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PREPARATION, BY CHEMICAL DEGRADATION OF HYALURONIC ACID, OF A SERIES OF EVEN- AND ODD-NUMBERED OLIGOSACCHARIDES HAVING A 2-ACETAMIDO-2-DEOXY-D-GLUCOSE AND A D-GLUCURONIC ACID RESIDUE, RESPECTIVELY, AT THE REDUCING END

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ABSTRACT

A series of even-numbered hyaluronate oligosaccharides (di- to octadeca-) having a 2-acetamido-2-deoxy-D-glucose residue at the reducing end was prepared by treatment of sodium hyaluronate with dimethyl sulfoxide containing 10% of 0.1M hydrochloric acid for 16 h at 95°. The mixture of the even-numbered oligosaccharides obtained was converted with 0.15M sodium carbonate for 6 h at 40° into a series of odd-numbered oligosaccharides (mono- to pentadeca-) having a D-glucuronic acid residue at the reducing end. Reaction of the hyaluronate tetrasaccharide with saturated calcium hydroxide gave β-D-Glc*p*A-(1→3)-β-D-Glc*p*NAc-(1→3)-D-*arabo*-trihydroxyglutaric acid besides the expected trisaccharide.

INTRODUCTION

Hyaluronic acid is a high-molecular-weight glycosaminoglycuronan which consists of alternating 2-acetamido-2-deoxy-β-D-glucose and β-D-glucuronic acid residues linked (1→3) and (1→4), respectively[1]. It has been usually depolymerized to even-numbered oligosaccharides by enzymes. Testicular hyaluronidase gave a series of oligosaccharides consisting of the disaccharide unit, β-D-Glc*p*A-(1→3)-β-D-Glc*p*NAc, and leech hyaluronidase a second group of oligosaccharides consisting of the reversed sequence β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*A[2,3]. Chemical depolymerization of the polysaccharide by acid hydrolysis or methanolysis gave hyalobiuronic acid or its methyl glycoside[4,5]. In our previous papers, it was reported that a sulfated glycosaminoglycuronan, chondroitin 6-sulfate (free acid or pyridinium salt) was desulfated and depolymerized to form a series of nonsulfated di- to octadeca-saccharides having a 2-acetamido-2-deoxy-D-galactose residue at the reducing end by treatment with dimethyl sulfoxide containing 10% of water[6],

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or methanol[7]. Furthermore, a nonsulfated glycosaminoglycuronan, hyaluronic acid (free acid), was depolymerized to some extent with the dimethyl sulfoxide reagents, and the addition of either pyridinium sulfate or pyridinium chloride greatly accelerated the reaction. Since the presence of pyridine was found not to be essential for the reaction, the addition of a small amount of inorganic acid was considered to be responsible for the enhancement of the reaction rate.

The present report describes the formation of a series of even-numbered oligosaccharides from hyaluronic acid by solvolysis with aqueous dimethyl sulfoxide containing a small amount of hydrochloric acid, the transformation of the oligosaccharides into a series of odd-numbered oligosaccharides by treatment with alkali, and a discussion of the alkali treatment.

RESULTS AND DISCUSSION

The reaction products, obtained by hydrolysis of sodium hyaluronate with 10mM hydrochloric acid or with dimethyl sulfoxide containing 10% of 0.1M hydrochloric acid for 16 h at 105°, were chromatographed on AG 1-X2 (Cl^-) anion-exchange resin[8]. Peaks 2, 4, 6, and 8 (Figs. 1a,b) were identified as di-, tetra-,

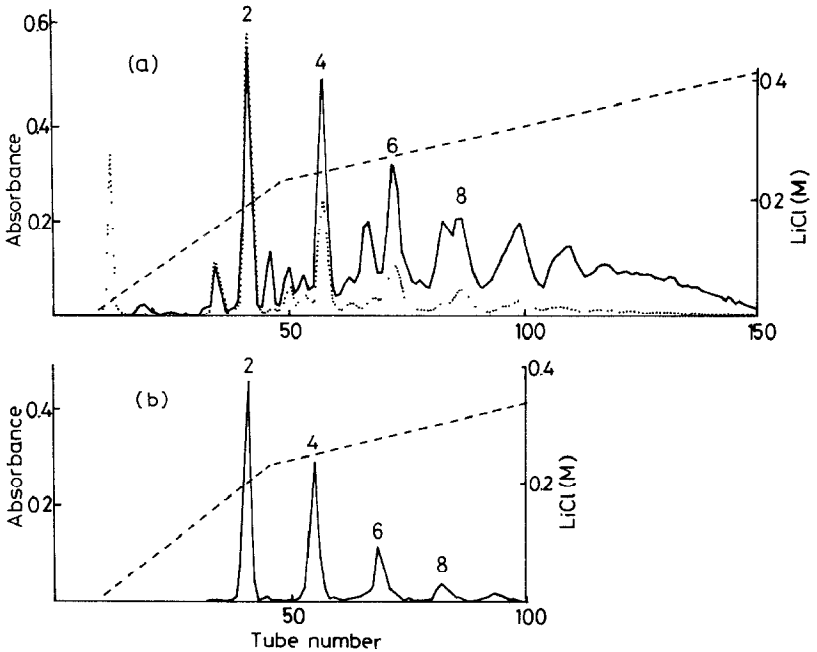

Fig. 1. Anion-exchange chromatography, on AG 1-X2 (Cl^-) resin, of the reaction products of hyaluronic acid treated for 16 h at 105° with: (a) 10mM hydrochloric acid, and (b) 1:9 (v/v) 0.1M hydrochloric acid–dimethyl sulfoxide; (——) carbazole reaction (absorbance at 530 nm), (·····) Morgan–Elson reaction (absorbance at 585 nm), and (------) concentration of lithium chloride. Unless otherwise noted, the concentration of the sample subjected to the carbazole reaction was the same as that subjected to the Morgan–Elson reaction

TABLE I

DEPOLYMERIZATION OF HYALURONIC ACID *(HA)* WITH DIMETHYL SULFOXIDE CONTAINING 10% OF ACID

Acid	*Reaction conditions*				*Reaction products*[a] *(%)*	
	Conc. of HA (mg/mL)	*Temp. (°)*	*Time (h)*	*pH*[b]	*Disaccharide*	*Higher mol.-wt. oligosaccharides*
0.2M HCl	2	105	15.5	2.54	95	5
0.1M HCl	2	105	15.5	2.93	48	52
50mM HCl	2	105	30	3.66	3	97
0.1M HCl	2	95	15.5	2.96	11	89
0.1M HCl	4	95	15.5	3.36	1	99
0.1M HCl	2	90	15.5	3.01	4	96
0.1M H_2SO_4	2	105	15.5	2.79	56	44
50mM H_2SO_4	2	105	15.5	3.30	4	96

[a]Proportion of each fraction-size of the reaction products separated on Sephadex G-25 (based on uronic acid determination). [b]The pH was measured after an equal volume of water had been added to the reaction mixture.

hexa-, and octa-saccharides having a 2-acetamido-2-deoxy-D-glucose residue at the reducing end. A free 2-amino-2-deoxyhexose residue was not detected in any fraction (Figs. 1a,b) by the trinitrophenylation method[9]. Accordingly, it is suggested that the peaks positive with the Morgan–Elson reagent, except those containing the even-numbered oligosaccharides (see Fig. 1a), contain odd-numbered oligosaccharides having a 2-acetamido-2-deoxy-D-glucose residue at the reducing end, and that the peaks negative with the reagent contain odd-numbered oligosaccharides having a D-glucuronic acid residue at the reducing end. The results illustrated in Figs. 1a,b suggest that, in dimethyl sulfoxide containing 10% of 0.1M hydrochloric acid, the rate of hydrolysis of the 2-acetamido-2-deoxy-D-glucosyl linkage in the polysaccharide is accelerated, and that of the D-glucuronosyl linkage is retarded probably by the solvent effect of dimethyl sulfoxide. The results of the depolymerization performed under various conditions indicate that the reaction primarily depends on the pH and temperature (Table I).

Sodium hyaluronate was treated, on a preparative scale, with dimethyl sulfoxide containing 10% of 0.1M hydrochloric acid for 16 h at 95°. Gel chromatography of an aliquot of the reaction product did not reveal the formation of free 2-acetamido-2-deoxy-D-glucose, which may have been liberated from the reducing end of the even-numbered oligosaccharides formed. A major part of the reaction product was fractionated on AG 1-X2 (Cl^-) anion-exchange resin into di- to octadeca-saccharide fractions (Fig. 2). The analytical data, summarized in Table II, are in good agreement with the values calculated for the respective even-numbered oligosaccharides.

It is known[10] that the reducing terminal 2-acetamido-2-deoxy-D-glucose residue of hyaluronate oligosaccharides is decomposed under alkaline conditions to form chromogens having a maximum absorption at 230 nm, and the stability of

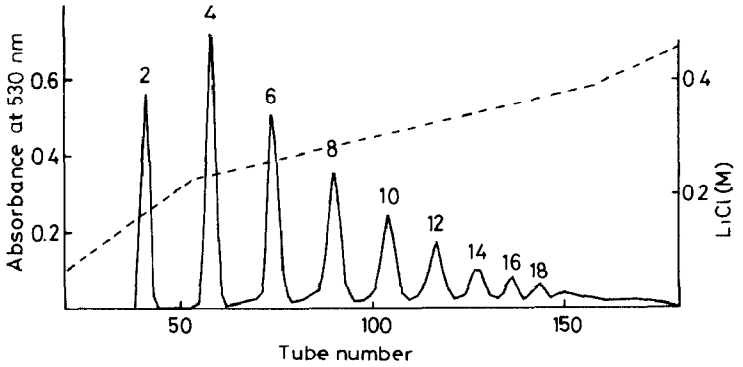

Fig. 2. Anion-exchange chromatography, on AG 1-X2 (Cl^-) resin, of the reaction product of hyaluronic acid treated with 1:9 (v/v) 0.1M hydrochloric acid–dimethyl sulfoxide for 16 h at 95°: (——) carbazole reaction, and (------) concentration of lithium chloride.

the even-numbered hyaluronate oligosaccharides was examined. Hyaluronate hexasaccharide was heated at 60° in buffer solutions of pH 6.0–9.0 and the absorbance at 235 nm measured at various time intervals. The results (see Fig. 3a) indicated that the rate of decomposition decreased with reduction of the pH of the solution and that decomposition proceeded slowly, even at pH 6.5. Therefore, the mixture obtained after depolymerization had to be treated at pH 5.5–6.0 in order

TABLE II

ANALYTICAL DATA OF EVEN-NUMBERED OLIGOSACCHARIDES (LITHIUM SALTS) PREPARED BY TREATMENT OF HYALURONIC ACID WITH DIMETHYL SULFOXIDE CONTAINING 10% OF 0.1M HYDROCHLORIC ACID FOR 16 h AT 95°

Oligo-saccharides[a]	*Yield*[b] *(mg)*	*Uronic acid (%)*	*2-Amino-2-deoxyhexose (%)*	*Ratio of 2-amino-2-deoxyhexose to uronic acid residues*		*Ratio of reducing 2-acetamido-2-deoxyhexose to uronic acid residues*[c,d]
				Before reduction	*After reduction*[d]	
2	67.3	41.76	38.64	1.00	0.05 (0)	1.00 (1.00)
4	91.8	43.23	40.45	1.01	0.50 (0.50)	0.50 (0.50)
6	80.0	44.39	40.87	1.00	0.67 (0.67)	0.33 (0.33)
8	64.7	46.97	43.43	1.00	0.74 (0.75)	0.24 (0.25)
10	46.6	46.75	43.48	1.01	0.79 (0.80)	0.19 (0.20)
12	34.7	46.20	43.12	1.01	0.83 (0.83)	0.16 (0.17)
14	25.0	47.47	44.06	1.01	0.88 (0.86)	0.13 (0.14)
16	14.0	47.40	44.00	1.01	0.89 (0.88)	0.11 (0.13)
18	8.2	47.25	42.74	0.98	0.88 (0.89)	0.10 (0.11)
>20	25.8	47.71	44.33	1.01	0.93 (>0.90)	0.08 (<0.10)

[a]Number of monosaccharide units. [b]Amount of product obtained from 640 mg of hyaluronic acid. [c]Expressed relative to the molar ratio of 2-acetamido-2-deoxyhexose to uronic acid residue in *N*-acetylhyalobiuronic acid. [d]In parentheses, calculated value for each oligosaccharide.

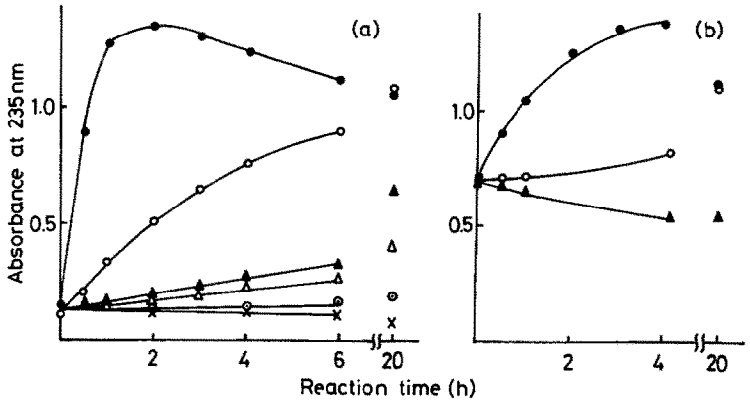

Fig. 3. Determination of absorbance of hyaluronate- (a) and chondroitin-hexasaccharide (b), in buffer solutions having various pH values at 60°: (●) pH 9.0, (○) pH 8.0, (▲) pH 7.0, (△) pH 6.5, (⊙) pH 6.0, and (×) pH 5.5.

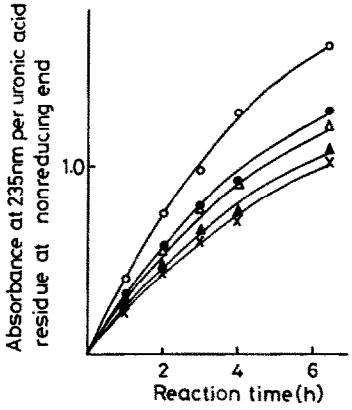

Fig. 4. Determination of absorbance of hyaluronate di- to tetradeca-saccharides in a buffer solution of pH 8.0 at 60°: (○) di-, (●) tetra-, (△) hexa-, (▲) deca-, and (×) tetradeca-saccharide.

to obtain the even-numbered hyaluronate oligosaccharides. On the contrary, the chondroitin hexasaccharide was much more stable than the hyaluronate hexasaccharide at pH 7.0–9.0 (Fig. 3b). To compare the stabilities in a buffer solution of pH 8.0, hyaluronate di- to tetradeca-saccharides were heated at 60°. The results (Fig. 4) showed that the smaller the oligosaccharide was, the more unstable it was in buffer solution.

In order to find the best conditions for preparing odd-numbered hyaluronate oligosaccharides from the parent even-numbered oligosaccharides by alkaline degradation, the hyaluronate tetrasaccharide was treated with various alkaline reagents. Treatment with 20mM carbonate buffer (pH 10.0) for 5 min at 100°, or 0.15M sodium carbonate[11] for 2 h at 37°, gave, on AG 1-X2 (Cl^-) anion-exchange column chromatography (elution diagrams not shown), a single peak that was posi-

TABLE III

^{13}C-N M R. DATA (δ) FOR *N*-ACETYLHYALOBIURONIC ACID AND TRISACCHARIDE-II

	β-D-*GlcpA*[a]	*β*-D-*GlcpNAc*[a]	N-*Acetyl-hyalobiuronic acid*			*Trisaccharide-II*		
			β-D-*GlcpA*	D-*GlcpNAc*		*β*-D-*GlcpA*	*β*-D-*GlcpNAc*	D-arabo-*Trihydroxy-glutaric acid*
				α	*β*			
C-1	104.1	102.9	103.7	92.0	95.7	103.9	101.4	
C-2	74.0	56.5 (55.5[b])	73.7	53.7	56.6	73.7	55.3	72.9 or 74.6
C-3	76.6	75.0 (84.0[b])	76.4	81.6	84.0	76.3	84.1	81.9
C-4	72.9	71.0 (70.0[b])	72.6	69.8	69.8	72.6	69.9	72.9 or 74.6
C-5	77.0	76.9	76.6	72.3	76.4	76.3	76.5	
C-6		61.8		61.7	61.7		62.2	
C=O	176.7	175.5	176.4	175.5	175.8	176.3	175.9	178.8, 179.6
CH_3		23.2		23.1	23.3		23.5	

[a]Values reported by Bociek *et al.*[15]. [b]In parentheses, predicted values due to additional substitution at C-3.

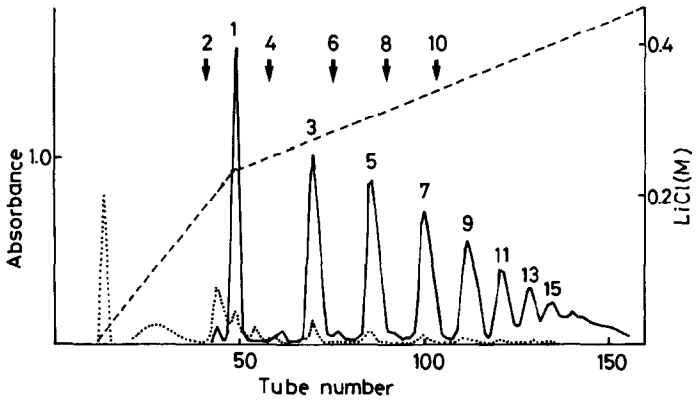

Fig. 5. Anion-exchange chromatography, on AG 1-X2 (Cl^-) resin, of the reaction products obtained by treatment of hyaluronic acid with 1:9 (v/v) 0.1M hydrochloric acid–dimethyl sulfoxide for 16 h at 95°, followed by treatment with 0.15M sodium carbonate for 6 h at 40°: (——) carbazole reaction (absorbance at 530 nm); (·····) Morgan–Elson reaction (absorbance at 585 nm), and (------) concentration of lithium chloride. Each of the fractions was subjected to the Morgan–Elson reaction without prior dilution. For uronic acid assay, each fraction was diluted twenty times with water, then subjected to the carbazole reaction. The arrows indicate the elution positions of hyaluronate di- to deca-saccharides.

tive to the carbazole reaction. The peak material was isolated and identified as the expected trisaccharide, β-D-Glc*p*A→β-D-Glc*p*NAc→D-Glc*p*A (Trisaccharide-I). Prolonged reaction (~30 min) under the former conditions or reaction at elevated temperature (~60°) under the latter conditions resulted in a decrease of the trisaccharide peak and appearance of small peaks at an earlier elution time, indicating that the trisaccharide *per se* is unstable to alkali. Treatment with M ammonium hydroxide for 2 h at 37° or with Dowex 1-X2 (HO^-) anion-exchange resin[12] for 24 h at room temperature was found to be unsuitable for degradation because of formation of by-products. Treatment with a saturated calcium hydroxide solution[13] afforded, on AG 1-X2 (Cl^-) anion-exchange column chromatography, two peaks positive to the carbazole reaction, one of which corresponded to Trisaccharide-I (elution diagram not shown). The second peak (Trisaccharide-II), which was eluted at a more retarded position, slightly increased under an oxygen atmosphere or under air free from carbon dioxide, and disappeared under a nitrogen atmosphere. Trisaccharide-I was converted into Trisaccharide-II, in 14% yield based on the carbazole reaction (experiments not described), upon treatment with saturated aqueous calcium hydroxide for 4 h at room temperature.

Trisaccharide-II did not show reducing power by the Park–Johnson method[14]. The ^{13}C-n.m.r. spectrum showed five signals at δ 72.9, 74.6, 81.9, 178.8, and 179.6, in addition to those due to the *N*-acetylhyalobiuronic acid unit; these signals (Table III) were assigned on the basis of the assignment reported by Bociek *et al.*[15]. The ^1H-n.m.r. spectrum of the methyl ester of Trisaccharide-II showed an intensity of the methyl ester signal (δ 3.79) corresponding to 3.27 moles per mole of acetamido signal (δ 2.00), which was probably over-estimated because of over-

TABLE IV

ANALYTICAL DATA OF ODD-NUMBERED OLIGOSACCHARIDES (LITHIUM SALTS) PREPARED BY TREATMENT OF HYALURONIC ACID WITH DIMETHYL SULFOXIDE CONTAINING 10% OF 0.1M HYDROCHLORIC ACID FOR 16 h AT 95°, FOLLOWED BY ALKALINE DEGRADATION WITH 0.15M SODIUM CARBONATE FOR 6 h AT 40°

Oligosaccharides[a]	*Yield*[b] *(mg)*	*Uronic acid (%)*	*2-Amino-2-deoxyhexose (%)*	*Ratio of 2-amino-2-deoxyhexose to uronic acid residues*		*Ratio of reducing 2-acetamido-2-deoxyhexose to 1000 uronic acid residues at nonreducing end*
				Before reduction[c]	*After reduction*	
1	34.2	78.82	1.26	0.02 (0)	0	17
3	58 5	57.86	26.00	0.49 (0.50)	0.84	21
5	63.9	51.09	30.61	0.65 (0.66)	0.77	19
7	44.9	50.41	34.22	0.74 (0.75)	0.75	19
9	43.5	51.12	37.70	0.80 (0.80)	0.88	21
11	32.9	51.14	39 35	0.83 (0.83)	0.83	21
13	20.6	49.23	39.15	0.86 (0.86)	0.89	22
15	7.3	49.40	39.40	0.86 (0.88)	0.90	20
>17	41.2	51.65	43.77	0.92 (>0.89)	0.95	17

[a]Number of monosaccharide units. [b]Amount of product obtained from 640 mg of hyaluronic acid. [c]In parentheses, calculated value for each oligosaccharide.

lapping with the signals of the ring protons. This suggested that Trisaccharide-II contains three carboxyl groups. Trisaccharide-II was treated with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride and then reduced with sodium borohydride. Reduced Trisaccharide-II was hydrolyzed and the product reduced with sodium borohydride to give the corresponding alditols, which were analyzed by g.l.c. after trimethylsilylation. The retention times of the two peaks obtained were identical with those of standard D-arabinitol and D-glucitol. Accordingly, the structure of Trisaccharide-II was established as β-D-Glc*p*A-(1→3)-β-D-Glc*p*NAc-(1→3)-D-*arabo*-trihydroxyglutaric acid.

On the basis of the aforementioned results, a series of odd-numbered oligosaccharides (mono- to pentadeca-) having a D-glucuronic acid residue at the reducing end was prepared from sodium hyaluronate. The elution diagram of the reaction product on AG 1-X2 (Cl^-) anion-exchange chromatography and the analytical data of the material from each peak are given in Fig. 5 and Table IV, respectively. Each peak corresponding to the mono- to pentadeca-saccharide was weakly positive with the Morgan–Elson reagent, showing ~0.02 mmol of 2-acetamido-2-deoxyhexose at the reducing end per mol of uronic acid residue at the nonreducing end. This is probably not due to the presence of unreacted even-numbered oligosaccharides, but to the known peeling-reaction under the alkaline conditions of the Morgan–Elson assay[16]. As seen in Fig. 5, each even-numbered oligosaccharide was eluted earlier than the odd-numbered oligosaccharide from which it derived. The molar ratios of 2-amino-2-deoxyhexose to uronic acid residue before reduction with sodium borohydride were in good agreement with the values

calculated for the corresponding odd-numbered oligosaccharide, but those obtained after reduction were not (see Table IV). This discrepancy among the values obtained is probably due to the slightly visible coloration given, in the carbazole reaction, by the L-gulonic acid residue resulting from the reduction of the reducing D-glucuronic acid residue.

EXPERIMENTAL

Materials. — Rooster comb hyaluronic acid (M_r 2 000 000, determined by viscometry) was obtained from Seikagaku Kogyo Co., Tokyo. Chondroitin hexasaccharide was obtained by solvolytic depolymerization of chondroitin 6-sulfate by the method previously reported[6].

Analytical methods. — The methods for determination of uronic acid, 2-amino-2-deoxyhexose, and 2-acetamido-2-deoxy-hexose, and for reduction of oligosaccharides with $NaBH_4$ were described previously[6]. ^{13}C-N.m.r. spectra were recorded at 22.50 MHz with a JEOL FX90Q n.m.r. spectrometer operated in the f.t. mode. Chemical shifts (δ) are expressed downfield from the signal of tetramethylsilane, calculated from the signal of 1,4-dioxane (δ 67.6). ^{1}H-N.m.r. spectra were recorded with a Varian EM-90 spectrometer at 22° for solutions in D_2O (99.95%). Chemical shifts (δ) are expressed downfield from the signal of sodium 4,4-dimethyl-4-silapentane-1-sulfonate, calculated from the signal of HOD (δ 4.65). G.l.c. was performed with a Shimadzu GC-4BM gas chromatograph, equipped with a flame-ionization detector, on a glass column (0.3 × 200 cm) packed with 3% SE-30 on Chromosorb W (80–100 mesh). Column temperature was programmed from 120 to 200° at 6°/min. N_2 was used as carrier gas at a flow rate of ~50 mL/min. Analytical gel chromatography on Sephadex G-25 was carried out as described previously[7].

Solvent effect of dimethyl sulfoxide on acid hydrolysis of sodium hyaluronate. — A solution of Na hyaluronate (160 mg for each experiment) in 10mM HCl or dimethyl sulfoxide containing 10% of 0.1M HCl (80 mL) was heated for 16 h at 105°. Each reaction mixture was cooled and diluted with an equal volume of water, and the pH was adjusted to 6.0 with 0.1M NaOH. The solution was evaporated *in vacuo* and the residue dissolved in water (5 mL) was applied to a column (2 × 90 cm) of AG 1-X2 (Cl^-, 200–400 mesh) anion-exchange resin. It was eluted at room temperature with linear gradients of 0–0.2M (0.6 L) and 0.2–0.4M LiCl (2.0 L) (Figs. 1a,b). The eluate was collected in 16-mL fractions and each fraction was analyzed by the carbazole and Morgan–Elson reactions, and by the trinitrophenylation method[9].

Depolymerization of sodium hyaluronate with dimethyl sulfoxide containing 10% of water in the presence of HCl or H_2SO_4. — A solution of Na hyaluronate (4 mg for each experiment) in dimethyl sulfoxide containing 10% of water (2 mL) and various amounts of HCl or H_2SO_4 was heated under various conditions, as shown in Table I, cooled, and diluted with an equal volume of water, and the pH adjusted

to 6.0 by the addition of 10mM NaOH. The solution was evaporated *in vacuo* and the residue, dissolved in 0.1M NaCl (1.0 mL), was examined by analytical gel chromatography on Sephadex G-25.

Preparation of a series of even-numbered oligosaccharides from Na hyaluronate with dimethyl sulfoxide containing 10% of 0.1M HCl. — A solution of Na hyaluronate (640 mg) in dimethyl sulfoxide containing 10% of 0.1M HCl (320 mL) was heated for 16 h at 95°, cooled, and diluted with an equal volume of water, and the pH was adjusted to 6.0 with 0.5M NaOH. The solution was evaporated *in vacuo* and the residue dissolved in water (5 mL). An aliquot (50 μL), diluted with 0.1M NaCl (1 mL), was examined by analytical gel chromatography on Sephadex G-25. The remaining was applied to a column (2 × 90 cm) of AG 1-X2 (Cl^-, 200–400 mesh) anion-exchange resin and the column eluted at room temperature with linear gradients of 0–0.2M (0.6 L), 0.2–0.4M (2.0 L), and 0.4–0.6M LiCl (1.0 L) (Fig. 2). The eluate was collected in 15.9-mL fractions and each fraction analyzed for uronic acid content. The material from each peak was pooled, lyophilized, and desalted on a column (2.5 × 70 cm) of Sephadex G-15 by elution with 10% ethanol. The desalted solution was lyophilized and the residue dissolved in a minimum volume of methanol, or methanol containing a few drops of water. The solution was filtered and the filtrate poured into cooled acetone with stirring to give a white precipitate. The precipitate was collected by centrifugation and dried in air, and then *in vacuo* in the presence of P_2O_5. The analytical data of the isolated oligosaccharides are reported in Table II.

Determination of the absorbance of hyaluronate hexasaccharide in buffer solutions having various pH values. — Hyaluronate hexasaccharide (1.5 mg for each experiment) was dissolved in 2 mL of 0.1M KH_2PO_4–$Na_2B_4O_7$ buffers of various pHs (5.5–9.0). The solutions were heated at 60°. At intervals, the solutions were cooled and the absorbance at 235 nm was measured (Fig. 3a). If necessary, the solution of pH 8 or 9 was diluted appropriately with the same buffer. The absorbance of chondroitin hexasaccharide was determined in the same manner (Fig. 3b).

Determination of the absorbance of even-numbered hyaluronate oligosaccharides (di- to tetradeca-) in a buffer solution of pH 8.0. — Di-, tetra-, hexa-, deca-, and tetradeca-saccharide (1.5 mg for each sample) were heated at 60° in 0.1M KH_2PO_4–$Na_2B_4O_7$ buffer (2.0 mL, pH 8.0). At intervals, the solution was cooled and the absorbance at 235 nm measured (Fig. 4). The uronic acid content of each oligosaccharide solution was determined by the carbazole reaction.

Alkaline degradation of hyaluronate tetrasaccharide. — Hyaluronate tetrasaccharide (4 mg for each experiment) dissolved in the alkaline medium (1 mL) was stirred in a stoppered glass tube under the following conditions: (a) 20mM Na_2CO_3 buffer (pH 10.0) for 5, 10, 30, and 45 min at 100°; (b) freshly prepared 0.15M Na_2CO_3 for 2, 4, and 6 h at 37°; (c) Dowex 1-X2 (HO^-, 0.2mL) anion-exchange resin for 24 h at room temperature; (d) M NH_4OH for 2 h at 37°; and (e) saturated $Ca(OH)_2$ solution for 2 and 4 h at room temperature, under N_2 or O_2 atmosphere, or under CO_2-free air. In (a) and (b), the mixture was made neutral by

the addition of Dowex 50W-X2 (H^+) cation-exchange resin. In (c), the mixture was filtered and the resin was washed with a small volume of 0.1M HCl; the combined filtrate and washing were made neutral with 0.1M NaOH. In (d), the mixture was evaporated to remove NH_3. In (e), the mixture was made neutral by bubbling CO_2. Each of the solutions was applied to a column (1 × 26 cm) of AG 1-X2 (Cl^-, 200–400 mesh) anion-exchange resin and eluted with a linear gradient of 0.2–0.3M LiCl (0.3 L). Each fraction (4 mL) was analyzed by the Morgan–Elson and carbazole reactions.

Degradation of hyaluronate tetrasaccharide with saturated $Ca(OH)_2$ on a preparative scale. — Hyaluronate tetrasaccharide (Li salt, 100 mg), dissolved in a saturated $Ca(OH)_2$ solution (25 mL), was stirred for 2 h at room temperature under CO_2-free atmosphere, and the mixture was made neutral with gaseous CO_2. The precipitate was filtered off and the filtrate evaporated *in vacuo*. The residue was applied to a column (1.4 × 40 cm) of AG 1-X2 (Cl^-, 200–400 mesh) anion-exchange resin and the column eluted at room temperature with a linear gradient of 0.2–0.3M LiCl (0.8 L). Each fraction (10 mL) was analyzed by the Morgan–Elson and carbazole reactions. The material from each peak reacting with carbazole was pooled, lyophilized, and desalted on a column (1.5 × 75 cm) of Sephadex G-15. Each of the desalted solutions was lyophilized to give Trisaccharide-I and -II in yields of 25 and 23 mg, respectively.

Preparation of the methyl ester of Trisaccharide-II. — A solution of diazomethane in diethyl ether (2 mL), prepared from 0.3 g of *N*-methyl-*N*-nitroso-4-toluenesulfonamide, was added to Trisaccharide-II (8.5 mg) dissolved in methanol (1.1 mL), and the mixture stirred for 20 min at 5°. After evaporation of the solvent, the residue was dissolved in a small volume of water and applied to a column (1 × 4 cm) of AG 1-X2 (Cl^-, 200–400 mesh) anion-exchange resin, which was washed with water. The combined effluent and washing were lyophilized to give the methyl ester of Trisaccharide-II (6.0 mg).

Preparation of alditols from Trisaccharide-II. — Trisaccharide-II (4.4 mg) was dissolved in water (1 mL), and the pH of the solution was adjusted to 4.75 by the addition of 0.1M HCl. To this solution was added 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (35 mg) in several portions at room temperature. The pH was kept at 4.5–5.0 by the addition of 0.1M HCl. After the hydrogen-ion uptake had ceased, 2M $NaBH_4$ (3 mL) was added to the solution in three portions. During the reaction (1 h), the pH was kept at ~8.5 by the addition of M HCl. The mixture was made acidic with M HCl, then neutral with M NaOH, and applied to a column (1.5 × 75 cm) of Sephadex G-15 for desalting. Fractions reacting with anthrone were collected and evaporated to dryness. The residue, dissolved in M HCl (1.5 mL), was heated for 2 h at 100° and the mixture evaporated to remove HCl. To the residue was added 1% $NaBH_4$ (5 mL) and the mixture kept for 30 min at room temperature. Excess $NaBH_4$ was removed by the addition of Dowex 50W-X2 (H^+) cation-exchange resin. The resin was filtered off, and the filtrate and washing were combined and repeatedly evaporated to dryness in the

presence of methanol. 1,1,1-Tris(hydroxymethyl)ethane (0.95 mg) was added to the residue and the mixture was dried *in vacuo* overnight at room temperature. After the addition of 1-(trimethylsilyl)imidazole (0.2 mL), the mixture was heated for 30 min at 80° and analyzed by g.l.c.

Preparation of a series of odd-numbered hyaluronate oligosaccharides having a D-*glucuronic acid residue at the reducing end.* — Sodium hyaluronate (640 mg) was depolymerized to give a series of even-numbered oligosaccharides according to the conditions described earlier. The mixture of even-numbered oligosaccharides was dissolved in freshly prepared 0.15M Na_2CO_3 (64 mL) and the solution kept for 6 h at 40°. The solution was made neutral by the addition of Dowex 50W-X2 (H^+) cation-exchange resin. The resin was filtered off, and the combined filtrate and washing were desalted on a column (2.5 × 75 cm) of Sephadex G-15. The desalted solution was lyophilized and the residue, dissolved in water (10 mL), was applied to a column (2 × 90 cm) of AG 1-X2 (Cl^-, 200–400 mesh) anion-exchange resin. The column was eluted at room temperature with linear gradients of 0–0.2M (0.6 L) and 0.2–0.4M LiCl (2 L). The eluate was collected in 16-mL fractions, which were analyzed by the carbazole and Morgan–Elson reactions (Fig. 5). The materials from each peak reacting with carbazole were isolated by the same procedure as described in the section of preparation of even-numbered oligosaccharides. Analytical data of the isolated oligosaccharides are reported in Table IV.

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