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Chemical change involved in the oxidative-reductive depolymerization of heparin *

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ABSTRACT

A solution of hog intestinal heparin (average M_r 12000, anti-clotting activity 168 USP units/mg) in 0.2 M phosphate buffer (pH 7.2), was incubated in the presence of Fe^{2+} for 20 h at 50° under an O_2 atmosphere to yield oxidative-reductively depolymerized heparin (ORD heparin, average M_r 3000, anti-clotting activity 34 USP units/mg). Chemical analysis of the ORD heparin showed a 22, 26, and 14% loss of hexosamine, uronic acid, and N-acetyl group, respectively, but no remarkable loss of both total and *N*-sulfate groups. ^1H and ^{13}C NMR spectroscopic analysis indicated no decrease in the amount of L-iduronic acid 2-sulfate, but a marked loss of nonsulfated uronic acid (73 and 39% loss of D-glucuronic acid and L-iduronic acids, respectively, the sum of which corresponds to the chemically determined loss of total uronic acid). The results indicated that the ORD reaction of heparin proceeds essentially by destruction of monosaccharide units, except L-iduronic acid 2-sulfate residues, due to oxygen-derived free radicals, followed by secondary hydrolytic cleavage of the resulting unstable residues.

INTRODUCTION

Macromolecular heparin (a heparin proteoglycan, M_r 1.1 MDa), isolated from pronase-digested rat skin, has been subjected to an oxidative-reductive depolymerization (ORD) with Cu^{2+}–ascorbic acid to obtain heparin chains from the proteoglycan[2]. Afterwards, commercial heparin was treated with Cu^{2+}–H_2O_2–ascorbic acid[3,4] to obtain low-molecular-weight heparin preparations. Recently, the ORD reaction of rat mast-cell heparin proteoglycan (M_r 0.75 MDa) with H_2O_2–Fe^{2+} (Fenton's reagent) was reported to cleave the proteoglycan into smaller molecules having a size similar to that of commercial heparin[5]. We reported the structure characterization of the products formed by the ORD reaction of hyaluronic acid (M_r 0.41 MDa) in the presence of Fe^{2+} for 24 h at 37° under an O_2 atmosphere[6].

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In this study, oxidative-reductively depolymerized heparin (ORD heparin, M_r 3 kDa) has been prepared from hog mucosal heparin (M_r 12 kDa) by the ORD reaction. The ORD heparin was cleaved separately by chemical (HNO_2 treatment) and enzymic (heparinase) treatment to give the smallest fragments participating in the ORD cleavage of heparin, each of which was separated, characterized, and quantitatively determined.

EXPERIMENTAL

Materials.—Hog mucosal heparin was purchased from V.G.F. Corporation (New York, NY 10170), and purified by the method of Rodén et al.[7] to remove contaminating dermatan sulfate. The purified heparin was shown, by NMR spectroscopy[8,9], to contain 2-deoxy-2-sulfoamino-D-glucose (GlcN*S*), 2-deoxy-2-sulfoamino-D-glucose 6-sulfate (GlcN*S*6*S*), 2 acetamido-2-deoxy-D-glucose 6-sulfate (GlcNAc6*S*), and L-iduronic acid-2-sulfate (IdoA2*S*), with a sulfate group ratio of 0.83 : 0.77 : 0.66. A part of the purified heparin was fractionated on Ultrogel AcA 44 (LKB-Produkter AB, Broma, Sweden), and a fraction having a larger molecular weight (M_r 16.6 kDa) was separated and used for the time course study of ORD of heparin. Heparinase from *Flavobacterium heparinum* (heparin lyase, EC 4.2.2.7) was obtained from Seikagaku Kogyo Co. Ltd. (Tokyo), and bovine liver β-D-glucuronidase (Type B-10, 10 300 units/mg, EC 3.2.1.31) from Sigma Chemical Co. (St. Louis, MO 63178). Reference heparin oligosaccharides (di to hexa) were prepared by cleavage[10] with HNO_2. A series of standard chondroitin 6-sulfates (sodium salt) having different M_r values (10, 8.5, 5, 4, 2.5, and 1 kDa) were described previously[11]. Water was deionized and then distilled in an all-glass apparatus.

Analytical methods.—The uronic acid content was determined by the method of Bitter and Muir[12], modified by increasing the borate concentration to 0.2 M, and using D-glucurono-6,3-lactone and 1,2-isopropylidene-L-idurono-6,3-lactone as standards[13], by the method of Dische[14], and by the orcinol method using a 20-min boiling time[15]. The hexosamine content was determined by a modification of the Gardell procedure[16] after hydrolysis with 3 M HCl for 15 h at 100°, the anhydromannose content by the indole–HCl method[17], the *N*-acetyl content by GLC after hydrolysis[18], the reducing value by the method of Park and Johnson[19], and the amount of total and *N*-sulfate groups by the methods previously reported[20,21].

Paper electrophoresis was performed on Toyo Roshi No. 51A paper (12 × 25 cm) in 5 : 1 : 5 : 250 (v/v) pyridine–acetic acid–butanol–water (pH 5.6) or 1.6 M formic acid (pH 1.7) at a potential of 30 V/cm for 1 h at 4°. Spots were detected with the 4-aminohippuric acid–phthalic acid reagent[22]. Analytical gel filtration on Sephadex G-100 was carried out by the same procedure as described previously[23], except for the eluent (0.2 M NaCl) and the flow rate (14 mL/h).

Anion-exchange HPLC was performed with a chromatography apparatus equipped with a liquid-delivery pump and autosampler (SP 8800 and 8780, Spec-

tra-Physics Co., San Jose, CA), a variable-wavelength UV detector (NS-310A, Nihon-Seimitsu Co., Tokyo) or a differential refractometer (ERC-7512, ERMA INC., Tokyo), and a column (2.0 × 25 cm) of NH_2-5251-N (5 μm, Senshu Pak, Senshu Kagaku Co., Tokyo). Gel permeation HPLC was performed with the same apparatus using a tandem Shodex KW 802.5 (double) plus KS 802 (single) column (0.8 × 30 cm each). Each sample (50–100 μg), dissolved in water (5–20 μL), was applied to the column, which was eluted at 60° with 0.2 M NaH_2PO_4 at a flow rate of 1.0 mL/min. The fractions were monitored by measuring the refractive index and UV absorption (232 nm). V_0 and V_t were determined by elution of 0.5% Blue Dextran and 0.1% NaN_3, respectively. GLC analysis for alditols was performed with a Shimadzu GC-4BM gas chromatograph, equipped with a flame-ionization detector, on a fused silica capillary column (SPB-1, 0.53 mm × 30 m). The column temperature was 200° and N_2 was used as the carrier gas at a flow rate of ~ 10 mL/min.

For the NMR spectroscopy of oligosaccharides, samples (1–3 mg) were treated several times with D_2O and finally dissolved in 99.95% D_2O (0.6 mL). ^1H and ^{13}C NMR spectra were recorded with a Varian XL-400 spectrometer at 20°. Chemical shift (δ) are expressed relative to 1,4-dioxane as the internal standard. Proton resonances were assigned by means of spin-decoupling difference experiments and homonuclear COSY-45.

Time course of ORD of heparin.—Ferrous sulfate (12 mg in 0.5 mL of water) was added to a solution of sodium heparin (12 mg, M_r 16.6 kDa) in 0.2 M phosphate buffer, pH 7.2 (6 mL). While the mixture was stirred at 60° under a stream of O_2 (10 mL/h), an aliquot of $FeSO_4$ (12 mg) in water (0.5 mL) was added every 2 h. Aliquots (2 mL) were withdrawn at intervals of 7.5, 15, and 22 h, and filtered through a membrane (Dismic-25 cs, Advantec Toyo Co., Tokyo). Each aliquot was concentrated in vacuo and subjected to analytical gel filtration on Sephadex G-100 as indicated in Fig. 1.

Preparation of ORD heparin.—To a solution of sodium heparin (250 mg, M_r 12 kDa) in 0.5 M phosphate buffer, pH 7.2 (125 mL), was added $FeSO_4$ (250 mg in 1.5 mL of water) and the mixture was stirred at 50° under a stream of O_2 (180 mL/h). Every 2 h, an aliquot of $FeSO_4$ (250 mg in 1.5 mL of water) was added during 20 h with continuous stirring. The mixture was centrifuged and the pellet was washed twice with water (total 25 mL). The supernatant and washings were concentrated in vacuo to a small volume, and desalted on a column (5 × 88 cm) of Sephadex G-25 in 10% EtOH. The eluent was lyophilized to give a crude preparation of ORD heparin (178 mg). To obtain the desired amount of ORD heparin, two more cycles of the reaction were repeated. The collected ORD heparin (555 mg) was dissolved in water (3 mL) and applied to a column (1.5 × 25 cm) of Chelex 100 (200–400 mesh, Na^+; Bio-Rad Lab., Richmond, CA), and the column was eluted with water (100 mL). The eluent, free from Fe^{3+} and Fe^{2+} ions, was lyophilized to give the sodium salt of ORD heparin [543 mg, average M_r 3 kDa (M_r 8.5–0.5 kDa), 34 USP units/mg].

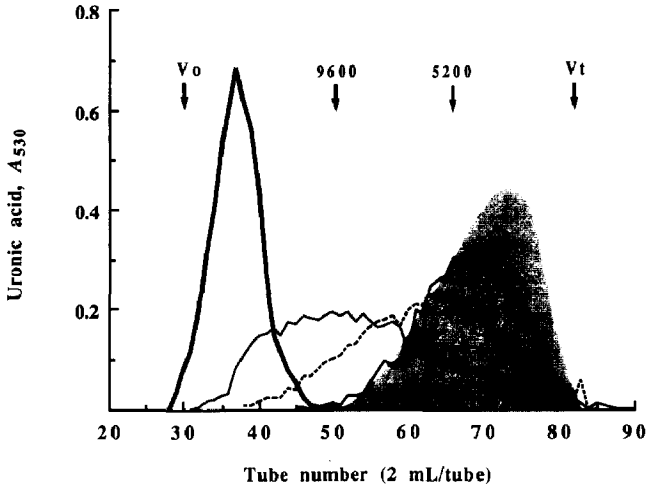

Fig. 1. Gel filtration of ORD products of heparin at different reaction periods on Sephadex G-100: starting heparin (M_r 16600) (——); each ORD product after 7.5 (·····), 15 (- - - - -), and 22 h (———). The *shaded* section is that of ORD heparin (M_r 3000). The *arrows* indicate the elution positions of chondroitin 6-sulfate standards.

Deaminative cleavage of ORD heparin with HNO_2 and separation of the products on Bio-Gel P-4.—To a solution of ORD heparin (50 mg) in water (0.5 mL), was added 1.0 mL of the HNO_2 solution (pH 1.5)[24], and the mixture was kept for 10 min at room temperature. The reaction was terminated by the addition of powdered Na_2CO_3, adjusting the pH to 9, and the solution was applied to a column (1.6 × 144 cm) of Bio-Gel P-4, prepared in 0.2 M NH_4HCO_3 (pH 8.0) and eluted with the same solvent; the flow-rate was 10 mL/h and 2.1-mL fractions were collected, each being assayed for uronic acid (A_{530}) and anhydromannose (A_{490}). The distribution among the mono-, di-, tri-, tetra-, and > hexa-saccharide-size fractions (ORD oligosaccharides) was calculated to be 12.4 (Fractions VII, VI), 52.3 (V, IV), 13.3 (III), 16.2 (II), and 5.8% (I), respectively, from each peak area (A_{530}) of the elution diagram (Fig. 2). The procedure was repeated 4 times, except the determination of the distribution among fractions, and the pooled mono- to tri-saccharide fractions (Fractions VII, VI, V, IV, and III) were freeze-dried to give each a white powder.

Characterization of ORD oligosaccharides, Fractions III and IV.—Separation by anion-exchange HPLC. Prior to separation, Fractions III (3.8 mg) and IV (7.1 mg) were separately reduced with $NaBH_4$ as reported previously[25]. The reduced Fractions III (~ 3.8 mg in 0.1 mL of water) and IV (~ 7.1 mg in 0.2 mL of water) were separately applied to a column of NH_2-5251-N anion-exchanger at 40°, followed by elution with a linear concentration gradient of ammonium acetate (0 → 0.2 M), pH 7.0 at 3.3 mM/min; the flow-rate was 2.5 mL/min and 2.5-mL fractions were collected, each of which was assayed for uronic acid. As indicated in Fig. 3, each fraction was freeze-dried, and the residue was subjected to gel

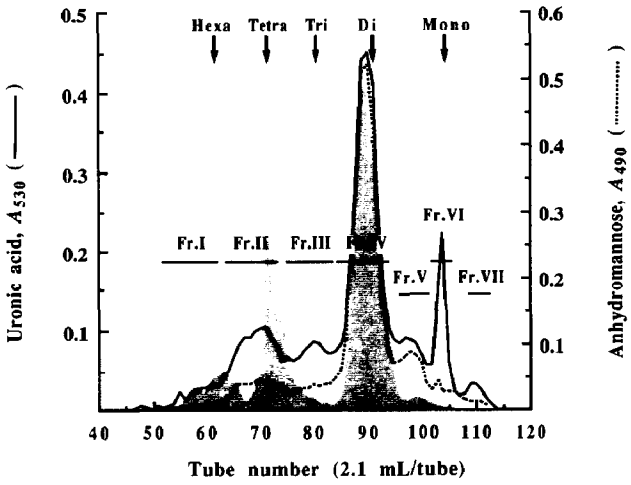

Fig. 2. Gel filtration of deamination-cleavage products of ORD heparin on Bio-Gel P-4. The *shaded* section (A_{530}) is that of the deamination products of starting heparin. The *arrows* indicate the elution positions of reference heparin oligosaccharides (di to hexa) and D-glucuronic acid.

filtration on Bio-Gel P-4, as described in the preceding paragraph. The eluent was freeze-dried to give Fractions III-1 (1.0 mg), III-2 (1.2 mg), IV-1 (1.0 mg), and IV-2 (4.8 mg), all of which were analyzed by NMR spectroscopy.

Determination of uronic acid residues in ORD oligosaccharide Fraction III-1. Oligosaccharide Fraction III-1 (1.0 mg) was carboxyl-reduced by the method of Taylor et al.[26] as follows. The product dissolved in M HCl (1 mL) was heated for 2 h at 100°, and the mixture evaporated in vacuo to remove HCl. The residue was reduced with $NaBH_4$ as described in the preceding paragraph, and then trimethylsilylated as described[6] and subjected to GLC. Trimethylsilyl derivatives of D-arabinitol and L-iditol were used as standards for GLC.

Determination of unusual sugar residues at the reducing and nonreducing ends of ORD oligosaccharide Fraction IV-1.—After the oligosaccharide Fraction IV-1 had been analyzed by NMR spectroscopy, the recovered sample (~ 1 mg) was treated with mercuric acetate by the method of Ludwigs et al.[27], and was processed as described previously[6], except that a Bio-Gel P-4 column (1.0 × 90 cm) in 0.2 M NH_4HCO_3 was used. The product, freed from oligosaccharide materials containing 4,5-unsaturated hexosyluronic acid residues, was subjected to NMR spectroscopy. The sample recovered from this analysis (~ 0.9 mg) was treated with acetic anhydride[28] and the product analyzed by NMR spectroscopy.

Characterization of Fraction V.—After separation, Fraction V weighed ~ 1 mg. A part of this material was analyzed by paper electrophoresis at pH 6.5, giving a single spot that had a mobility identical with that of a mixture of L-idosyluronic acid → 2,5-anhydro-D-mannose 6-sulfate (IdoA → anMan6*S*) and L-idosyluronic acid 2-sulfate → 2,5-anhydro-D-mannose (IdoA2*S* → anMan) used as reference. The residual amount of the material was analyzed by ^1H NMR spectroscopy,

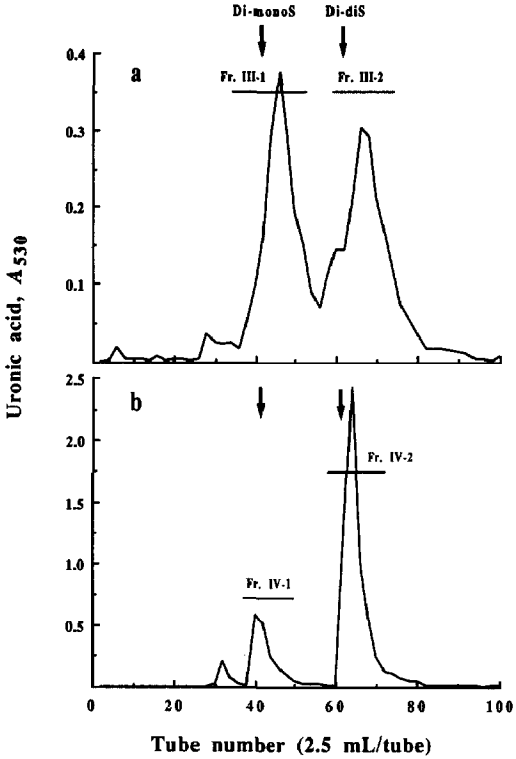

Fig. 3. Separation of ORD oligosaccharide Fractions III and IV by anion-exchange HPLC. Fractions III (3.8 mg) (a) and IV (7.1 mg) (b) were separately chromatographed on a column of NH_2-5251-N anion-exchanger by use of a linear concentration gradient (0 → 0.2 M ammonium acetate, pH 7.0, at 3.3 mM/min). The *arrows* indicate the elution positions of reference compounds: L-idosyluronic acid 2-sulfate → 2,5-anhydro-D-mannitol 6-sulfate (Di-di*S*), and a mixture of L-idosyluronic acid → 2,5-anhydro-D-mannitol 6-sulfate and L-idosyluronic acid 2-sulfate → 2,5-anhydro-D-mannitol (Di-mono*S*). The *brackets* indicate each fraction range of Fractions III-1 and -2 (a), and IV-1 and -2 (b), subjected to further isolation procedure.

showing the main signals [δ 3.49 (H-2) and 4.10 (H-6) for IdoA → anMan6*S*, and 4.13 (H-2) and 3.80 (H-6) for IdoA2*S* → anMan, respectively], in agreement with the data reported by Huckerby et al.[29].

Characterization of ORD monosaccharides, Fractions VI and VII.—Fractions VI (1.7 mg) and VII (0.8 mg) were separated. Paper electrophoreses at pH 1.7 and 5.6 indicated that Fraction VI was substituted with one sulfate group, but Fraction VII was not. The carbazole-to-orcinol ratio of Fractions VI and VII was 0.44 and 1.41, respectively, suggesting that they contain L-iduronic acid and D-glucuronic acid[30], respectively. To confirm this, Fractions VI (0.8 mg) and VII (0.6 mg) were separately subjected to carboxyl group reduction[26], followed by hydrolysis in M HCl for 2 h at 100°, products were trimethylsilylated for GLC analysis.

Examination of reducing terminal residues of ORD heparin.—ORD heparin (50 mg) was carboxyl-reduced[26], the product (34 mg) hydrolyzed in 3 M HCl (4 mL) at

100° for 15 h under an N_2 atmosphere, the reaction mixture evaporated in vacuo to dryness, the residue dissolved in water (1 mL) and applied to a column (1 × 7 cm) of Dowex 1-X8 (HO^-; 200–400 mesh) anion-exchange resin, and the column eluted with water (20 mL), as described by Yamaguchi et al.[31]. Since the retention times of hexitol (9.91 min) and hexosaminitol (9.88 min) are close, the alditol plus hexosaminitol fraction was divided into two equal fractions. One was lyophilized and trimethylsilylated; the other was passed through a column (1 × 5 cm) of Dowex 50W-X8 (H^+, 50–100 mesh) cation-exchange resin, lyophilized, and trimethylsilylated. GLC of these fractions was performed at a column temperature of 200° with the trimethylsilyl derivatives of D-arabinitol, D-glucitol, and 2-amino-2-deoxy-D-glucitol as references.

Determination of L-iduronic acid and L-iduronic acid 2-sulfate residues located at nonreducing ends of ORD heparin.—Digestion of ORD heparin with heparinase and fractionation of the products on Bio-Gel P-4. To a solution of ORD heparin (3 mg) in water (0.2 mL) was added heparinase (300 mU) in 20 mM NaOAc–2 mM $Ca(OAc)_2$ buffer, pH 7.0 (0.6 mL), and the mixture was incubated for 2 h at 37°. After heating at 100° for 1 min, the mixture was centrifuged, and the supernatant was loaded on a column (1.0 × 90 cm) of Bio-Gel P-4 in 0.2 M NH_4HCO_3 (pH 8.0). The column was eluted with the same solvent at a flow rate of 4.4 mL/h, and each fraction collected (0.54 mL) was analyzed for uronic acid and A_{232} (the inset to Fig. 4). The disaccharide fraction, which is termed "ORD disaccharide (heparinase)", was separated and lyophilized (960 μg).

Treatment of ORD disaccharide (heparinase) with mercuric acetate and analysis of the products by gel permeation HPLC. A solution of ORD disaccharide (heparinase) (440 μg) in 50 mM Tris · acetate buffer, pH 7.5 was treated with mercuric acetate by the method of Ludwigs et al.[27]. The product was analyzed by gel permeation HPLC as described in *Analytical methods.*

Examination of nonreducing D-glucuronic acid ends of ORD heparin.—ORD heparin (5 mg) was digested with *exo*-β-D-glucuronidase under essentially the same conditions as described[10], and release of the nonreducing, terminal D-glucuronic acid residues by the enzyme was examined by gel filtration on a Bio-Gel P-4 column (1.0 × 90 cm) in 0.2 M NH_4HCO_3.

Preparation of low-molecular-weight heparin samples.—Fractionation of ORD heparin on Sephadex G-75. A solution of ORD heparin (20 mg) in water (1 mL) was applied to a column (1.0 × 95 cm) of Sephadex G-75 prepared in 0.2 M NH_4HCO_3, and the column was eluted with the same solvent. The flow-rate was 20 mL/h and 1.4-mL fractions were collected, each of which was assayed for uronic acid. The product of elution was separated into three fractions having an average M_r 8000, 5000, and 3000, weighing 1.6, 1.4, and 2.1 mg, respectively, after freeze-drying.

Partial cleavage of heparin with HNO_2 and fractionation of the product on Sephadex G-75. To a solution of heparin (20 mg) in water (0.57 mL) was added an HNO_2 solution (30 μL, pH 1.5)[24], and the mixture was kept for 10 min at room temperature. The reaction was terminated by addition of M Na_2CO_3 (110 μL);

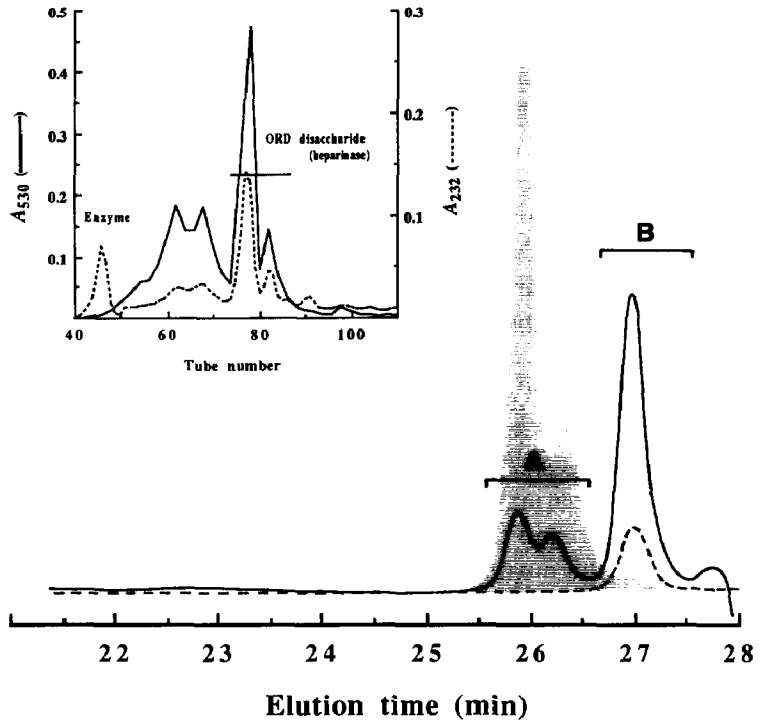

Fig. 4. Gel permeation HPLC analysis of products obtained by treatment of ORD disaccharide (heparinase) with mercuric acetate. The *shaded* section is that of intact ORD disaccharide (heparinase). Bracket A: elution diagram of unaltered ORD disaccharides; bracket B, elution diagrams of monosaccharide(s) formed from unsaturated hexosyluronic acid disaccharides by the mercuric acetate treatment. Detection: refractive index (———) and A_{232} (- - - - -). The elution diagrams on Bio-Gel P-4 of the products obtained by digestion of ORD heparin with heparinase are shown in the *inset*.

0.25 M $NaBH_4$ in M NaOH (0.2 mL) was added and the mixture was kept at 50° for 30 min. After neutralization with M acetic acid, the mixture was fractionated as described in the previous paragraph to give 3.3 (M_r 8000), 2.1 (5000), and 2.8 mg (3000).

Affinity chromatography of low-molecular-weight heparins on antithrombin III-Sepharose.—The procedure applied was essentially that described by Laurent et al.[32]. Solutions (each 1.0 mg in 0.2 mL) of the low-molecular-weight heparins obtained by two different methods (Table III) were separately applied to a column (0.85 × 3.6 cm) of antithrombin III-Sepharose 4B (protein content, 9 mg/mL of gel) in 50 mM NaCl–50 mM Tris · HCl buffer (pH 7.4) at 4°, followed by elution with the same buffer (10 mL) and a linear NaCl concentration gradient up to 3.2 M (40 mL). The flow rate was 38 mL/h and 0.9-mL fractions were collected, each of which was assayed for uronic acid and ionic strength. The areas of A_{530} of the nonadsorbed fraction, the low-affinity fraction, and the high-affinity fraction, which were eluted at the NaCl concentrations of 0.05, 0.05–1.1, and 1.1–3.0 M,

TABLE I

Analytical data of heparin and ORD heparin

	Heparin	ORD Heparin
M_r	12000	3000
Uronic acid (%)	37.0	27.3
Hexosamine (%)	28.5	22.3
Total S (mol) [a]	2.25	2.20
N-Sulfate (mol)	0.83	0.80
N-Acetyl (mol)	0.125 [b]	0.108 [b]
Relative signal intensity (^{1}H NMR) of		
IdoA2*S* (H-1)(δ 5.2)	100.0	100.0
IdoA (H-1)(δ 5.0)	13.4	8.2
GlcA (H-2)(δ 3.4)	39.8	10.6
GlcNAc ($NCOCH_3$)(δ 2.0)	27.0	23.5

[a] Mol/mol of hexosamine. [b] GLC data.

respectively, were measured. The ratio of the A_{530} areas of each fraction to the total A_{530} area of the low molecular weight heparin applied was expressed as a percentage.

RESULTS

The time course showed a steady fragmentation of the heparin chain under the conditions used for hyaluronan[6], except at a reaction temperature of 50 or 60°, which suggested that the sulfated glycosaminoglycans are more resistant to radical-induced oxidation than the nonsulfated glycosaminoglycans (Fig. 1). In this experiment, either exclusion of oxygen or addition of radical scavengers, such as D-mannitol, prevented completely the progress of the reaction; the accelerating effect of phosphate ions and the role of L-ascorbic acid were also confirmed, as observed in the case of hyaluronan[6]. Chemical and GLC analyses of the ORD heparin showed a 22, 26, and 14% loss of hexosamine, uronic acid, and *N*-acetyl group, respectively, but no loss of both total and *N*-sulfates (Table I). ^{1}H and ^{13}C NMR data showed 73 and 39% loss of nonsulfated D-glucuronic and L-iduronic acid residues, respectively, the sum of which corresponds to the chemically determined loss of total uronic acid (Table I). The signal for olefinic protons (δ 5.73), due to 4,5-unsaturated hexosyluronic acid residues, which was found for Fraction IV-1a obtained by deaminative cleavage of ORD heparin, described later on, was not detected in the spectrum of the ORD heparin.

The ORD heparin was expected to be chemically modified at the reducing and nonreducing ends as found for ORD hyaluronan[6,33,34]. The degree of disaccharide formation by heparinase digestion of ORD heparin was ~ 50% (see Fig. 4), as compared to 60–65% for intact heparin. Since ORD heparin is a nonasaccharide on average, the results suggest that the internal sequence structure remained fairly intact.

Deaminative cleavage of ORD heparin with nitrous acid gave the smallest oligosaccharides bearing the modified terminal groups (ORD oligosaccharides) in addition to the native heparin di- and tetra-saccharides probably formed from the internal sequence. The products were fractionated on a Bio-Gel P-4 column (Fig. 2) into tetra- and higher oligo-saccharides (Fractions I and II), trisaccharide (III), disaccharide (IV and V), and monosaccharide (VI and VII). Anion-exchange HPLC of Fraction II indicated several unknown components besides some of the native heparin tetra-saccharides previously reported[29,35], and the yield of Fraction I was too small for analysis; accordingly, Fractions I and II were not investigated further. Fraction V, eluted between the di- and mono-saccharide fractions from the column (Fig. 2), consisted mainly of a mixture of IdoA → anMan6*S* and IdoA2*S* → anMan, which resulted from the internal sequence and the reducing ends. Monosaccharide fractions VI and VII, each of which contained a single component reacting positively to both the carbazole and Park-Johnson reactions, originated from the reducing ends of ORD heparin. Fractions VI and VII contained L-iduronic acid 2-sulfate and D-glucuronic acid, respectively, as shown by the carbazole-to-orcinol ratio[30], paper electrophoresis at pH 1.7 and 5.6, and GLC of the corresponding alditols. The ratios of the L-iduronic acid 2-sulfate and of D-glucuronic acid residues at the reducing ends to the total reducing ends of ORD heparin were calculated to be ~17 and ~7%, respectively, from the yields of Fractions VI and VII, and an average M_r of 3000 for ORD heparin.

As shown in Fig. 2, the elution curve does not indicate clearly the presence of 2,5-anhydro-D-mannose and its 6-sulfate. Further inspection by paper electrophoresis at pH 1.7 and 5.6 showed no evidence for these compounds (electrophoretograms not shown), indicating the absence of 2-sulfamino-2-deoxy-D-glucose and its 6-sulfate residues at the nonreducing ends of ORD heparin.

Fraction III was reduced with sodium borohydride prior to separation by anion-exchange HPLC in order to achieve a good separation into Fractions III-1 and III-2 (Fig. 3 and Table II). Fraction III-1 was homogeneous in saccharide size by filtration on Bio-Gel P-4 (data not shown), but anion-exchange HPLC (Fig. 3a) indicated several minor components, in addition to the main component which exhibited, in the NMR spectrum, a strong signal at δ 2.0 due to the methyl protons of *N*-acetyl groups. The uronic acid units of Fraction III-1 were reduced to the corresponding alditols and trimethylsilylated[6,25]. GLC gave two peaks with retention times identical with those of D-arabinitol (5.85 min) and L-iditol (9.91 min), and approximately equivalent peak areas *. A previous study on ORD hyaluronan[6] indicated the formation of ORD fragments bearing a modified sequence, -β-D-GlcA-(1 → 3)-β-D-GlcNAc-(1 → 3)-D-*arabino*-penturonic acid at the reducing end. The D-arabinitol detected by GLC originated probably from the assumed D-

* The 2-acetamido-2-deoxy-D-glucose unit of the main component of Fraction III-1 was converted into 2-amino-2-deoxy-D-glucitol, which was removed by subsequent treatment with Dowex 50W-X2 (H^+) cation-exchange resin.

TABLE II

Structural characteristics of oligosaccharide Fractions III-1 and -2, and IV-1 and -2, formed by deamination cleavage of ORD heparin

	ORD oligosaccharide fraction			
	III-1	III-2	IV-1	IV-2 [a]
Weight (mg) [b]	1.0	1.2	1.0	4.8
2,5-Anhydro-D-mannose residue [c]	–	–	+	+
N-Acetyl group [d]	+ [e]	– [e]	–	–
Olefinic-H [d]	–	–	+	–
D-*arabino*-Penturonic acid residue [f]	+	[g]	[g]	[g]

[a] Identified as IdoA2*S* → anManOH6*S* by reference to the ^1H and ^{13}C NMR data reported by Huckerby et al.[29]. [b] Yield from the ORD heparin (250 mg) cleaved by deamination with HNO_2. [c] Estimated from the elution diagram (A_{490}) in Fig. 2. [d] By ^1H NMR spectroscopy. [e] (+) An indication of detection at a main component level; (–) an indication of no detection. [f] The oligosaccharide Fraction III-1 was carboxyl group reduced, as described by Taylor et al.[26], and acid hydrolyzed. The product was reduced with $NaBH_4$, and then converted to the trimethylsilyl derivative for GLC analysis. [g] Not determined.

arabino-penturonic acid residues of the main component of Fraction III-1, as in the case of ORD hyaluronan; these residues may be formed by ORD of the D-glucuronic acid residues linked to C-1 of the reducing 2-acetamido-2-deoxy-D-glucose residues in the native heparin, suggesting for the main component of Fraction III-1 a sequence α-L-IdoA-(1 → 4)-β-D-GlcNAc-(1 → 3)-D-*arabino*-penturonic acid. The ratio of D-*arabino*-penturonic acid residues at the reducing ends to the total reducing residues of ORD heparin was estimated at 5.5% from the yield of the pentitol obtained by GLC and an average M_r of 3000 for ORD heparin.

Fraction III-2 was shown to be fairly homogeneous by gel filtration on Bio-Gel P-4 (data not shown), but anion-exchange HPLC indicated three to four components, the ionic properties of which are close to each other (Fig. 3a). After separation, NMR spectroscopy showed multiple, broad H-1 signals, and the fraction was not investigated further.

The $NaBH_4$-reduced Fraction IV material was resolved into two main peaks, IV-1 and IV-2, and a small peak eluted earlier (Fig. 3b). Fraction IV-2 was shown to be homogeneous by gel filtration on Bio-Gel P-4. ^1H and ^{13}C NMR data (not shown) of Fraction IV-2 indicated the presence of IdoA2*S* anManOH6*S*[29]. This disaccharide derived mainly from the internal sequence and from the nonreducing ends (as described later on) of ORD heparin by deaminative cleavage. Fraction IV-1 was shown to be composed of two components by ^1H and ^{13}C NMR spectroscopy, and COSY-45 two-dimensional, homonuclear correlation experiment. One of them (Fraction IV-1a), which accounts for 25% of Fraction IV-1, was shown to be possibly a 4,5-unsaturated hexosyluronic acid → anManOH6*S*, as indicated by the resonances at δ 5.45 (H-1), 100.6 (C-1), 3.75 (H-2), 70.8 (C-2), 4.23 (H-3), 66.9 (C-3), 5.73 (H-4), and 108.7 (C-4) of the unsaturated hexosyluronic acid residue, and by the resonances of the 2,5-anhydro-D-mannitol 6-sulfate residue at

the reducing end[29]. The existence of the 4,5-unsaturated uronic acid residue was confirmed by treatment of Fraction IV-1 with the mercuric acetate reagent[27] which removed the unsaturated uronic acid residue, as shown by ^{1}H and ^{13}C NMR. The ratio of the 4,5-unsaturated uronic acid residues at the nonreducing ends to the total nonreducing ends of ORD heparin was ~2.4% as estimated by NMR spectroscopy and a M_r of 3000 for ORD heparin. Another component of Fraction IV-1 (termed Fraction IV-1b), which accounted for ~50% of Fraction IV-1, was shown to contain a 4-substituted 2-amino-2-deoxy-D-glucose residue at the reducing site, as indicated by the resonances at δ 5.27 (H-1), 95.5 (C-1) 3.54 (H-2), 51.9 (C-2), 3.47 (H-3), 53.5 (C-3), 4.35 (H-4), and 65.1 (C-4). This was confirmed by treatment of Fraction IV-1 with mercuric acetate, followed by *N*-acetylation[28], and ^{1}H NMR spectroscopy, which indicated the presence of a distinct *N*-acetyl signal having the intensity of the signals of a 4-substituted 2-acetamido-2-deoxy-D-glucose residues. Because the 2-amino-2-deoxy-D-glucose residues of various saccharides are known to be resistant to deaminative cleavage under the conditions used[24], the existence of these residues at the reducing ends of the ORD heparin is possible. The ratio of the reducing 2-amino-2-deoxy-D-glucose residues to the total reducing ends of ORD heparin was calculated to be ~5% from the yield estimated by NMR spectroscopy and a M_r of 3000 for ORD heparin.

Carboxyl group reduction[26], followed by strong hydrolysis converted the hexosamine-containing terminal residues of ORD heparin into 2-amino-2-deoxy-D-glucitol, and the reducing, terminal uronic acid residues into the corresponding alditols. On the other hand, the nonreducing, terminal residues and the residues in the internal sequence remained as 2-amino-2-deoxy-D-glucose, or were converted into aldoses derived from the component uronic acids. The alditols formed were separated from the aldoses by chromatography[31], after increasing the column size. GLC of the trimethylsilylated alditol fraction revealed the presence of pentitol (5.85 min), hexitol (9.91 min), and hexosaminitol (9.88 min), in the proportions of 13.8, 29.6, and 56.5%, respectively. These results indicated the presence of the respective parent residues at the reducing ends of ORD heparin.

The presence of uronic acid residues unsubstituted at C-4 could not be obtained by NMR spectroscopy of ORD heparin. Accordingly, to ascertain the presence of uronic acid residues located at the nonreducing ends (other than the 4,5-unsaturated uronic acid residues, Fraction IV-1a), ORD heparin was digested with heparinase, and the product was subjected to gel filtration on Bio-Gel P-4 (the inset to Fig. 4). The disaccharide fraction was separated and treated with the mercuric acetate reagent[27] to remove the 4,5-unsaturated hexosyluronic acid residues, analyzed by gel permeation HPLC, which indicated the presence of two components eluted at the positions of heparin disaccharide tri- and di-sulfate, respectively (Fig. 4). These components, which were the digestion products with heparinase and were resistant to the mercuric acetate treatment, are the native heparin disaccharides: IdoA2*S* → GlcN*S*6*S*, and a mixture of IdoA → GlcN*S*6*S*

TABLE III

Biological properties of low-molecular-weight heparins obtained by different depolymerization methods

Preparation	$M_r \times 10^{-3}$	Anticoagulant activity (USP units/mg)	Distribution of the fractions separated on antithrombin III-Sepharose (%)		
			Nonadsorbed	Low affinity	High affinity
Heparin	12	164	3.3	50.1	46.6
Low-molecular-weight heparin obtained by: Free radical-induced cleavage (ORD heparin)					
I	8	18	47.6	33.4	19.0
II	5	7	67.6	21.3	11.1
III	3	5	78.1	13.5	8.4
Nitrous acid cleavage (HNO_2 heparin)					
I	8	64	29.9	31.5	38.6
II	5	10	56.8	22.2	21.0
III	3	6	73.3	16.0	10.7

and IdoA2*S* → GlcN*S*, respectively. The ratio of L-iduronic acid plus L-iduronic acid 2-sulfate residues at the nonreducing ends to the total nonreducing ends of ORD heparin was calculated to be ~ 51% from the gel permeation HPLC and a M_r of 3000 for ORD heparin. The proportion of L-iduronic acid 2-sulfate residues is probably more than two thirds of the content of L-iduronic acid residues as judged from the elution diagram (Bracket A in Fig. 4). To determine the possible presence of nonreducing D-glucuronic acid end units, ORD heparin was digested with *exo*-β-D-glucuronidase and the product analyzed by gel filtration (not shown), which indicated the absence of D-glucuronic acid residues.

To test the clinical interest of the ORD heparin fractions with average molecular weights of 8000, 5000, and 3000, as antithrombotic agents[36], their biological activities were assayed and compared with those of the corresponding low-molecular-weight heparin fractions prepared by deaminative cleavage of native heparin. As indicated by affinity chromatography on antithrombin III-Sepharose (Table III), the samples obtained by ORD had a lower activity than those obtained by deaminative cleavage. Likewise, the anticlotting activity of the former samples was generally lower than that of the latter samples.

DISCUSSION

The proportion of L-iduronic acid 2-sulfate residues remained unchanged after ORD of heparin, indicating a resistance of these residues to the attack of oxygen-derived free radicals, and the residues were found to occupy 35–51 and 17% of the nonreducing and reducing ends of ORD heparin, respectively. On the

other hand, a marked loss of nonsulfated uronic acid residues, especially of D-glucuronic acid residues, suggested that they were susceptible to attack by free radicals and would undergo a breakdown resulting in the cleavage of the polysaccharide chain. The L-iduronic acid 2-sulfate residues are known to form more sulfated regions of the heparin molecule in association with 2-deoxy-2-sulfamino-D-glucose and its 6-sulfate residues, and the D-glucuronic acid residues are known to form the less sulfated regions of the molecule with the 2-acetamido-2-deoxy-D-glucose and its 6-sulfate residues[37]. The results reported herein agree with the suggestion of Bianchini et al.[3] that ascorbic acid–H_2O_2–Cu^{2+} oxidizes preferentially less sulfated regions.

The *O*- and *N*-sulfate contents of ORD heparin remained nearly unchanged, but it contained 22 and 14% less hexosamine and *N*-acetyl group, respectively, suggesting that most of the lost hexosamine residues were the 2-sulfoamino- and 2-acetamido-2-deoxy-D-glucose, and their 6-sulfate residues, which underwent a partial structural change (but not desulfation) by the attack of free radicals. This would result in the formation of modified residues at the reducing and nonreducing ends; however, the present study failed to prove the presence of any modified residues derived from the 2-amino-2-deoxy-D-glucose-containing residues described above, mainly owing to a poor separation of Fraction III-2 into components by anion-exchange HPLC. The 2-amino-2-deoxy-D-glucose-containing residues, most of which are probably 2-sulfoamino-2-deoxy-D-glucose and its 6-sulfate, were found located at the reducing ends of ORD heparin in a ratio of ~57% to the total reducing ends. The existence of 2-deoxy-2-sulfoamino-D-glucose 6-sulfate and L-iduronic acid 2-sulfate residues at the reducing ends, and of L-iduronic acid 2-sulfate residues at the nonreducing ends are in agreement with the proposal of Casu[38], and partly with that of Bianchini and Mascellani[4]. On the other hand, no evidence of 2-sulfoamino-2-deoxy-D-glucose and its 6-sulfate residues at the nonreducing ends was found, in contradiction with a previous proposal[38]. The presence of D-glucuronic acid residues at the nonreducing end of ORD heparin (nonasaccharide on average) was not confirmed by the *exo*-β-D-glucuronidase digestion, which had been successfully used for the determination of the nonreducing, terminal D-glucuronic acid residues in the hexasaccharide (units 3–8) derived from the antithrombin III-binding octasaccharide (units 1–8)[10].

GLC analysis of the alditols derived specifically from the reducing terminal residues of ORD heparin, which had been originally planned to determine the proportion, at the reducing ends, of hexosamine-containing residues, such as 2-deoxy-2-sulfoamino-D-glucose, 2-acetamido-2-deoxy-D-glucose, 2-amino-2-deoxy-D-glucose (cf. Fraction IV-1b), and their 6-sulfates, exhibited peaks of hexitol (29.6%) and pentitol (13.8%), besides that of hexosaminitol (56.5%). The amount of hexitol fraction, which would originate from the component uronic acids and their 2-sulfates, approximately matched with the sum of the L-iduronic acid 2-sulfate (~17%) and D-glucuronic acid (~7%) recovered in Fractions VI and VII, respectively. The pentitol fraction would be derived from the penturonic acid

residues (D-*arabino*- and L-*xylo*-penturonic acid) by carboxyl reduction. Thus, the D-*arabino*-penturonic acid (~ 5.5%) recovered in Fraction III-1 is probably a part of the total penturonic acid (13.8%) determined by GLC of the reducing ends of ORD heparin. The existence of the D-glucuronic acid (Fraction VII, ~ 7%) and penturonic acid (Fraction III-1, ~ 5.5%) residues at the reducing ends of ORD heparin suggested that the D-glucuronic acid residues linked to C-1 of the 2-deoxy-2-sulfoamino-D-glucose or its 6-sulfate residues would be resistant to attack by free radicals, and those linked to C-1 of the 2-acetamido-2-deoxy-D-glucose or its 6-sulfate residues would be susceptible to attack.

As a result of minor chemical changes from the ORD of heparin, 4,5-unsaturated hexosyluronic acid residues (Fraction IV-1a, ~ 2.4%) and 2-amino-2-deoxy-D-glucose residues (Fraction IV-1b, ~ 5%) were detected at the nonreducing and reducing ends, respectively. As far as we are aware, the formation of unsaturated uronic acid residues in ORD of heparin has not been reported until now, nor the formation of such residues in the deamination process of heparin. The existence of 4,5-unsaturated uronic acid residues indicated that a minor part of the 2-sulfoamino (or 2-acetamido)-2-deoxy-α-D-glucosyl bonds in heparin are cleaved by the ORD process, probably through β-elimination which involves the loss of H-5 from the uronic acid unit, because the resulting radical would be stabilized by resonance interaction with the carboxyl group[39,40].

The biological activity data of the ORD heparin fractions indicated that heparin underwent a preferential destruction of the D-glucuronic acid residues, which are one of the essential constituents of the antithrombin-binding saccharide sequence, and this change in the structure caused a marked decreased in the affinity for the protein and also in the related anticoagulant activity.

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