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PREPARATION OF METHYL GLYCOSIDES OF DI- AND HIGHER OLIGOSACCHARIDES FROM GLYCOSAMINOGLYCURONANS BY SOLVOLYSIS WITH DIMETHYL SULFOXIDE CONTAINING METHANOL

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ABSTRACT

Solvolysis of chondroitin 4- or 6-sulfate (pyridinium salt) with dimethyl sulfoxide containing 10% of methanol for 18 h at 95° resulted in the cleavage of the 2-amino-2-deoxy-D-glucoside bonds together with initial desulfation to give methyl β-glycosides of *N*-acetylchondrosine as a main product and, in addition, higher oligosaccharides, without any loss of uronic acid. Dermatan sulfate was also depolymerized to yield methyl glycosides of di- and higher oligo-saccharides under the same conditions. Hyaluronic acid (free acid) was depolymerized by the same solvent in the presence of an equimolar amount of pyridine–sulfur trioxide or pyridinium sulfate per disaccharide unit to give methyl glycosides of di- and higher oligo-saccharides. In contrast, *N*-desulfated, *N*-acetylated heparin was stable under these solvolytic conditions and did not yield heparin oligosaccharides.

INTRODUCTION

Acid hydrolysis of glycosaminoglycuronans yields corresponding repeating disaccharides, such as chondrosine from chondroitin sulfates[1] and hyalobiuronic acid from hyaluronic acid[2], while methanolysis of hyaluronic acid with methanolic hydrogen chloride followed by successive acetylation and saponification yields the methyl α-glycoside of *N*-acetylhyalobiuronic acid[3]. On the other hand, dermatan sulfate is hydrolyzed more easily than the aforementioned polysaccharides to afford a larger proportion of free hexosamine and uronic acid at an early stage of the hydrolysis[4]. Generally, acid hydrolysis or methanolysis of *N*-acetylated glycosaminoglycuronans results in splitting of the glycoside bonds accompanied by *N*-deacetylation, which makes subsequent cleavage of the 2-amino-2-deoxyglycoside linkages in the polysaccharides difficult owing to the liberation of free amino groups, and, therefore, the loss of a part of the uronic acid is unavoidable under the conditions used.

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As reported in our previous paper[5], we observed that solvolytic desulfation of chondroitin 4- and 6-sulfates with dimethyl sulfoxide containing 10% of methanol at 80° was accompanied by considerable methanolysis. The present paper reports the results of depolymerization of chondroitin sulfates, dermatan sulfate, and hyaluronic acid with dimethyl sulfoxide containing 10% of methanol, and a new method for the preparation of repeating disaccharides and higher oligosaccharides from these polysaccharides.

EXPERIMENTAL

Materials. — Sodium chondroitin 6-sulfate (mol. wt. 65 000 by the light-scattering method), sodium chondroitin 4-sulfate (mol. wt. 64 500 by the Nelson–Somogyi method), sodium hyaluronate from rooster comb (mol. wt. 2 000 000 by viscosity measurement), and sodium dermatan sulfate from rooster comb were obtained from Seikagaku Kogyo Co., Tokyo. *N*-Desulfated heparin (found: total sulfate, 8.65%; *N*-sulfate, 0.42%) was prepared by treatment of hog mucosal heparin with dimethyl sulfoxide containing 5% of water for[6] 90 min at 50°, and its *N*-acetylation was performed by the method of Danishefsky *et al.*[7] to yield *N*-desulfated, *N*-acetylated heparin, which showed no free amino group as determined by 2,4,6-trinitrophenylation[8]. Chondroitin 6-sulfate (free acid) was prepared by passing a 1% solution of sodium chondroitin 6-sulfate in water through a column of Dowex 50-W X-8 (H^+) cation-exchange resin, and lyophilizing the acidic effluent. Hyaluronic acid (free acid) was prepared as just described, except decreasing the concentration of sodium hyaluronate to 0.1%. Bovine liver β-D-glucuronidase (type B-10, 10 000 units per mg) was a product of Sigma Chemical Co. (St. Louis, Mo 63178). Dimethyl sulfoxide was purified by heating the commercial product (1 L) containing potassium hydroxide (1 g) for 1 h at 125°, followed by distillation of the dried material under reduced pressure at 72°, and storage over molecular sieves[9].

Analytical methods. — The methods for the quantitative determination of uronic acid content and the measurement of reducing power have been reported previously[6]. Paper electrophoresis was performed on Toyo Roshi No. 51 paper in a buffer solution (pH 5.8) of 5:1:5:250 (v/v) pyridine–acetic acid–1-butanol–water at a potential of 23 V/cm for 30 min. Thin-layer chromatography was performed on cellulose plates developed with 2:1:1:2 (v/v) ethyl acetate–pyridine–ethanol–water (solvent *A*) or 2:1:1 (v/v) 1-butanol–acetic acid–water (solvent *B*), and on silicic acid plates developed with 2:1 (v/v) acetone–methanol. Paper or thin-layer cellulose plates were stained with the *p*-aminohippuric acid reagent[10], and the silicic acid plates were exposed to iodine vapor. N.m.r. spectra were recorded at 22° with a JNM-PS-100 n.m.r. spectrometer operated at 100 MHz for solutions in [$^2H_6$]dimethyl sulfoxide containing tetramethylsilane as the internal standard.

Gel chromatography. — The samples (~3 mg), dissolved in 0.1M sodium chloride (1 mL), were applied to a column (1.6 × 84 cm) of Sephadex G-25 and eluted with the same solvent at a flow rate of 12 mL/h. The eluate was collected in

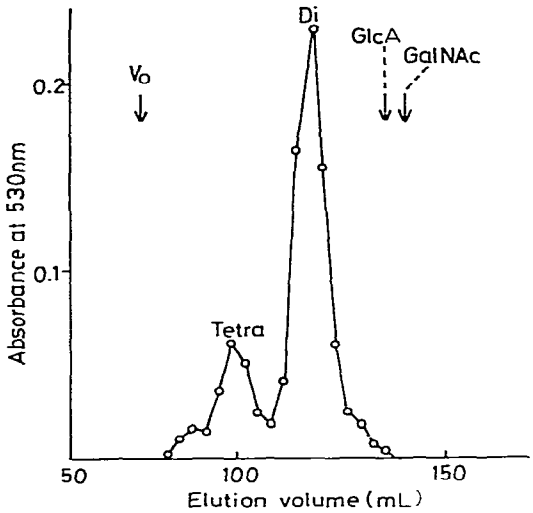

Fig. 1. Gel filtration, on Sephadex G-25, of the reaction products of chondroitin 6-sulfate treated with 1:9 (v/v) methanol–dimethyl sulfoxide for 18 h at 95°.

3-mL fractions, and each fraction was analyzed for uronic acid content. For Sephadex G-75 chromatography, the samples (~5 mg) in 0.1M sodium chloride (1 mL) were applied to a column (1.6 × 93 cm) and treated by the same method.

Preparation of methyl glycosides of the disaccharide and tetrasaccharide from chondroitin 6-sulfate (pyridinium salt) with dimethyl sulfoxide containing 10% of methanol. — The pyridinium salt of chondroitin 6-sulfate (500 mg) was dissolved in dimethyl sulfoxide containing 10% of methanol (200 mL). After the solution had been kept for 18 h at 95°, the content was cooled, diluted with an equal volume of water, and the pH adjusted to 6.8 by the addition of 0.5M sodium hydroxide. The solution was concentrated under reduced pressure to ~5 mL and diluted with an equal volume of water. A portion (0.05 mL) of the reaction mixture, dissolved in 0.1M sodium chloride (1 mL), was chromatographed on Sephadex G-25 (Fig. 1). The remaining reaction-mixture was applied to a column (2 × 92 cm) of AG-1 X-4 (Cl^-, 200–400 mesh), anion-exchange resin, and eluted with a linear gradient of 0–0.2M lithium chloride (1.8 L) at a flow rate of 75 mL/h. The eluate was collected in 12.4-mL fractions, and each fraction was analyzed for uronic acid content and pooled as indicated in Fig. 2.

The pooled fractions corresponding to Fraction 3 (Fig. 2) were lyophilized, and desalted by passage through a column (2.5 × 90 cm) of Sephadex G-15, which was eluted with 10% ethanol. The fractions freed from the salt were collected and lyophilized to give a pale-yellow powder (170 mg, 43.0%). A solution of the powder in a small volume of methanol was applied to a column (3 × 30 cm) of silicic acid, which had been prepared with 4:1 (v/v) acetone–methanol, and the column was successively eluted, stepwise, with 4:1 (150 mL), 3:1 (450 mL), and 2:1 (all v/v)

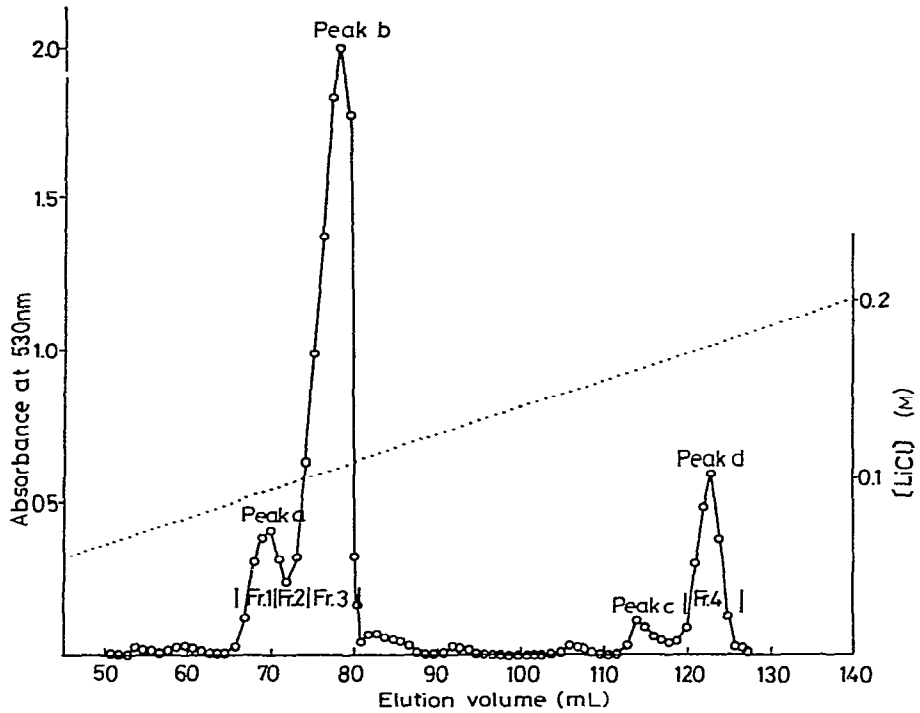

Fig. 2. Anion-exchange chromatography, on AG-1 X-4 resin, of the reaction products of chondroitin 6-sulfate treated with 1:9 (v/v) methanol–dimethyl sulfoxide for 18 h at 95°.

(1 L) acetone–methanol. Fractions (50 mL each) were collected, concentrated under diminished pressure, and analyzed by t.l.c. The major fractions giving a positive reaction with the *p*-aminohippuric acid reagent were eluted with 2:1 (v/v) acetone–methanol and evaporated, and the residue was dissolved in a small volume of methanol. This solution was treated with activated carbon, and precipitated by the addition of 1:1 (v/v) acetone–ether to give a white powder. The precipitation was repeated twice to give the final product (Compound I; 126 mg, 31.9%), $[\alpha]_D^{29}$ −120.7° (*c* 0.675, methanol).

Anal. Calc. for $C_{15}H_{24}LiNO_{12} \cdot H_2O$: C, 41.39; H, 6.02; N, 3.22. Found: C, 41.18; H, 5.92; N, 3.33.

The pooled fractions corresponding to Fraction 4 (Fig. 2) were desalted by the method just described and lyophilized to give a white powder (41 mg, 10.4%). It was dissolved in a small volume of methanol and precipitated by the addition of acetone. The precipitation was repeated twice to give the final product (Compound II; 35 mg, 8.9%), $[\alpha]_D^{29}$ −47.9° (*c* 0.772, methanol).

Anal. Calc. for $C_{30}H_{44}Li_2N_2O_{23} \cdot 2H_2O$: C, 41.54; H, 5.77; N, 3.34. Found: C, 41.86; H, 6.00; N, 3.37.

The pooled fractions corresponding to Fractions 1 and 2 (Fig. 2) were desalted

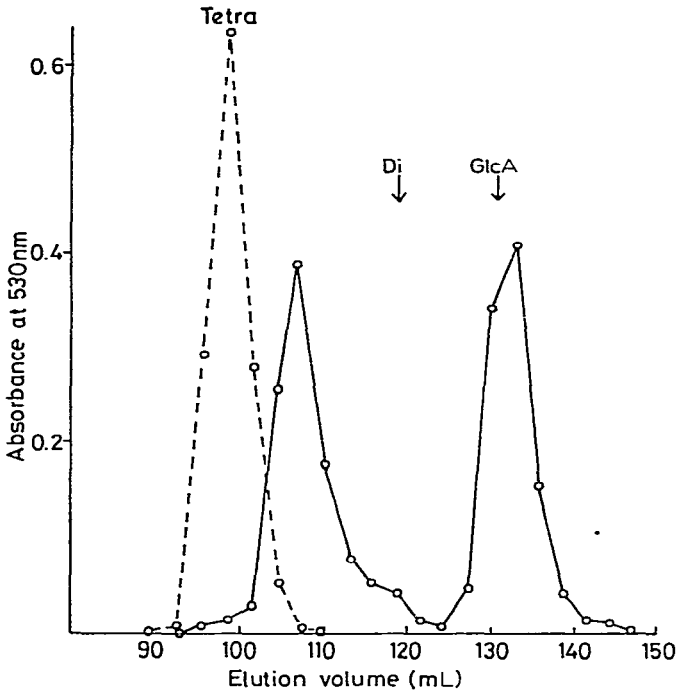

Fig. 3. Gel filtration, on Sephadex G-25, of the β-D-glucuronidase digest of a tetrasaccharide obtained from chondroitin 6-sulfate: (-----) tetrasaccharide and (———) digest of tetrasaccharide.

by gel-filtration on Sephadex G-15, and gave 31 mg and 37 mg, respectively (total yield of Fractions 1–4, 70.6%).

Acid hydrolysis of Fraction 1 and Compound I. — Fraction 1 (1 mg) and Compound I (1 mg) in M hydrochloric acid (0.2 mL) were heated for 1 h in a boiling-water bath. After the solution had been lyophilized, the residue was dissolved in water (1 drop) and examined by t.l.c. on cellulose with chondrosine as a standard [R_F of chondrosine: 0.18 (A), 0.11 (B)].

Digestion of Compounds I and II with β-D-glucuronidase. — Compound II (0.80 mg) in 0.05M acetate buffer, pH 5.0 (1 mL), was digested[11] with bovine liver β-D-glucuronidase (0.22 mg). After incubation for 14.5 h at 37°, the digest was analyzed by gel chromatography on a column (1.6 × 86 cm) of Sephadex G-25, which was eluted with 0.1M sodium chloride (Fig. 3). A control incubation with the boiled enzyme was carried out simultaneously and analyzed by a similar procedure.

Compound I (0.59 mg), in 0.05M acetate buffer, pH 5.0 (1 mL), was digested with bovine liver β-D-glucuronidase (0.61 mg). After incubation for 14.5 h at 37°, the digest was analyzed by gel chromatography as just described.

Depolymerization of chondroitin 6-sulfate (pyridinium salt) with absolute methanol. — The pyridinium salt of chondroitin 6-sulfate (10 mg), suspended in absolute methanol (5 mL), was heated for 18 h at 95°. The suspension was cooled, and

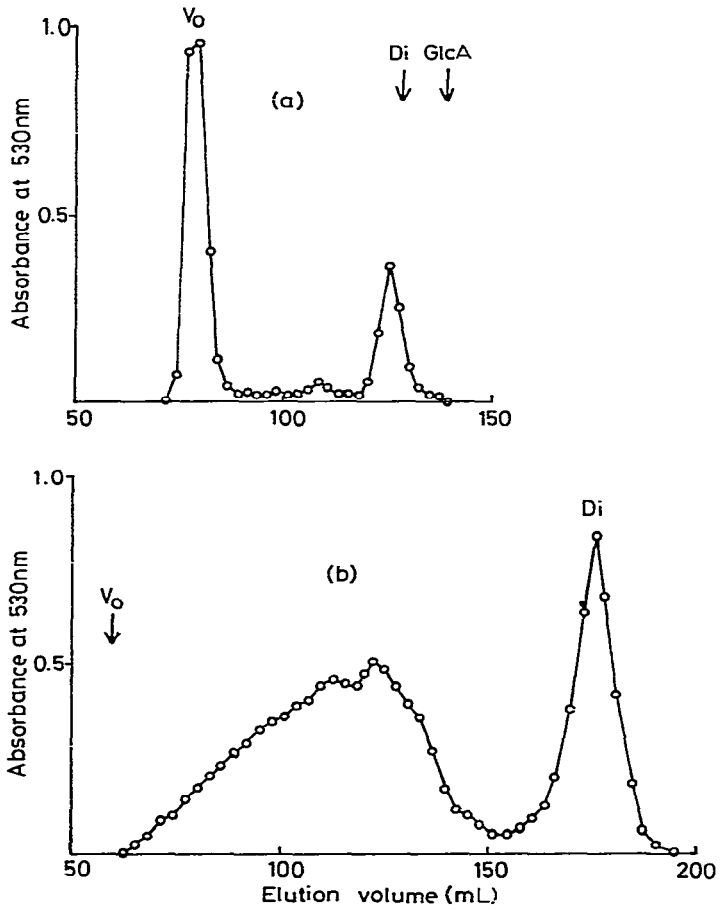

Fig. 4. Gel filtration, on (a) Sephadex G-25 and (b) Sephadex G-75, of the reaction products of chondroitin 6-sulfate treated with methanol for 18 h at 95°.

diluted with an equal volume of water, and the pH was adjusted to 6.8 by the addition of 0.5M sodium hydroxide. The solution was evaporated to dryness under diminished pressure, and the residue was dissolved in 0.1M sodium chloride (1.5 mL). A portion (0.5 mL) of this solution was analyzed on a column of Sephadex G-25 (Fig. 4a), and the remaining solution was analyzed on a column of Sephadex G-75 (Fig. 4b).

Depolymerization of chondroitin 6-sulfate (free acid) with dimethyl sulfoxide containing 10% of methanol. — A solution of chondroitin 6-sulfate (free acid, 5 mg) in dimethyl sulfoxide containing 10% of methanol (2.5 mL) was heated for 18 h at 95°, cooled, treated by the method described above, and chromatographed on Sephadex G-25.

Depolymerization of dermatan sulfate (pyridinium salt) with dimethyl sulfoxide containing 10% of methanol. — A solution of the pyridinium salt of dermatan sulfate (5 mg) in dimethyl sulfoxide containing 10% of methanol (2.5 mL) was heated for

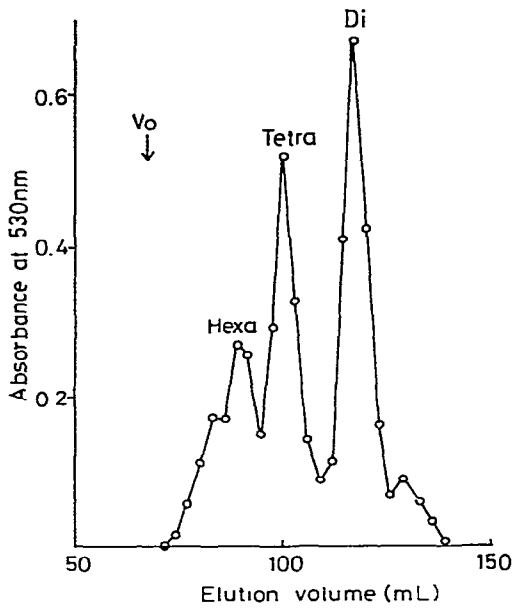

Fig. 5. Gel filtration, on Sephadex G-25, of the reaction products of dermatan sulfate treated with 1:9 (v/v) MeOH–Me_2SO for 18 h at 95°.

18 h at 95°, cooled, treated by the method described above, and chromatographed on Sephadex G-25 (Fig. 5).

Depolymerization of hyaluronic acid (free acid) with dimethyl sulfoxide containing 10% of methanol in the presence of various acidic substances. — To prepare a 50mM pyridinium sulfate solution in methanol, pyridine–sulfur trioxide (79.58 mg) was dissolved in water (5 mL) with gentle heating, the solution was lyophilized, and the lyophilizate was dissolved in methanol (10 mL). Hyaluronic acid (free acid, 3 mg for each sample) was weighed accurately into four test tubes. To each was added dimethyl sulfoxide (1.8 mL) and, after mixing, absolute methanol (0.2 mL), 50mM hydrogen chloride in methanol (0.2 mL), 50mM pyridine–sulfur trioxide in methanol (0.2 mL), or pyridinium sulfate in methanol (0.2 mL), to form a homogeneous solution. Hyaluronic acid (3.79 mg) was weighed into another test tube, and 5mM hydrogen chloride in methanol (2 mL) was added. All test tubes were heated for 24 h at 100°, and the content of each test tube was cooled, diluted with water (2 mL), neutralized with 0.1M sodium hydroxide, and dialyzed against running tap-water for 20 h and then against distilled water for 20 h. The nondialyzable fractions obtained were each diluted with water to a volume of 50 mL, and an 0.5-mL aliquot of each was analyzed for uronic acid (Table II).

Depolymerization of hyaluronic acid (free acid) with dimethyl sulfoxide containing 10% of methanol in the presence or absence of pyridine–sulfur trioxide. — Methanol (0.5 mL) or 50mM pyridine–sulfur trioxide in methanol (0.5 mL) was added to a solution of hyaluronic acid (free acid, 10 mg) in dimethyl sulfoxide

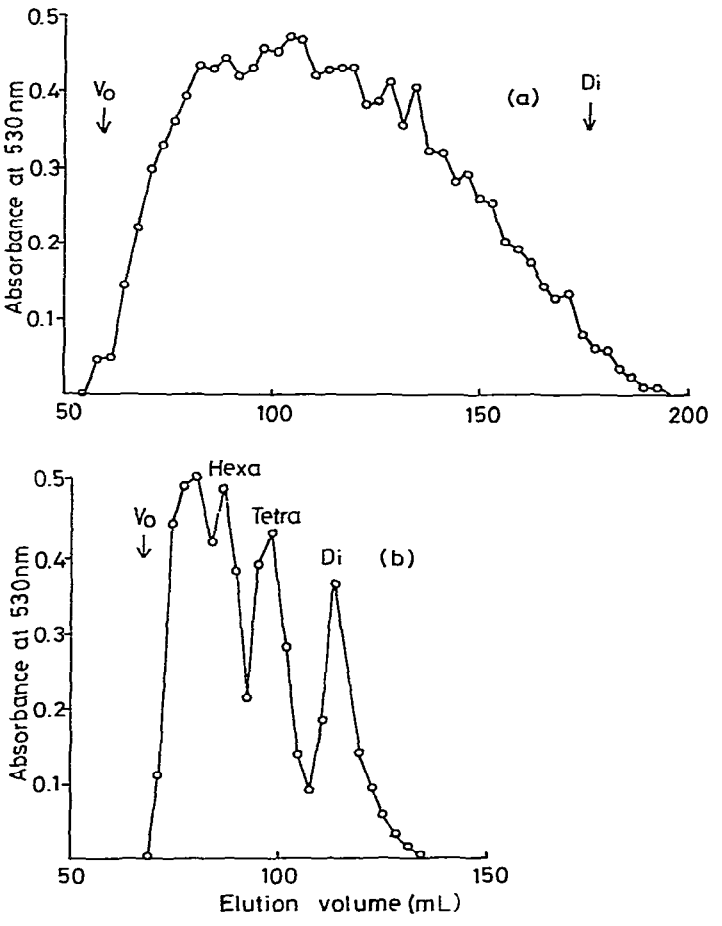

Fig. 6. Gel filtration, on (a) Sephadex G-75 and (b) Sephadex G-25, of the reaction products of hyaluronic acid treated with 1:9 (v/v) MeOH–Me_2SO for 18 h at 95°: (a) in the absence and (b) in the presence of an amount of $C_5H_5N \cdot SO_3$ equimolar to the disaccharide unit.

(4.5 mL). The reaction mixture was treated by the method described in the section on chondroitin 6-sulfate, and chromatographed on Sephadex G-75 or G-25 (Figs. 6a and 6b).

Depolymerization of N-*desulfated,* N-*acetylated heparin* (*pyridinium salt*) *with dimethyl sulfoxide containing 10% of methanol.* — A solution of the pyridinium salt of *N*-desulfated, *N*-acetylated heparin (90 mg) in dimethyl sulfoxide containing 10% of methanol (20 mL) was heated for 18 h at 95°, cooled, diluted with an equal volume of water, and the pH adjusted to 6.8 by the addition of 0.5M sodium hydroxide. The solution was concentrated under diminished pressure to a small volume and diluted with 10% ethanol (5 mL). A portion (0.1 mL) of the solution was chromatographed on Sephadex G-25 (Fig. 7a), and another portion (0.2 mL) on Sephadex G-75 (Fig. 7b). The residual solution was loaded on a column (2.5 × 90 cm) of Sephadex

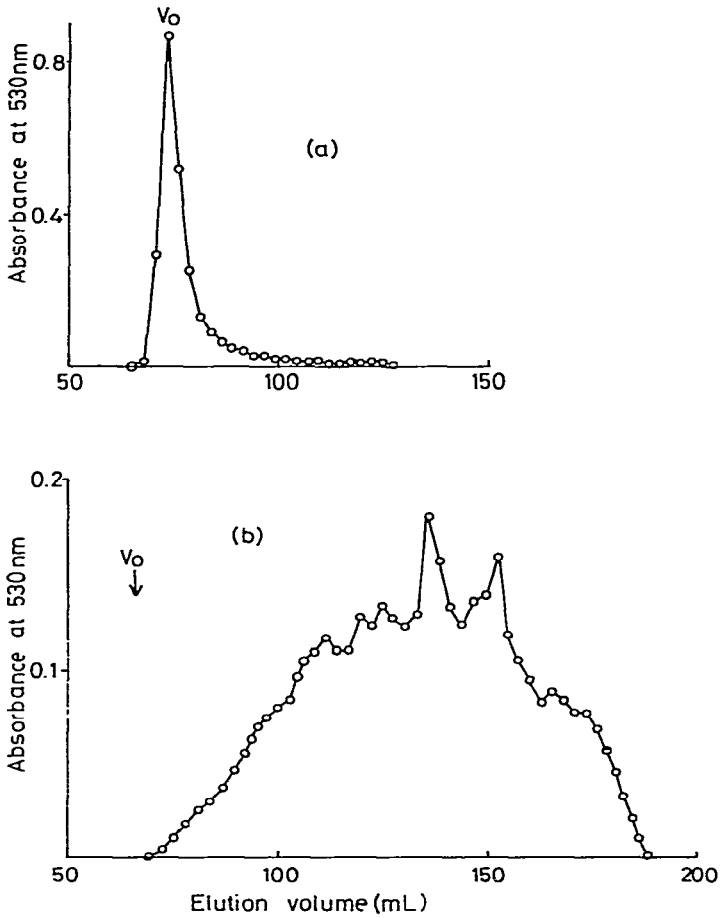

Fig. 7. Gel filtration, on (a) Sephadex G-25 and (b) Sephadex G-75, of the reaction products of *N*-desulfated, *N*-acetylated heparin treated with 1:9 (v/v) MeOH–Me_2SO for 18 h at 95°.

G-15, and the column was eluted with 10% ethanol. The fractions eluted in the excluded volume were combined and lyophilized to give a pale-yellow powder (40 mg). The n.m.r. spectrum of this powder was measured on a solution in deuterium oxide.

RESULTS AND DISCUSSION

The pyridinium salt of chondroitin 6-sulfate was treated with dimethyl sulfoxide containing 10% of methanol for 18 h at 95°. Gel filtration of the reaction products on a column of Sephadex G-25 gave the results shown in Fig. 1. The amount of uronic acid recovered in the reaction products was almost the same as the uronic acid content of the starting material used. Neither positive ninhydrin reaction nor reducing power was detected for any of the peaks in the elution diagram. The reaction products were

fractionated on anion-exchange resin into separate uronic acid-containing fractions (Fig. 2); the first and second peaks (a and b) and the fourth peak (d) were characterized as those of disaccharides and a tetrasaccharide, respectively. The ratio of di- (Peak b) to tetra-saccharides (Peak d) was ~4.6:1, as estimated from the total color-intensity of the carbazole reaction. The third peak (c) could not be examined further due to its minute amount. The proportion of higher oligosaccharides increased for a lower reaction-temperature. The ratios of di-, tetra-, and hexa-saccharides in the reaction products at 90° were ~3.3:1.9:1 (the elution diagram is not shown).

Fraction 3 (see Fig. 2) was desalted by gel filtration on Sephadex G-15 and gave one major and two minor spots having similar R_F values on thin-layer cellulose plates with solvents *A* and *B*. After purification on a column of silicic acid, Fraction 3 afforded Compound I, which showed a single spot on silicic acid or cellulose thin-layer plates, although it was difficult to crystallize. On paper electrophoresis, Compound I migrated toward the anode at a rate a little lower than that of D-glucuronic acid, and gave a negative result both with the ninhydrin test and for reducing power. Hydrolysis of Compound I (M hydrochloric acid, boiling-water bath, 1 h) gave chondrosine alone, as characterized by thin-layer chromatography. The n.m.r. spectrum of Compound I showed two singlet-peaks of three protons at δ 1.84 and 3.22, attributed to an *N*-acetyl and a methoxyl group, respectively. The anomeric protons resonated at δ 4.25 (*J* 7 Hz) and 4.63 (*J* 2 Hz). The latter signal of the anomeric protons corresponded to that at δ 4.66 of the tetrasaccharide (see later), which was assigned to an equatorial anomeric proton of the reducing residue, based on its relative integral to *N*-acetyl protons and a narrow spacing. Therefore, the latter signal was assigned to H-1 of the 2-acetamido-2-deoxy-D-galactose residue, and the former signal to H-1 of the D-glucuronic acid residue (Table I). The highly negative value of the optical rotation of Compound I suggested a β-D-configuration for the reducing 2-acetamido-2-deoxy-D-galactose residue and consequently a $^1C_4$(D) conformation. From these results and the results of elemental analysis, the structure of Compound I is methyl 2-acetamido-2-deoxy-3-*O*-(lithium β-D-glucopyranosyl-

TABLE I

N.M.R. DATA (δ) FOR COMPOUNDS I AND II DERIVED FROM CHONDROITIN 6-SULFATE[a]

Compound	*Solvent*	*Acetyl group*	*Methoxyl group*	*H-1 of GlcA*	*H-1 of reducing GalNAc*
I	[$^2H_6$]Me_2SO	1.84 (3 H)	3.22 (~ 3 H)	4.25 (*J* 7)	4.63 (1 H) (*J* 2)
	[$^2H_4$]MeOH	1.94 (3 H)	3.34	4.46 (1 H) (*J* 7.2)	4.80 (1 H) (*J* 1.5)
II	[$^2H_6$]Me_2SO	1.84 (6 H)	3.20	4.27	4.66 (1 H)

[a] *J* values in Hz.

uronate)-β-D-galactopyranoside. Compound I as well as its reducing disaccharide, *N*-acetylchondrosine, were not digested by β-D-glucuronidase of bovine liver under the conditions reported[12].

Peak a (Fig. 2, Fraction 1) was eluted in earlier fractions than Compound I, and gave a negative reaction with the ninhydrin reagent and for reducing power. Its positive optical rotation ($[\alpha]_D^{29}$ +48.8°, *c* 0.736, methanol) and formation of chondrosine by hydrolysis (M hydrochloric acid, boiling-water bath, 1 h) indicated that it contained the α anomer of Compound I.

Peak d (Fig. 2, Fraction 4) was desalted by gel filtration on Sephadex G-25 and showed a single spot on thin-layer cellulose plates with solvents *A* and *B*. The powder obtained from Fraction 4 was dissolved in a small volume of methanol and precipitated by the addition of acetone to give Compound II as a white powder. Compound II was digested by β-D-glucuronidase of bovine liver, and the products formed were fractionated on a column of Sephadex G-25 to afford two peaks that emerged from the column in the positions expected for a trisaccharide and glucuronic acid (Fig. 3). The negative optical rotation and the presence of the *N*-acetyl and methoxyl groups as shown by n.m.r. spectrometry (Table I) indicated that Compound II was the methyl β-glycoside of a tetrasaccharide consisting of two *N*-acetylchondrosine units.

The sodium salt of chondroitin 6-sulfate was neither desulfated nor depolymerized with dimethyl sulfoxide containing methanol, whereas its free acid was desulfated and depolymerized at a rate a little higher than that of the pyridinium salt; the uronic acid-containing material formed was recovered in a good yield (data not shown). The pH at the end of the reaction of free chondroitin 6-sulfate was 3.17, whereas that for the reaction of the pyridinium salt was 3.70. The presence of pyridine, therefore, does not seem to be essential for this reaction. On the other hand, the solvent effect of dimethyl sulfoxide was found to be considerable, as can be seen in a more limited depolymerization of the pyridinium salt of chondroitin 6-sulfate heated in methanol alone for 18 h at 95° (Figs. 4a and 4b). The elution diagrams of Figs. 4a and 4b indicate that only a partially solubilized portion of the pyridinium salt of chondroitin 6-sulfate in methanol was depolymerized to the disaccharide level under the conditions used. In the case of the pyridinium salt of chondroitin 4-sulfate, approximately the same desulfation and depolymerization took place in dimethyl sulfoxide containing 10% of methanol (data not shown). Consequently, treatment of chondroitin 4- and 6-sulfates (pyridinium salts or free acids) with dimethyl sulfoxide containing 10% of methanol results in the solvolytic desulfation of the ester sulfates at the initial stage, followed by methanolysis of the 2-acetamido-2-deoxy-D-galactosyl linkages accelerated by the solvent effect of dimethyl sulfoxide under mild acidic conditions.

The pyridinium salt of dermatan sulfate was also depolymerized under the same conditions at a rate a little lower than those of chondroitin sulfates to give di-, tetra-, and hexa-saccharides, as shown in Fig. 5. It is remarkable that no monosaccharides were produced, in contrast to the previous report that acid hydrolysis

TABLE II

DEPOLYMERIZATION OF HYALURONIC ACID (FREE ACID) WITH DIMETHYL SULFOXIDE CONTAINING 10% OF METHANOL IN THE PRESENCE OF VARIOUS ACIDIC SUBSTANCES[a] FOR 24 H AT 100°

Reaction medium	*Nondialyzable fraction (A_{530}/mg)*	*Uronic acid content of nondialyzable fraction/ initial uronic acid content (%)*
Water	0.2054	100
1:9 (v/v) MeOH–Me_2SO	0.1606	82
$C_5H_5N \cdot SO_3$[a] in 1:9 (v/v) MeOH–Me_2SO	0.0687	37
$C_5H_5N \cdot H_2SO_4$[a] in 1:9 (v/v) MeOH–Me_2SO	0.0770	38
HCl[a]–MeOH	0.1625	80
HCl[a] in 1:9 (v/v) MeOH–Me_2SO	0.1827	94

[a]In an amount equimolar to the disaccharide unit.

of dermatan sulfate yields a product containing approximately equal amounts of mono- and oligo-saccharides[4].

Although hyaluronic acid (free acid) was poorly depolymerized with methanolic hydrogen chloride or dimethyl sulfoxide containing 10% of methanol under the conditions used, addition of an equimolar amount of pyridine–sulfur trioxide or pyridinium sulfate per disaccharide unit to the reaction mixture containing dimethyl sulfoxide–10% methanol considerably accelerated the reaction rate, as indicated in Table II and Figs. 6a and 6b. The pH at the end of the reaction in the presence or absence of pyridine–sulfur trioxide was 3.60 or 3.74, respectively. The results of the depolymerization of hyaluronic acid (Table II and Fig. 6), together with those of chondroitin 6-sulfate and dermatan sulfate, suggest that the solvolytic depolymerization is greatly accelerated by the presence of the sulfate and methyl sulfate ions that are released during the reaction or had been added at the beginning of the reaction.

The pyridinium salt of *N*-desulfated, *N*-acetylated heparin was treated with dimethyl sulfoxide containing 10% of methanol for 18 h at 95°, and most of the reaction product was eluted in the excluded volume from the column of Sephadex G-25 (Fig. 7a). Gel filtration of the product on Sephadex G-75 showed a marked heterogeneity in molecular-size distribution (Fig. 7b). The n.m.r. data indicate that very few of the *N*-acetyl groups of *N*-desulfated, *N*-acetylated heparin were hydrolyzed during the reaction (data not shown). Therefore, it is unlikely that the free amino groups formed contributed to the stabilization of the 2-acetamido-2-deoxy-D-glucoside linkages of the polysaccharide. The marked stability of *N*-desulfated, *N*-acetylated heparin to the solvolytic conditions may be due to the stability of the 2-acetamido-2-deoxy-α-D-glucopyranoside bonds themselves. To confirm this hypothesis, methyl 2-acetamido-2-deoxy-α-D-glucopyranoside and its β-D anomer were treated with dimethyl sulfoxide containing 10% of water at 95° in the presence of an equimolar amount of pyridine–sulfur trioxide, and the reducing power of the

reaction mixture was measured at regular intervals. It was observed that the glycoside bond of the α-D anomer was much more stable than that of the β-D anomer under the conditions used (data not shown). These results are consistent with the data reported by Shively and Conrad[13], together with our previous results indicating that the conditions used in the solvolytic desulfation of heparin do not fragment the polysaccharide chain to any great extent, although a part of the amino groups of native heparin is acetylated[14,15].

As described in the preceding paragraphs, solvolysis with dimethyl sulfoxide containing 10% of methanol results in the cleavage of the ester sulfate groups and the 2-amino-2-deoxyglycoside bonds of glycosaminoglycuronan sulfates consisting of 2-acetamido-2-deoxy-β-D-hexose and uronic acid residues to give mainly methyl D-glycosides of disaccharides and higher oligosaccharides. For hyaluronic acid, a similar solvolytic depolymerization proceeds in the presence of an amount of pyridine–sulfur trioxide or pyridinium sulfate equimolar to the disaccharide unit. It should be noted that the uronic acid components are neither degraded nor esterified, and that the *N*-acetyl groups are markedly stable under these solvolytic conditions.

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