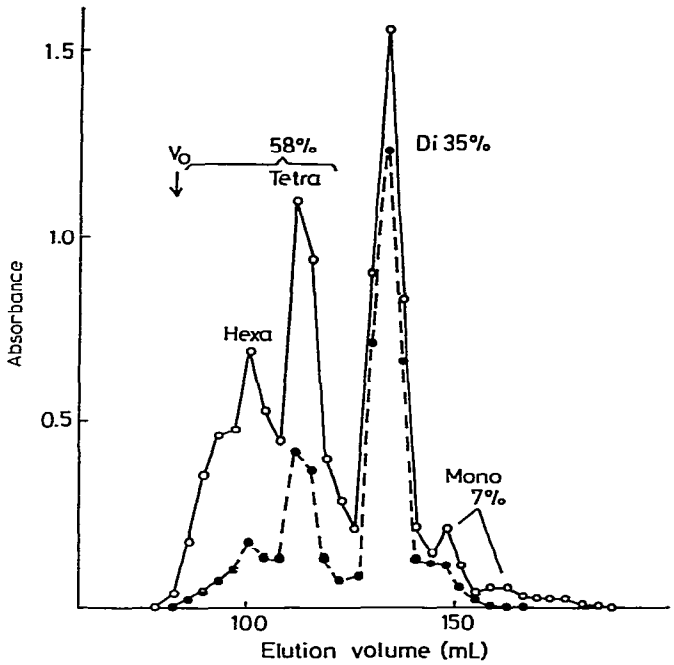

Fig. 8. Gel filtration, on Sephadex G-25, of the reaction products of dermatan sulfate of rooster comb treated with 1:9 (v/v) water–dimethyl sulfoxide for 18 h at 105°: (——) carbazole reaction (absorbance at 530 nm) and (– – – –) Morgan–Elson reaction (absorbance at 585 nm).

The pyridinium salt of dermatan sulfate of rooster comb was depolymerized at a rate slightly lower than that of chondroitin 6-sulfate, but more free uronic acid was produced from the former polysaccharide under the same conditions (18 h, 105°) (Table I and Fig. 8). The pyridinium salt of dermatan sulfate was treated with dimethyl sulfoxide containing 10% of water for 30 h at 105°, and the reaction products were fractionated on AG 1-X4 (HCO_2^-) anion-exchange resin. Peaks A and B on the elution diagram (Fig. 3) were characterized as *N*-acetyldermosine and *N*-acetylchondrosine, respectively, based on identification of their hydrolysis products by t.l.c. Peak C (Fig. 3) was identified as L-iduronic acid, and Peak D was assumed to be a mixture of tetrasaccharides from the R_F value (0.14) on thin-layer plate with solvent *B*, and R_{GlA} value (0.87) in paper electrophoresis. The free acids of *N*-acetyldermosine and *N*-acetylchondrosine were obtained by lyophilization of the pooled fractions corresponding to Peaks A and B (see Fig. 3), respectively, and they were converted to the barium salts, which were purified by precipitation with ethanol. As shown in Table III, these disaccharides were isolated in a fairly good yield, considering the concomitant formation of higher oligosaccharides and free L-iduronic acid during the reaction. The carbazole–orcinol ratios were consistent with the values reported by Fransson *et al.*[4]. Pig-skin dermatan sulfate, which has a high content of L-iduronic acid[3], yielded more free uronic acid (mainly L-iduronic acid) than did dermatan

sulfate of rooster comb, which contains a higher proportion of D-glucuronic acid (Table I, Figs. 3 and 4a). The formation of a larger amount of free uronic acids from both dermatan sulfates seems to be due to the lability of the L-idosyluronic linkages in the polysaccharides under the conditions of solvolysis described herein, as well as under those reported for the acid hydrolysis of pig-skin dermatan sulfate[3,17]. A small amount of pyridine was added to the reaction mixture of pig-skin dermatan sulfate to decrease free uronic acid formation. The pH of the reaction mixture was 3.61 without addition of pyridine, whereas the pH values of the reaction mixtures of dimethyl sulfoxide containing 10% of 0.3% pyridine in water, and 1% pyridine in water, were 3.97 and 4.67, respectively. The elution patterns (Figs. 4a, b, and c) indicate that the addition of pyridine caused a small rise in the pH of the reaction mixtures, resulting in decreasing formation of free uronic acids with increasing amount of higher oligosaccharides. Therefore, a higher pH is desirable for the depolymerization of pig-skin dermatan sulfate. The results obtained by the solvolytic depolymerization of dermatan sulfates of rooster comb and of pig-skin (Figs. 3 and 4a) imply the possible formation of mono- and di-saccharide fractions from the polysaccharides as the sole final products.

Although the solvolytic depolymerization of hyaluronic acid (free acid) in dimethyl sulfoxide containing water proceeded, to some extent, without addition of any acidic substance, the addition of either pyridinium sulfate or pyridinium chloride greatly accelerated the reaction (Fig. 5). Our previous results, however, indicated that the solvolysis of hyaluronic acid in dimethyl sulfoxide containing 10% of methanol was accelerated by the addition of pyridine–sulfur trioxide or pyridinium sulfate[6], but not accelerated by the addition of hydrogen chloride. The reason for the different actions of these acidic substances in dimethyl sulfoxide containing methanol or water is not clear.

The pyridinium salt of *N*-desulfated, *N*-acetylated heparin was treated with dimethylsulfoxide containing 10% of water for 30 h at 105°, and most of the reaction products were eluted, from the Sephadex G-25 column (data not shown), in the excluded volume. This indicates that the 2-acetamido-2-deoxy-α-D-glycosyl linkages in the polysaccharide were resistant to solvolysis with dimethyl sulfoxide containing either water or methanol[6].

Depolymerization of the pyridinium salts of chondroitin 6-sulfate and dermatan sulfate of rooster comb with hot water. — The solvent effect of dimethyl sulfoxide on depolymerization of the glycosaminoglycuronans has been recognized to be considerable, as indicated by a limited reaction of the pyridinium salt of chondroitin 6-sulfate in methanol alone[6]. The solvent effect of dimethyl sulfoxide in the present study was examined anew in detail. The pyridinium salts of chondroitin 6-sulfate and dermatan sulfate of rooster comb were dissolved in water, and each of the solutions was heated for 18 h at 105°. The sulfur content of the reaction products of chondroitin 6-sulfate and dermatan sulfate was 5.51% (originally 6.36%) and 4.08 (originally 6.44%), respectively, indicating that most of the sulfate groups in the polysaccharides had not been hydrolyzed. Gel filtration of the reaction products of the polysacchar-

ides on Sephadex G-25, indicated the formation of free uronic acids and a mixture of smaller oligosaccharides carrying sulfate groups, as judged from the elution diagrams in Figs. 6a and b. Anion-exchange chromatography, on AG 1-X4 resin, of the reaction product of dermatan sulfate of rooster comb revealed peaks corresponding to 2-acetamido-2-deoxy-D-galactose, two types of uronic acid, and a mixture of sulfated oligosaccharides (Fig. 7). Some of the peaks showed both positive carbazole and Morgan–Elson reactions, whereas others showed only a positive carbazole reaction. The former peaks seem to be those of the oligosaccharides having a nonsulfated 2-acetamido-2-deoxy-D-galactose residue at the reducing end, and the latter peaks seem to be those of the oligosaccharides having a uronic acid or 2-acetamido-2-deoxy-D-galactose 4-sulfate residue at the reducing end. These findings indicate that the pyridinium salt of dermatan sulfate of rooster comb (and probably also chondroitin 6-sulfate) is only partially desulfated in the medium without dimethyl sulfoxide, and that there is no distinct difference, in the rate of cleavage, between the ester sulfate groups, the glycosidic linkages of the uronic acid, and of 2-acetamido-2-deoxy-D-galactose residues under the reaction conditions used. Consequently, dimethyl sulfoxide may play an essential role in the solvolysis, which includes both desulfation and selective cleavage of 2-acetamido-2-deoxy-β-D-hexosyl linkages of the polysaccharides. To examine the effect of aprotic solvents other than dimethyl sulfoxide, the pyridinium salt of chondroitin 6-sulfate was treated with *N*,*N*-dimethylformamide containing 10% of water for 18 h at 105°. Most of the reaction products were excluded from Sephadex G-25 gels, indicating some inhibiting effect of *N*,*N*-dimethylformamide on the solvolysis (data not shown).

Thus, the essential feature of the solvolysis described herein is the rapid, initial cleavage of the sulfate groups of glycosaminoglycuronan sulfates under moderate acid-conditions. This probably results from the solvation between protons and dimethyl sulfoxide molecules in the reaction medium*, the 2-acetamido-2-deoxy-β-D-hexosyl linkages of the resulting desulfated glycosaminoglycuronans being preferentially cleaved without any marked decomposition of the uronic acid residues. Consequently, a series of nonsulfated, even-numbered oligosaccharides having a 2-acetamido-2-deoxy-D-hexose residue at the reducing end are obtained in fairly good yield.

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*Although no data are shown, it was observed throughout all experiments that the pH of a reaction mixture without dimethyl sulfoxide was lower than that of the same mixture containing dimethyl sulfoxide.

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