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# STUDY OF STRUCTURALLY DEFINED OLIGOSACCHARIDE SUBSTRATES OF HEPARIN AND HEPARAN MONOSULFATE LYASES*

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## ABSTRACT

The rapid preparation of multimilligram quantities of five heparin-derived oligosaccharides (**1–5**) is described. These oligosaccharides are the final poducts obtained from the action of heparin lyase (heparinase, E.C. 4.2.2.7) at its primary sites in the heparin polymer. Five oligosaccharides comprise from 75–85 wt% of commercial porcine mucosal heparins and are recovered in good yield and high purity. Four of these five oligosaccharides were further acted upon at much lower rates by prolonged treatment with heparin lyase or heparan monosulfate lyase (heparitinase, E.C. 4.2.2.8), revealing the subspecificities of these enzymes. These oligosaccharides were used as defined substrates for heparin lyase and heparan monosulfate lyase and their kinetic constants were obtained. Potential applications for these oligosaccharides include their use as defined substrates for purification of heparin monosulfate lyases, and for establishing the catalytic purity of enzyme preparations.

## INTRODUCTION*

Polysaccharide lyases (or eliminases) comprise an important class of enzymes that act as specific glycosidic linkages in certain acidic polysaccharides[1]. *Flavobacterium heparinum* is a major source of eliminases including: chondroitinases (chondroitin ABC[2], AC[3], B[4], and C[5] lyases); heparitinases[6,7] (heparan monosulfate lyase I and II); and heparinase[8] (heparin lyase). The flavobacterial lyases can be divided into two classes: the chondroitin lyases, which act on (1→4)-glycosidically linked alternating copolymers of substituted uronic acid (1→3)-linked to hexosamine, and the heparin/heparan monosulfate lyases, which act on a (1→4)-glycosidically linked alternating copolymer of uronic acid (1→4)-linked to hexosamine[1]. Assays have been designed to measure and study these enzymic activities using

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**Abbreviations:* — SAX, strong anion exchange; h.p.l.c., high-pressure liquid chromatography; ΔUA*p*, 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid; GlcN*p*, glucosamine; IdoA*p*, iduronic acid; GlcA*p*, glucuronic acid; S, sulfate; Ac, acetate; and d.p., degree of polymerization.

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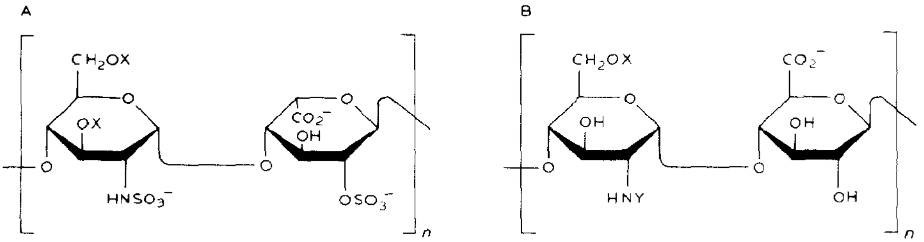

Fig. 1. Primary site of action of heparin lyase (A) and heparan monosulfate lyase(s) (B), where $n \geq 3$ and X = $SO_3^-$ or H and Y = $SO_3^-$ or $COCH_3$.

polymeric substrates consisting of fractionated glycosaminoglycans[1]. These polymeric substrates usually contain a major repeating-structure, for which a single lyase is specific, as well as secondary sites at which other lyases can act.

Flavobacterial lyases that act on heparin and heparan monosulfate have found a wide variety of applications. The major site of action of these enzymes is shown in Fig. 1. Heparinase has been used to remove heparin, an anticoagulant drug, from the blood[9], and to prepare new anticoagulants[10,11], anti-tumor[12], anti-complementary[13,14], and anti-atherosclerotic agents[15]. Heparin lyase and heparan monosulfate lyase have also found use in sequencing heparin and heparan monosulfate polymers[15–17]. These applications require catalytically pure preparations of heparin lyase and heparin monosulfate lyase(s), but only heparin lyase has been purified to the criterion of homogeneity demonstrated by gel electrophoresis[18]. All of these enzymes can act on the commercial polymeric substrates, heparin and heparan monosulfate, allowing for minor, contaminating enzymic activity to go undetected. Substrates are described in this paper that contain a single site which can only be cleaved by a single lyase. These substrates may be used in a simple absorbance assay or a strong anion exchange–high-pressure liquid chromatography (SAX-h.p.l.c.) assay to detect minor catalytic contaminants in a lyase preparation.

This paper provides a detailed examination of the primary specificity of heparin lyase. Action of this enzyme at these primary sites results in a small number of different, final oligosaccharide products[16,19]. Exhaustive treatment with heparin lyase or heparan monosulfate lyase results in cleavage of these oligosaccharide products at secondary sites, affording additional oligosaccharides. The kinetic parameters associated with the action of these enzymes on secondary sites are also reported. A complete understanding of the specificity (both primary and secondary) of these enzymes, as well as their mode of action[20], is required for their effective use as tools in sequence analysis of heparin and heparan monosulfate. The main structural oligosaccharide components of porcine mucosal heparin may also find utility as standards in the commercial preparation and analysis of heparin, or as synthons of new heparin-like drugs.

## MATERIALS

Heparin sodium salt, from porcine intestinal mucosa (160 U/mg), was obtained from Hepar Industries, Franklin, OH. Heparan monosulfate (heparan sulfate or heparitin) sodium salt, from bovine kidney, was obtained from ICN Immunobiologicals, Lisle, IL. Heparin lyase (E.C. 4.2.2.7) was purified from *Flavobacteria heparinum* (5 m-units/$\mu$g) (1 m-unit = 1 nmol product formed/min) or purchased (11 m-units/$\mu$g) from Sigma Chemical Company, St. Louis, MO. Heparan monosulfate lyase, (heparitinase), E.C. 4.2.2.8 (capable of cleaving 2-acetamido- and 2-deoxy-2-sulfoamido-$\alpha$-D-glucosyl-(1$\rightarrow$4)-D-glucuronate linkages with 6-sulfate being present, according to the manufacturer, Seikagaku Kogyo Co., Ltd., Tokyo, Japan)[21] with a specific activity on heparan monosulfate of 17 m-units/$\mu$g, was obtained from ICN Immunobiologicals. Sephadex G-10 was purchased from Pharmacia Biochemicals, Piscataway, NJ. All other chemicals were reagent grade.

SAX-h.p.l.c. was performed using two LDC-Milton Roy (Riviera Beach, FL) Constametric III) pumps with gradient control by digital-to-analog interface using an Apple IIe microcomputer and Chromatochart software from Interactive Microware Inc., State College, PA. The system was equipped with a Rheodyne (Cotati, CA) 7125 injector, and an ISCO (Lincoln, NE) Model 1840 variable-wavelength u.v. detector. Preparative separations were performed on a 1 cm × 25 cm, 5-$\mu$m particle size, Spherisorb strong-anion-exchange (SAX) h.p.l.c. column, and analytical separations on a 4.6 mm × 25 cm column of identical packing, both from Phase Separations, Norwalk, CT.

U.v. spectroscopy was performed with a Shimadzu (Tokyo, Japan) Model UV-160 spectrophotometer. Infrared spectroscopy was performed with a Nicolet (Madison, WI) Model 5DxB Fourier-transform i.r. spectrophotometer. Mass spectrometry was performed with a VG Analytical ZAB-HF spectrometer (f.a.b.) or on a BIN-10K (Bio-Ion Nordic AB) spectrometer ($^{252}$Cf plasma desorption).

## METHODS

*Heparin depolymerization.* — Heparin (100 mg at 16 mg/mL) was depolymerized at 30° with 62 m-units/mL heparin lyase in a solution of 0.2M sodium chloride and 5mM sodium phosphate at pH 7.0. The reaction was monitored by removing aliquots and measuring the product absorbance at 232 nm after a 1:100 dilution into 0.03M hydrochloric acid. When the reaction approached a constant absorbance (10 h), the enzyme was thermally inactivated at 100° for 1 min. The sample was processed 100 mg at a time by applying it to a Sephadex G-10 desalting column (2.5 × 50 cm) eluted with distilled water at a flow rate of 3.3 mL/min. Fractions (3.3 mL) were collected and their absorbance was measured at 232 nm. The heparin-derived oligosaccharides eluted at 80 mL, and the buffer salts eluted at 120 mL. The tubes containing the heparin-derived oligosaccharide peak were pooled, frozen, and freeze-dried.

*Kinetic analysis of heparin depolymerization by SAX-h.p.l.c.* — During the course of the depolymerization reaction, aliquots (100 μL) were removed at 60, 120, 180, 240, 300, and 600 min and quickly frozen on Dry Ice. The aliquots were then collectively heated in a water bath for 1 min at 100° to thermally inactivate the enzyme. The percent reaction-completion of each aliquot was determined from absorbance at 232 nm after adding 10 μL of each to 990 μL of 0.03M hydrochloric acid. SAX-h.p.l.c. analysis was performed by injecting 10 μL (160 μg) of sample and 10 μL (8 μg) of diluted (1:20 in distilled water) sample into a 200-μL sample loop attached to the preparative SAX-h.p.l.c. column. The column was pre-equilibrated with 0.2M sodium chloride and eluted by using a linear gradient of concentration Y [(Y in molarity) at any time (X in sec) = 0.0001X + 0.2] sodium chloride, pH 3.5, at a flow rate of 1.5 mL/min.

*Prepartive SAX-h.p.l.c. of heparin-derived oligosaccharides.* — The desalted heparin-derived oligosaccharides were dissolved in 500 μL of distilled water and 160 μL (33 mg) was injected into a 200-μL injection loop. The preparative SAX-h.p.l.c. column was pre-equilibrated with 0.2M sodium chloride at pH 3.5 and the flow rate was set at 1.5 mL/min. After sample injection, a linear gradient of concentration Y [(Y in molarity) at any time (X in sec) = 0.0002 X + 0.2] of sodium chloride, pH 3.5, at 1.5 mL/min was used to elute the oligosaccharides. The separation was monitored by measuring absorbance at 232 nm with 2 absorbance units full scale (AUFS) and 1-mL fractions were collected with a fraction collector. The complete separation required three 33-mg injections. Like fractions from each injection were combined, frozen, freeze-dried, redissolved in 5 mL of water, desalted on a Sephadex G-10 column, pooled, frozen, and freeze-dried.

*Purity, yield, and characterization.* — Purity of the five major heparin-derived oligosaccharides was established by injection of each oligosaccharide (10 μg in 50 μL of water) onto an analytical SAX-h.p.l.c. column pre-equilibrated with 0.2M sodium chloride (pH 3.5) at a flow rate of 1.5 mL/min. After injection a linear gradient of concentration Y [(Y in molarity) at any time (X in sec) = 0.0002 X + 0.2] of sodium chloride, pH 3.5, at 1.5 mL/min, was used to effect the separation, which was monitored by absorbance at 232 nm with 0.02 AUFS. The area under the major component as well as the minor components were measured by computer-assisted integration and used to calculate purity of each fraction.

The expected yield of each of the five major compounds was determined from its concentration in the depolymerization mixture, using analytical SAX-h.p.l.c. Injection of a 4- and a 40-μg sample permitted the accurate analysis of both major and minor components. Computer-assisted integration of the chromatograms was used to measure the area under each of the elution peaks.

Calibration curves (μg of sample injected *vs.* peak area) were constructed for compounds **1–5**. Purified oligosaccharide (>95% pure following a second fractionation on preparative SAX-h.p.l.c.) was dissolved in 0.03M hydrochloric acid and made up to a final absorbance of 0.9 absorbance units at 232 nm. SAX-h.p.l.c. analysis was performed by applying each of the five samples separately at 15, 30, and 45 μL, and the areas under the peaks were determined by computer-assisted integration. The analysis was performed in triplicate for each oligosaccharide and calibration curves were constructed from a linear regression of the averaged values.

Molar absorptivities were determined for each of the five compounds by carefully weighing the desalted, dried, pure samples and dissolving 1 mg of sample into 1 mL of 0.03M hydrochloric acid and determining the absorbance at three dilutions. The analysis was performed in triplicate and the molar absorptivity was determined from the slope of the plot of absorbance *vs.* molarity, utilizing the molecular weight calculated from the chemical structure. Molar absorptivity of the disaccharide was also measured in 0.2M sodium acetate 2.5mM calcium acetate, pH 7.0.

*Kinetic constants of heparin lyase and heparan monosulfate lyase on polymeric purified substrates.* — Each of the oligosaccharides **1**–**5** at concentrations of 160μM were treated individually with either heparin lyase (40 m-units/mL) at 30° or heparan monosulfate lyase (40 m-units/mL) at 43° or 30° for 12 h. The time-course of the reaction was monitored by using analytical SAX-h.p.l.c.

Heparin ($M_r$ av. 14,000) and heparan monosulfate ($M_r$ av. 20,000) at concentrations of 14μM were used to determine the specific activity of heparin lyase prepared in our laboratory and to check the manufacturers activities for heparin lyase and heparan monosulfate lyase. The kinetic constants of heparin lyase and heparan monosulfate lyase were measured by using between 0.1 and 2μM heparin or 0.1 and 5μM heparan monosulfate at 30° and 43°, respectively.

Tetrasaccharide **2** was at concentrations of between 20 and 160μM and tetrasaccharide **4** between 6 and 40μM for the kinetic studies. Heparin lyase (2.5 m-units) and tetrasaccharide **2** or **4** were prepared in 700 μL of acetate solution (200mM sodium acetate, 2.5mM calcium acetate, pH 7.0). The absorbance of the reaction, performed at 30°, was monitored continuously at 232 nm to obtain a curve of initial reaction velocity.

Tetrasaccharide **3** at concentrations between 6 and 35μM and hexasaccharide **5** at concentrations between 7.14 and 115μM were used as substrates for heparan monosulfate lyase. Heparan monosulfate lyase (2.5 m-units) and substrate were prepared in 700 μL of acetate solution as already described. The absorbance of the reaction mixture, performed at 30° or 43°, was monitored continuously in the u.v. at 232 nm to obtain a curve of initial reaction-velocity. The initial velocity-data obtained was fitted to a hyperbolic curve using Hyper[22], a computer program, to obtain optimized $K_M$ and $V_{max}$ values.

The temperature optimum of the reaction rate was determined in a separate experiment in which the initial velocity was measured at 25°, 30°, 37°, and 43° using a saturating concentration of each substrate and 2.5 m-units of heparin lyase or heparan monosulfate lyase, in 700 μL of acetate solution by monitoring the reaction continuously at 232 nm.

## RESULTS

Depolymerization of heparin by heparin lyase results in the reproducible production of oligosaccharides containing a 4-deoxy-2-sulfo-α-L-*threo*-hex-4-enopyranosyluronic acid (ΔUA*p*2S) residue at their nonreducing end. Porcine mucosal heparins contain predominantly five oligosaccharides derived by the action of heparin lyase[14] (Fig. 2). The most abundant of these (35–50 wt %), corresponding to the major repeating unit in the heparin polymer, is disaccharide **1** having an observed mass of 663.89260 (663.89130 calculated for [M + 4Na − 5H$^-$], where M is the fully protonated acid form of **1**) by high-resolution f.a.b.-m.s. consistent with a molecular formula[23] of $C_{12}H_{15}NNa_4S_3O_{19}$ for **1**. Carbon and proton n.m.r. spectra have been fully assigned[24] and are identical to previously published spectra[25,26]. The structure of this trisulfated disaccharide is ΔUA*p*2S(1→4)-α-D-GlcN*p*2S6S.

Three tetrasaccharides, **2**, **3**, and **4** were isolated from the final product-mixture. Sulfate analysis of tetrasaccharide **4** showed that it contained 6 sulfate groups[24]. Low resolution Cf p.d.-m.s. gave a molecular ion consistent with the molecular formula[27] $C_{24}H_{31}N_2Na_8S_6O_{38}$. Carbon and proton n.m.r. spectra have

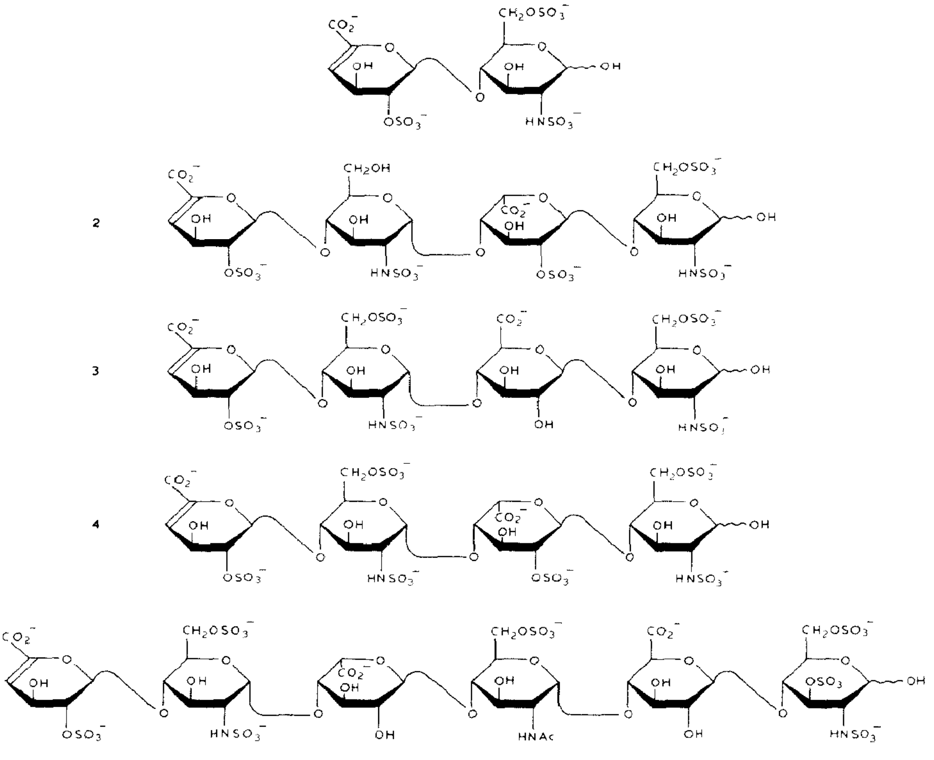

Fig. 2. Final oligosaccharides (1–5) derived by the action of heparin lyase (EC 4.2.2.7) on heparin.

been fully assigned[24]. The proton spectrum is identical to one previously reported[25] and clearly demonstrates the internal uronic acid residue to be 2-sulfated iduronic acid (H-1, 5.24 p.p.m.). Exhaustive treatment of **4** with heparin lyase results in two equivalents of a trisulfated disaccharide giving an identical SAX-h.p.l.c. retention time, proton n.m.r. spectrum and f.a.b.-m.s. analysis as **1**. The structure of **4** can be definitively assigned as ΔUA*p*2S(1→4)-α-D-GlcN*p*2S6S(1→4)-α-L-IdoA*p*2S-(1→4)-α-D-GlcN*p*2S6S.

Tetrasaccharide **3** contained five sulfate groups by sulfate analysis[24] and had a mass of 1227 ($[M + 7Na - 8H]^-$) by low-resolution f.a.b.-m.s., consistent with a molecular formula[23] of $C_{24}H_{30}N_2Na_7S_5O_{35}$. Carbon and proton spectra have been fully assigned[24]. The proton spectrum, identical to one previously reported[25], clearly identifies the internal uronic acid as glucuronic acid (H-1, 4.51 p.p.m.). The lack of sulfation at this internal glucuronic acid is demonstrated by its sensitivity towards periodate oxidation[24]. On treatment with heparan monosulfate lyase, **3** is completely converted into one equivalent of trisulfated disaccharide and one equivalent of disulfated disaccharide. The trisulfated disaccharide co-elutes with **1** on SAX-h.p.l.c. while the disulated disaccharide elutes earlier on SAX-h.p.l.c. The structure of **3** was assigned as ΔUA*p*2S(1→4)-α-D-GlcN*p*2S6S(1→4)-β-D-GlcA*p*(1→4)-α-D-GlcN*p*2S6S.

Tetrasaccharide **2** contained 5 sulfates by sulfate analysis[28] using ion chromatography[29] and had a mass of 1227 ($[M + 7Na - 8H]^-$) by low-resolution f.a.b.-m.s., consistent with a molecular formula[28] of $C_{24}H_{30}N_2Na_7S_5O_{35}$. Carbon and proton signals for the reducing and non-reducing end sugars are virtually identical with those observed[24,28] for **1**, **3**, and **4**. The internal uronic acid was assigned as a 2-sulfated iduronic acid from an observed H-1 shift of 5.22 p.p.m. Both internal and reducing-end glucosamine residues were *N*-sulfated, as indicated by two upfield signals at 59.2 and 60.3 p.p.m. assigned to the 2-position carbons. The 6-position of the internal glucosamine is unsulfated, showing a carbon signal at 62 p.p.m. as compared to the 69.6 p.p.m. signal for this carbon in the 6-sulfated glucosamine residue of **4**[24,28].

On exhaustive treatment with heparin lyase, **2** breaks down completely into one equivalent of trisulfated disaccharide and disulfated disaccharide. The trisulfated disaccharide co-elutes with **1** on SAX-h.p.l.c. and gives an identical mass by f.a.b.-m.s. analysis. The disulfated disaccharide, coeluting with **2′** on SAX-h.p.l.c., gave a molecular ion of 562 ($[M + 3Na - 4H]^-$) by low-resolution f.a.b.-m.s., consistent with a molecular formula of $C_{12}H_{16}NNa_3S_2O_{16}$. The structure of **2** was assigned as ΔUA*p*2S(1→4)-α-D-GlcN*p*2S(1→4)-α-L-IdoA*p*2S(1→4)-α-D-GlcN*p*2S6S.

Hexasaccharide **5** contained seven sulfate groups by sulfate analysis[11]. Low resolution Cf p.d.-m.s. gave a molecular ion consistent with the molecular formula[27] $C_{38}H_{49}N_3Na_{10}S_7O_{52}$. Both carbon and proton n.m.r. were almost completely assigned by using 2-dimensional $^1H$-homonuclear COSY and carbon–hydrogen correlation spectroscopy[11]. On exhaustive treatment of **5** with heparan monosulfate lyase, one equivalent of trisulfated disaccharide and one equivalent of tetrasulfated tetrasaccharide are formed. Compound **5** contains two linkages at which a lyase might cleave, both giving rise to a trisulfated disaccharide and a tetrasulfated tetrasaccharide. Cf p.d.-m.s. gave spectra consistent with molecular formulas of $C_{12}H_{15}NNa_4S_3O_{19}$, and $C_{26}H_{34}N_2Na_6S_4O_{32}$, indicating that the tetrasaccharide was *N*-acetylated but not establishing at which internal glycosidic linkage heparan monosulfate lyase cleaved[28]. Proton n.m.r. of the tetrasaccharide clearly identified its internal uronic acid as iduronic from its shift at 5.0 p.p.m., establishing that the enzyme acted at →4)-α-D-GlcNAc*p*6S(1→4)-β-D-GlcA*p*(1→[11,28]. The structure of **5** was unequivocally established as ΔUA*p*2S(1→4)-α-D-GlcN*p*2S6S(1→4)-α-L-IdoA*p*(1→4)-α-D-GlcNAc*p*6S(1→4)-β-D-GlcA*p*(1→4)-α-D-GlcN*p*2S3S6S. The structure of **5** has recently been confirmed by other workers[35].

These five oligosaccharides (**1–5**) are liberated from the heparin polymer at comparable rates and define the primary sites of heparin lyase action (Fig. 3). The depolymerization products of heparin were quantitatively analyzed by direct injection on analytical SAX-h.p.l.c. at two concentrations, to obtain all of the five major peaks on scale (Fig. 4, labeled **1–5**). Calibration curves were constructed by plotting peak area *vs.* mass in micrograms for each individual purified compound. The samples of **1–5** used in these calibration curves were rechromatographed by semipreparative SAX-h.p.l.c. to an estimated (by analytical SAX-h.p.l.c.) purity of >95%. The slope [peak area (A)/μg], intercept [peak area (A)] and correlation ($r^2$) for these curves are: **1**, 162465A/μg, 8454A, and 0.999; **2**, 77343A/μg, and 1227A, and 0.999; **3**, 86530A/μg, −23717A, and 0.999; **4**, 93590A/μg, −14573A, and 0.999; and **5**, 58575A/μg, −10946A, and 0.999. These standard curves were then used to calcualte an expected yield and isolated yield (on a weight basis) for each of these five compounds.

Preparative fractionation of depolymerized heparin was achieved by first desalting the reaction product on a Sephadex G-10, concentrating it by freeze-drying, and directly injecting it into a semi-preparative 5-μm particle-size SAX-h.p.l.c. column. The five major compounds were eluted in the same order as in the analytical separation. It was observed that up to 33 mg of desalted, depolymerized heparin could be injected onto this semi-preparative column with an acceptable resolution of the five major compounds. Optimum separation was achieved at a flow rate of 1.5 mL/min with a gradient slope of 0.1mM/sec. Consecutive injections were reproducible, provided that the column was pre-equilibrated with 0.2M sodium chloride (pH 3.5) before each injection.

Combination of like fractions obtained from 100 mg of depolymerized heparin (three injections), followed by desalting using Sephadex G-10, and freeze drying, resulted in yields of **1–5** ranging from 65 to 95% of expected yield, with product purity of from 81 to >99% (Table I). Re-application of any single product to the preparative SAX-h.p.l.c. column resulted in the isolation of products that were >95% pure, as evaluated by analytical SAX-h.p.l.c. Sample identity was confirmed by analytical SAX-h.p.l.c. co-elution with previously prepared and charac-

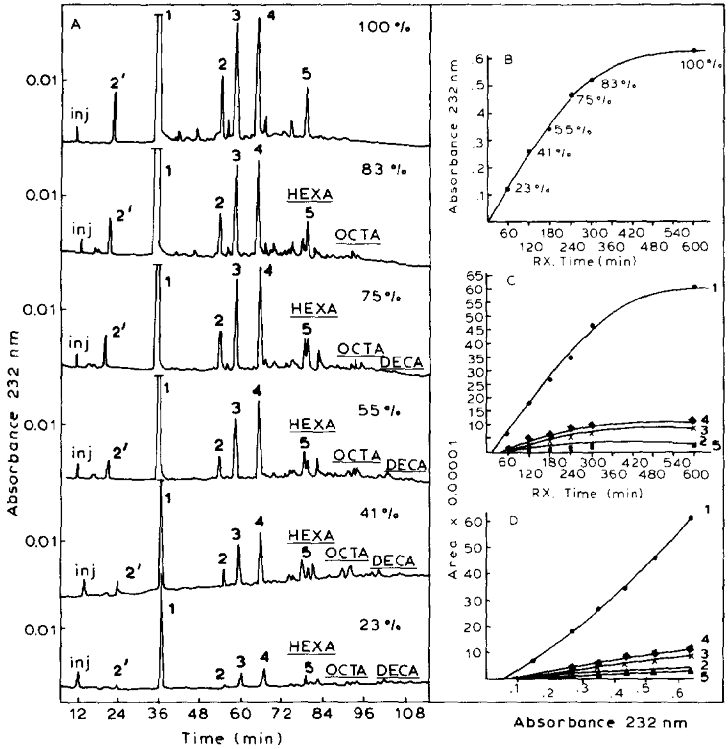

Fig. 3. Analysis of heparin lyase-catalyzed depolymerization of heparin. (A) SAX-h.p.l.c. analysis of heparin lyase acting on heparin. Chromatograms are of 23, 41, 55, 75, 83, and nearly 100% reaction completion. Peaks corresponding to **1**–**5** and transient oligosaccharides are indicated. (B) Time course of heparin lyase-catalyzed heparin depolymerization monitored by u.v. 232 nm after 1:100 dilution in 0.03M hydrochloric acid. (C) Time course of the formation of oligosaccharides **1**–**5** by integrated area under each peak measured throughout the heparin depolymerization reaction. (D) Crossplot of peak area for **1**–**5** *vs.* extent of reaction (C *vs.* B) at 23, 41, 55, 75, 83, and nearly 100% reaction completion.

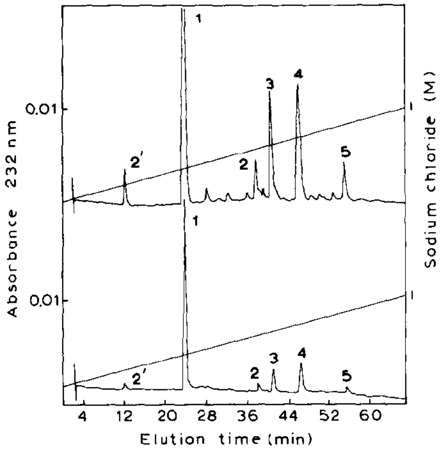

Fig. 4. Analysis of preparative oligosaccharide product-mixture at nearly 100% completion by injection of 40 μg (A) and 8 μg (B) onto the analytical SAX-h.p.l.c. used to calculate the expected yield of **1**–**5**.

TABLE I

PURITY AND YIELD OF HEPARIN-DERIVED OLIGOSACCHARIDES ISOLATED BY A SINGLE SEMI-PREPARATIVE SAX-h.p.l.c. STEP

| *Oligosaccharide identity*[a] | *Expected yield*[b] *(mg)* | *Yield*[c] *(%)* | *Purity*[d] *(%)* |
|---|---|---|---|
| **1** | 41.4 | 70 | 100 |
| **2** | 2.02 | 94 | 86 |
| **3** | 11.9 | 66 | 90 |
| **4** | 14.6 | 76 | 88 |
| **5** | 5.78 | 95 | 81 |

[a]The molecular weight of the sodium salt of **1**–**5** and the molar absorptivity ($M^{-1}cm^{-1}$) of each in 0.03M hydrochloric acid is: **1**, 665, 5063 ±10% (lit.[34] 5200); **2**, 1228, 5331 ±0.6%; **3**, 1228, 5066 ±3.7%; **4**, 1330, 5657 ±1.4%; and **5**, 1883, 5275 ±7%. [b]Amount determined by analytical SAX-h.p.l.c. to be present in 100 mg of depolymerized heparin. [c]Determined as amount isolated (by u.v.$_{232}$ absorbance) divided by theoretical yield. [d]Determined by analytical SAX-h.p.l.c. A second fractionation by semi-preparative SAX-h.p.l.c. increased the purity of **2**–**5** to >95%.

terized oligosaccharides **1**–**5**. These oligosaccharides also compared well with authentic standards when examined by diffuse reflectance Fourier-transform i.r. spectroscopy[11].

Each of the five oligosaccharides was tested for its ability to act as substrates for heparin lyase and heparan monosulfate lyase. Comparable rates of reaction were obtained when measured by either increase in absorbance at 232 nm or SAX-h.p.l.c. The trisulfated disaccharide showed no susceptibility to either heparin lyase or heparan monosulfate lyase. Minor sulfatase activity in the heparin lyase preparation was detected, by the formation of an undersulfated disaccharide from **1** when the enzyme was assayed in acetate solution. Contaminating sulfatase activity could be markedly diminished if the reaction was performed in 0.2M sodium chloride, 5mM sodium phosphate buffer at pH 7.0. No reverse catalysis, the formation of higher oligomers from disaccharide, was detected by SAX-h.p.l.c., even when high substrate concentrations were used.

Of the five oligosaccharides, only tetrasaccharides **2** and **4** were susceptible to heparin lyase as demonstrated by analytical SAX-h.p.l.c., analysis of the reaction products. Heparin lyase treatment of tetrasaccharide **2** resulted in a molar equivalent of trisulfated disaccharide **1**, and a molar equivalent of disulfated disaccharide (**2'**), ΔUA*p*2S(1→4)-α-D-GlcN*p*2S, derived from the non-reducing end. Tetrasaccharide **4** was cleaved into two equivalents of trisulfated disaccharide **1**. These reactions were also studied in the spectrophotometer by direct u.v. analysis at 232 nm (Table II). The initial-velocity data obtained on these two heparin lyase substrates shows an apparent $K_M$ of 77μM for tetrasaccharide **2** and an apparent $K_M$ of 8.0μM for tetrasaccharide **4**. The $V_{max}$ for tetrasaccharides **2** and **4** were 0.083 nmol/min and 0.055 nmol/min, respectively. The temperature optimum for the maximum initial rate reaction for tetrasaccharide **4** was 25–30° (Table II).

Of the five compounds, only pentasulfated tetrasaccharide **3** and hepta-

TABLE II

KINETIC CONSTANT AND TEMPERATURE OPTIMA FOR HEPARIN LYASE AND HEPARAN MONOSULFATE LYASE ACTING ON VARIOUS SUBSTRATES

| *Substrate/enzyme* | *Apparent* $K_M$ ($\mu$M)[c] | $V_{max}$[c,d] *(nmol/min)* | *V/K* | *Relative initial rate (%) at different temperatures*[e] | | | |
|---|---|---|---|---|---|---|---|
| | | | | *25°* | *30°* | *37°* | *43°* |
| Heparin/heparin lyase[a] | 0.293 | 3.29 | 11.2 | 54 | 80 | 100 | 57 |
| **2**/Heparin lyase[a] | 77.6 | 0.083 | 0.0011 | n.d.[f] | n.d. | n.d. | n.d. |
| **4**/Heparin lyase[a] | 7.9 | 0.055 | 0.0070 | 100 | 97 | 46 | 25 |
| Heparan | | | | | | | |
| Heparan/monosulfate lyase[b] | 5.7 | 3.57 | 0.626 | 53 | 63 | 93 | 100 |
| **3**/Heparan monosulfate lyase[b] | 13.5 | 0.166 | 0.012 | 64 | 100 | 71 | 13 |
| **5**/Heparan monosulfate lyase[b] | 15.3 | 0.102 | 0.007 | 62 | 79 | 100 | 99 |

[a] Determined using heparin lyase purchase from Sigma. The enzyme concentration was 2.5 m-units/700 μL buffer. The enzyme specific activity was 11 m-units/μg as determined by the manufacturer[21]. [b] Determined using heparan monosulfate lyase purchased from Miles (ICN). The enzyme concentration was 2.5 m-units/700 μL buffer. The specificity activity was 17 m-units/μg as determined by the manufacturer. [c] Kinetic constants were determined from computer hyperbolic fit-optimized data[22]. [d] Determined using a molar absorptivity of $3800\text{M}^{-1}$, determined in 0.2M sodium acetate 2.5mM calcium acetate for the disaccharide **1**. [e] Determined in a temperature-controlled spectrophotometer. The enzyme concentration and substrate concentration were constant. [f] Not determined.

sulfated hexasaccharide **5**, containing the required glucuronic acid residues[1], were found sensitive to heparan monosulfate lyase. Tetrasaccharide **3**, which was shown to be resistant to cleavage by heparin lyase, was acted upon by commercial heparan monosulfate lyase to form trisulfated disaccharide **1** and a disulfated disaccharide, ΔUA*p*(1→4)-α-D-GlcN*p*2S6S, which co-eluted on SAX-h.p.l.c. with the disulfated disaccharide (**2′**) formed in the heparin lyase cleavage of tetrasaccharide **2**. Kinetic analysis of this substrate using the spectrophotometric assay (Table II) showed an apparent $K_M$ of 13.5μM and $V_{max}$ of 0.166 nmol/min. The temperature optimum for maximal rate of reaction was 30° (Table II).

The heptasulfated hexasaccharide **5** had been previously reported to be a substrate for heparan monosulfate lyase, resulting in a tetrasulfated tetrasaccharide, ΔUA*p*2S(1→4)-α-D-GlcN*p*2S6S(1→4)-α-L-IdoA*p*(1→4)-α-D-GlcNAc*p*6S, and a trisulfated disaccharide ΔUA*p*(1→4)-α-D-GlcN*p*2S3S6S, formed by cleavage of the →4)-α-D-GlcNAc*p*6S(1→4)-β-D-GlcA*p*(1→ linkage[11]. Direct spectrophotometric assay resulted in an apparent $K_M$ = 15.3μM and the $V_{max}$ = 0.102 nmol/min. The temperature optimum for the maximum initial reaction-rate was 37–43° (Table II).

The polymeric substrates, heparin and heparan monosulfate, were also studied using heparin lyase and heparan monosulfate lyase. Heparin showed an apparent $K_M$ of 0.29μM and a $V_{max}$ of 3.29 nmol/min when acted on by heparin lyase at 30°. Heparan monosulfate showed an apparent $K_M$ of 5.7μM and a $V_{max}$ of 3.57 nmol/min when acted on by heparan monosulfate lyase at 43°.

## DISCUSSION

Treatment of porcine heparin with commercial heparin lyase results in a reproducible mixture of oligosaccharides. Five oligosaccharides comprise >80% of this mixture. Each of these oligosaccharides (**1**–**5**) arises at nearly a constant rate throughout the course of the reaction (Fig. 3), indicating that each must result from the action of heparin lyase on sites with equivalent reactivity (primary sites) shown in Fig. 1. Oligosaccharides **1**–**5** were each shown to be stable under the reaction conditions. The slightly higher rate of formation of **1**, late in the reaction, probably results because heparin lyase fails to behave in an ideal random endolytic fashion[20,36] rather than from the cleavage of secondary sites in **2** and **4**.

From 100 mg of porcine mucosal heparin, each of these five final oligosaccharide products was recovered in yields ranging from 70–95%, and at purity of >81% by fractionation on a semi-preparative SAX-h.p.l.c. column and desalting on a low-pressure Sephadex G-10 column. A second SAX-h.p.l.c. step was used to obtain oligosaccharides **1**–**5** in a purity of >95% as determined by analytical SAX-h.p.l.c. Each oligosaccharide also showed a single band on gradient polyacrylamide gel electrophoresis[16]. A preparative SAX-h.p.l.c. column (2 cm × 25 cm) of the same packing material has been used successfully to fractionate 100 mg of heparin oligosaccharides in a single injection.

Two of the tetrasaccharides, **2** and **4**, contain linkages that are sensitive to heparin lyase. Their production throughout the reaction and recovery intact from the depolymerization products suggests that **2** and **4** result from heparin lyase acting at primary sites in the heparin polymer, and that their remaining heparin lyase-sensitive linkages constitute secondary sites. The cleavage of these two tetrasaccharides by heparin lyase was demonstrated by direct u.v. assay and SAX-h.p.l.c. Tetrasaccharides **2** and **4** bind with lower affinity to heparin lyase than does heparin, with apparent $K_M$ (heparin) = $0.29\mu\mathrm{M}$, apparent $K_M$ (**2**) = $78\mu_{\mathrm{M}}$ and apparent $K_M$ (**4**) = $8\mu\mathrm{M}$. This binding is also less productive than it is for the polymeric substrate, heparin, as suggested by the lower $V_{\max}$ values for **2** and **4**, possibly because of their decreased size, making these secondary sites for the action of heparin lyase. Comparison of $V/K$ values for **2** and **4** to the $V/K$ value of heparin demonstrates a 1600-fold decrease in substrate reactivity, explaining the stability of **2** and **4** under the conditions for heparin depolymerization. When heparin depolymerization was followed by SAX-h.p.l.c., the rate of formation of **2′** (data not shown) was similar to that observed for oligosaccharides **1**–**5** (Fig. 3A). This suggests that the →4)-α-D-GlcN*p*2S(1→4)-α-L-IdoA*p*2S(1→ linkage in polymeric or higher oligomeric substrates (d.p. ≥ 6), corresponding to the secondary site in **2**, comprises a primary site of action for heparin lyase.

The structures of the final oligosaccharide products are also useful in defining the substrate requirements of heparin lyase (Fig. 1 and 2). As suggested earlier by others[30], the presence of the 6-sulfate in the glucosamine residue is not an absolute requirement but rather enhances substrate binding, as is evident from the 10-fold difference in the apparent $K_M$ for **2** and **4**. The 2-sulfate in the iduronic acid residue is required for heparin lyase action, as demonstrated by the failure of the enzyme to act on the →4)-α-D-GlcN*p*2S6S(1→4)-α-L-IdoA*p*(1→ linkage in **5** (Table III and Fig. 2).

The heparan monosulfate lyase activity is considerably more complex, as the commercially available enzyme is described[21] as having activities consistent with a mixture of heparan monosulfate lyase I and II[7,31]. Although these activities were described by several groups[6,7,8,32,33], neither the specificity nor the number of different forms of heparan monosulfate lyase is clear. Because of the lack of defined substrates, it has been difficult to purify and to characterize the specificities of the heparan monosulfate lyases. The use of uncharacterized commercial enzyme preparations has two major pitfalls. The first is that this approach does not permit the full exploitation of these enzymes' cross-specificities, required for examination of the fine structure of the heparan monosulfate and heparin polymers. The second is the underlying assumption that the heparan monosulfate lyases display identical activity optima (*i.e.*, temperature, pH, ionic strength, etc.). Both lyase I and II reportedly[26] have activity temperature optima at 43°. Using defined tetrasaccharide **3** and hexasaccharide **5** as substrates with the commercial enzyme preparation, a different activity temperature optimum was determined for each substrate, **3** at 30° and **5** at 37–43°.

The different temperature optima observed for **3** and **5** (Table II) could be associated with the presence of two different heparan monosulfate lyases in the commercial preparation, consistent with earlier observations[7,31,32]. Alternatively, one heparan monosulfate lyase having a broad specificity may act on both **3** and **5** at different optimum temperatures, similar to the different temperature optima observed for heparin lyase acting on oligomeric **4** and polymeric heparin (Table II). To examine this question, the thermal stability of the activities in this enzyme preparation was assessed. Heparan monosulfate lyase was incubated for 6 h at 43° before assaying it for activity towards **3** and **5** at 30° and 43°, respectively. Activity on both substrates was still observed, consistent with a single enzyme (in the commercial preparation) acting on both **3** and **5**.

Commercially obtained heparan monosulfate lyase shows a requirement for a →4-α-D-GlcN*p*2S (or GlcNAc*p*)(1→4)-β-D-GlcA*p*(1→ linkage. Our substrates do not permit determination of whether 6-sulfation of the glucosamine residue is required for activity or whether 2-sulfation of the glucuronic acid residue would interfere with the action of the commercial heparan monosulfate lyase preparation. Additional defined substrates will be required to further establish the specificities of this and other heparan monosulfate lyases.

These defined oligomeric substrates should be useful in studying the substrate specificity and action pattern[20] of both heparin lyase and heparan monosulfate lyases. These five oligosaccharides may be prepared in good purity and in multi-mg quantities in a single day. Two of these oligosaccharides, tetrasaccharide **4** and hexasaccharide **5**, have biological activity as inhibitors of complement activation[13,14].

An alternative to the complete enzymic depolymerization of heparin is its partial depolymerization, which can be achieved by early termination of the reaction. We have recently described the analytical SAX-h.p.l.c. separation of higher-molecular-weight oligosaccharides having a degree of polymerization[16] between 8 and 20. The high-molecular-weight oligosaccharides are formed early in the reaction and then are further acted upon by heparin lyase, eventually breaking down to the final oligosaccharide products (**1**–**5**). These oligosaccharides are detected as clusters of peaks corresponding to hexasaccharide-, octasaccharide-, and decasaccharide-sized components observed in the time-course study of the depolymerization reaction (Fig. 3). They have primary sites and are cleaved at rates comparable to heparin, and may be thus termed transient oligosaccharides. Included among these transient oligosaccharides are a hexasaccharide and an octasaccharide comprised entirely of →4)-α-D-GlcN*p*2S6S(1→4)-α-L-IdoA*p*2S(1→ linkages corresponding to primary sites for heparin lyase[14]. Our laboratory is now applying preparative SAX-h.p.l.c. to prepare milligram quantities of these higher-molecular-weight heparin oligosaccharides. In the future these may serve as improved enzyme substrates or as a class of potent new heparin-like drugs.

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