Resistance of Heparin-derived Heparin Fragments to Biotransformation*

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The biotransformation of heparin-derived heparin fragments was examined via a combined approach using 35S-labeled heparin fragments as well as unlabeled chemically defined heparin fragments. Rats dosed with either [35S]di-, tetra-, hexa-, or octasaccharide fragments (2 mg/kg body weight, intravenously) excreted 63–69% of the injected radioactivity into the urine within 24 h with two-thirds being excreted during the first 6 h. Gel permeation chromatography of the urine samples showed that the tetra- and octasaccharides have undergone minor (≈5%) depolymerization whereas no change was observed for the di- and hexasaccharides. No N-desulfation was demonstrated for any of the substances. The hexa- and octasaccharide metabolites present in the urine 24 h after dosing exhibited the same antiaggregant activity as that of the injected material. A chemically defined trisulfated disaccharide and a hexasulfated tetrasaccharide were prepared and dosed in a similar manner. Only one metabolite was recovered from animals dosed with disaccharide. This compound was characterized by anion exchange chromatography, proton nuclear magnetic resonance spectroscopy, Fourier transform infrared spectrometry, and mass spectrometry and shown to be identical to the injected disaccharide. Five metabolites were isolated from the urine of rats dosed with the hexasulfated tetrasaccharide. The major metabolite, consisting of at least 65% of the total, was characterized as described for the disaccharide and shown to be identical to the injected compound. The remaining material appeared to be disaccharides and, possibly, a tetrasaccharide conjugate. Taken together, our results show that the heparinase-derived heparin fragments are very resistant to biotransformation compared with heparin and endogenous heparin fragments. These fragments may therefore be useful in defining structure activity relationships in vivo.

Heparin is a sulfated copolymer of glucosamine and uronic acid. The major repeating unit in the heparin polymer is 2-O-sulfated-L-iduronic acid-N-sulfated-6-O-sulfated-D-glucosamine (Comper, 1981), with heterogeneous regions containing D-glucuronic acid, nonsulfated-L-iduronic acid and N-acetylated-D-glucosamine (for review, see Casu, 1985). Heparin is also heterogenous with respect to molecular weight (Laurent et al., 1978). These variations have complicated the understanding of the structure-activity relationship of heparin's various biological activities. Heparinase (heparin lyase, EC 4.2.2.7) catalyzes an eliminase reaction which results in the production of di-, tetra-, hexa-, and oligosaccharides containing a 4-5 site of unsaturation in their nonreducing end (Linker and Hovingh, 1972, 1984). Immobilized heparinase has been used to eliminate heparin's anti-thrombin activity in extracorporeal circuits (Langer et al., 1982). Studies have shown that heparinase-derived heparin fragments as small as tetrasaccharides are biologically active as measured by anticoagulant activity (Linhardt et al., 1982; Larsen et al., 1984) and by antithrombin and anticoagulant activity (Linhardt et al., 1988). Heparinase-derived heparin fragments of hexasaccharide size, when combined with cortisone, prevent tumor growth in various tumor systems. In this respect the hexasaccharides were even more potent than their parent compound, heparin (Folkman et al., 1983).

We have recently shown that heparin fragments present in the plasma of rats dosed with oral heparin are extensively degraded and excreted into the urine as mono- and disaccharides void of biological activity (Larsen et al., 1986a). This suggests that the study of the activity of heparin fragments in vitro is very complex. For example, lack of activity could be due to either in vivo deactivation or the failure to have the correct structure-activity relationship.

In the present study, we have examined the metabolism of the heparinase-derived heparin fragments. The rationale for conducting this study was 2-fold. First, the structure-activity relationships of heparinase-derived heparin fragments is of considerable interest in a number of areas as discussed above. If such fragments are metabolized that would be important in determining what structure is ultimately responsible for biological activity. Second, immobilized heparinase has been proposed for use in removing heparin in a variety of clinical situations, and the degradation products of this reaction have been shown to be nontoxic in a number of tests (Langer et al., 1982). However, if such products are biotransformed in vivo, such toxicological tests would have limited value. Determining whether such products are biotransformed is impor-
Biotransformation of Heparin Fragments

tant in eventually assessing the safety of clinical procedures involving heparinase.

For this metabolic study we have developed a combined approach using [$^{35}$S]heparin fragments as well as unlabeled chemically defined heparin fragments. The biotransformation of the heparin fragment was followed by analyzing the metabolites excreted into the urine since earlier studies have shown that only a minor part (<1%) is excreted into the urine (Larsen et al., 1984, 1986b). Finally, we have examined how the metabolism affects the biological activity of the heparin fragments as well as of heparin.

MATERIALS AND METHODS

Chemicals—Heparin, sodium salt (153 units/mg) from porcine intestinal mucosa was obtained from Hepar Industries, Franklin, OH. (N-sulfonated) heparin (specific activity 21 mCi/mg; 1 Ci = 37 GBq) and [$^{35}$S]sulfate (carrier-free) were purchased from American Corp. while [H]$^1$glucosamine was from Du Pont-New England Nuclear. The manufacturer states that sulfate-labeled heparin is prepared by N-deacetylation of N-desulfated material and that the resulting heparin shows no difference in optical rotation, viscosity, sulfur content, infrared data as well as in vitro and in vivo anticoagulant activity as compared with the starting material. However, ≤1% of the O-sulfate is irreversibly removed during the N-desulfation procedure (Levy and Petracek, 1982). Carbazole, H$_2$O (99.99% atom %), 3-(trime thylsilyl)-1-propanol and sodium salicylate and potassium bromide were from Aldrich. Trigonoldichromylmethylammonium chloride was from Polysciences, Inc. (Warrington, PA).

Heparinase Preparation—Heparinase prepared fermentatively from Flavobacterium heparinum was purified by hydroxyapatite batch chromatography followed by anion exchange chromatography on the QAE-Sephadex (Pharmacia LKB Biotechnology Inc.) (Yang et al., 1985). This results in high recovery of heparinase that is nearly free of contaminating enzyme activities which could further act on the heparin degradation products (Yang et al., 1985). Immobilization of heparinase to cyanogen bromide activated Sepharose 4B (Pharmacia LKB Biotechnology Inc.) was performed by a variation (Langer et al., 1982) of the procedure of March et al. (1974).

Preparation of $^{35}$S-Labeled Heparin Fragments—Porcine mucosal heparin was desulfated with immobilized heparinase at 37 °C until the degradation was complete (Lindhurst et al., 1982). Heparinase was used to eliminate those claves certain a-glycosidic linkages in heparin, resulting in the generation of approximately 50 mol % disaccharides, 25% tetrasaccharides, 15% hexasaccharides, and 10% oligosaccharides (Lindhurst, 1978; Linhardt et al., 1982). These fragments contain a 4-5 site of unsaturation in their nonreducing end which can be detected by its UV absorbance at 232 nm (Hoving and Linker, 1970). This suspension was filtered to remove the immobilized enzyme. The same procedure was used to prepare [$^{35}$S]heparin fragments. The heparin fragments were then separated on a 1.5 × 110-cm Fractogel TSK HW-40 (S) (MCB Manufacturing Chemists, G cbst, NJ) column, eluted with 0.5 M ammonium bicarbonate buffer. The elution was performed at 4 °C using a flow rate of 0.1 ml/min. Fractions of 1 ml were collected, and the eluent was examined by measuring the absorbance at 232 nm or, for [$^{35}$S]heparin fragments, by liquid scintillation spectrometry. The heparin fragments were identified by comparing their elution profiles with those of diheparin, tetraheparin, and octasaccharide markers (Folkmann et al., 1983; Larsen et al., 1984). [H]$^1$glucosamine, [$^{35}$S]sulfate (carrier-free), and blue dextran. The octa-, hexa-, tetra- and di- fractions were collected and freeze-dried. Each fraction was then desalted on a 2.5 × 45-cm Sephadex G-10 (Pharmacia LKB Biotechnology Inc.) column eluted with distilled water at 4 ml/min at 4 °C. Unlabeled and labeled heparin fragments were then mixed to yield a solution containing 1.2 mg/ml and 1.2 μCi/ml.

Preparation of Pure Trisulfated Disaccharide and Hexasulfated Tetrasaccharide—Heparin, 100 mg, was degraded with heparinase as described above. The reaction mixture was then pressure filtered and concentrated to 3 ml in an Amicon unit containing a YCG (300 mV, cut off) membrane. The sample, 1 ml at 30 mg/ml, was then subjected to strong anion exchange-high pressure liquid chromatography (SAX-HPLC). The system consists of a Rhodyne 7125 injector connected to dual LDC Consametric III pumps and gradient mixer with microprocessor gradient control and data collection. The reaction mixture was injected onto a 1.0 × 25-cm SAX preparative column containing Spherosorb 5 Å-silica particles (Phase Separations, SAX-HPLC, CT) which had been equilibrated with 0.2 M sodium chloride at pH 3.5. Elution was performed using a 700-ml linear gradient from 0.2 to 1.6 M of sodium chloride, pH 3.5, at a flow rate of 3.0 ml/min. Detection was by absorbance at 232 nm and 2.0 absorbance units full scale. The two largest peaks in the chromatogram (peak a and peak b) were termed the trisulfated disaccharide (34 min) and the hexasulfated tetrasaccharide (72 min) which were collected (Merchant et al., 1985; Rice et al., 1987). An authentic trisulfated disaccharide (4-deoxy-α-L-threo-4-enopyranosyluronic acid-2-sulfate and 4-deoxy-α-D-threo-4-enopyranosyluronic acid-2-sulfate) was identified by HPLC and mass spectrometry. An authentic tetrasulfated disaccharide (4-deoxy-α-L-threo-4-enopyranosyluronic acid-2-sulfate) was also identified by HPLC and mass spectrometry.

Figure 1 shows the trisulfated disaccharide to be >95% pure. Drug Administration and Sample Collection—Male Wistar rats (200–350 g) were anesthetized by intraperitoneal injection of sodium pentobarbital (Nembutal, 50 mg/kg body weight) and the right jugular vein was cannulated as previously described (Larsen et al., 1984). These animals were then injected with heparin fragments at a dose of 2 mg/kg body weight, a dose within the clinical range, and placed in Nalgene metabolic cages (Nalgene Company, Rochester, NY). At least two rats were studied separately in each experiment. Urine samples were collected from the metabolic cages 6 and 24 h after injection, immediately frozen, and stored at −20 °C until examined.

Preparation of Unlabeled Metabolites—The urine samples were filtered and adjusted to pH 3.0 with 5 M HCl before application to a SP Sephadex column (1.5 × 10 cm) (Pharmacia LKB Biotechnology Inc.) and subjected to gel permeation chromatography on a Fractogel column as described above. The elution profiles were then compared to that of injected material of markers.

Preparation of Pure Trisulfated Disaccharide and Hexasulfated Tetrasaccharide—The reaction mixture was then pressure filtered and concentrated to 3 ml in an Amicon unit containing a YCG (300 mV, cut off) membrane. The sample, 1 ml at 30 mg/ml, was then subjected to strong anion exchange-high pressure liquid chromatography (SAX-HPLC). The system consists of a Rhodyne 7125 injector connected to dual LDC Consametric III pumps and gradient mixer with microprocessor gradient control and data collection. The reaction mixture was injected onto a 1.0 × 25-cm SAX preparative column containing Spherosorb 5 Å-silica particles (Phase Separations, SAX-HPLC, CT) which had been equilibrated with 0.2 M sodium chloride at pH 3.5. Elution was performed using a 700-ml linear gradient from 0.2 to 1.6 M of sodium chloride, pH 3.5, at a flow rate of 3.0 ml/min. Detection was by absorbance at 232 nm and 2.0 absorbance units full scale. The two largest peaks in the chromatogram (peak a and peak b) were termed the trisulfated disaccharide (34 min) and the hexasulfated tetrasaccharide (72 min) which were collected (Merchant et al., 1985; Rice et al., 1987). An authentic trisulfated disaccharide (4-deoxy-α-L-threo-4-enopyranosyluronic acid-2-sulfate) was identified by HPLC and mass spectrometry. An authentic tetrasulfated disaccharide (4-deoxy-α-L-threo-4-enopyranosyluronic acid-2-sulfate) was also identified by HPLC and mass spectrometry.

The abbreviations used are: SAX-HPLC, strong anion exchange-high pressure liquid chromatography; FT-IR, Fourier transform-infrared spectrometry.

FIG. 1. Structure of the trisulfated disaccharide (A) and the hexa-sulfated tetrasaccharide (B).
fraction at 232 nm. Major peaks were pooled and freeze dried. Each sample was dissolved in 1 ml of water and desalted on a Sephadex G-10 column where all gave a single peak at 232 nm eluting at the column's void volume. The peak was collected, freeze dried, and reconstituted with 2 ml of distilled water.

Analysis of Metabolized Samples by UV Spectroscopy—An absorbance spectrum between 200 and 300 nm was taken on each sample utilizing a Shimadzu UV 160 UV-visible spectrometer after diluting 20–140 µl of desalted sample up to 1 ml with 0.08 N HCl.

Quantitation of Metabolized Samples—The uronic acid present in each sample was quantitated by a carbazole assay (Bitter and Muir, 1962). The standard curve was constructed using 0–10 µg of glucuronolactone. The uronic acid content of each sample was determined at two concentrations and the data were averaged.

Characterization of Metabolites by Gel Permeation Chromatography—Each of the desalted samples was analyzed by gel permeation chromatography-HPLC using a S100 Polygel 3-µm particle column of 0.95 × 25 cm as described (Rice et al., 1985). A sample was dissolved in 100 µl with 0.2 M NaCl, pH 3.5, injected onto the column and eluted at flow rate of 0.8 ml/min with 0.2 M sodium chloride, pH 3.5. The detector was set at 232 nm with 0.02 absorbance units full scale, and retention times were measured to ±0.1 s. The K, of each sample was determined using blue dextran and sodium azide to measure void and total volume. Authentic disaccharide, tetrasaccharide, hexaxosaccharide, and heptaxosaccharide were used to calibrate the column (Rice et al., 1987).

Characterization of Metabolites by SAX-HPLC—Each of the desalted samples was analyzed by SAX-HPLC using an analytical 0.46 × 25-cm 5-µm particle size column. A sample dissolved in 100 µl of 0.2 M NaCl, pH 3.5, was injected onto a column which had been equilibrated with 0.2 M sodium chloride at pH 3.5. Elution was performed using a 50-ml linear gradient from 0.2 to 1.0 M of sodium chloride, pH 3.5, at a flow rate of 1.5 ml/min. In addition to being injected by itself, each sample was spiked with a known amount of added trisulfated disaccharide or hexa- and tetrasulfated tetrasaccharide standard. Detection was by absorbance at 232 nm and 0.02 absorbance units full scale.

Proton NMR of Metabolites—The major metabolites isolated from the administration of the trisulfated disaccharide and hexa- and tetrasulfated tetrasaccharides were analyzed by 80 MHz proton NMR on an IBM NR 80 spectrometer. The samples, 0.85 mg of trisulfated disaccharide metabolite and 3 mg of hexa- and tetrasulfated tetrasaccharide metabolite, were prepared separately. Each desalted sample was freeze dried and dissolved in 1 ml of D$_2$O (9996 atom %), frozen, and freeze dried. This D$_2$O exchange was performed twice on each sample before finally dissolving each in 0.3 ml of D$_2$O containing 0.1% 3-[(trimethyl)1-propanesulfonic acid sodium salt. The proton NMR spectra were obtained for both samples.

FT-IR of Metabolites—The major metabolites isolated from the administration of the trisulfated disaccharide and hexa- and tetrasulfated tetrasaccharides were each analyzed by FT-IR on a Nicolet Fourier transform infrared spectrophotometer. Each desalted, freeze-dried sample (100 µg) was mixed thoroughly with 20 µg of dry potassium bromide, and a spectra was obtained by diffuse reflectance (Linhardt et al., 1986).

Mass Spectrometry of Metabolites—The sodium salt of the major metabolite isolated from the administration of the trisulfated disaccharide was prepared in thioglycerol at a concentration of 40 µg/µl and deposited on a gold-tipped direct-insertion probe. The fast atom bombardment (FAB) mass spectra was collected in the negative ion mode by using nitrogen gas, the gun operating at 1.5-Ma tube current at an energy of 7 Kev (Merchant et al., 1985). The sodium salt of the major metabolite isolated from the administration of the hexa- and tetrasulfated tetrasaccharide (50 µg) was bound to an aluminiized Mylar foil impregnated with tridecyl methylammonium chloride. The Waters 252 California plasma desorption mass spectra was obtained (McNeal et al. 1986).

Anticoagulant Activity—The anticoagulant activity was determined by a factor Xa chromogenic assay (Kabi Diagnostica, Stockholm, Sweden). The procedure followed was that recommended by the manufacturer. In this assay, the decrease in absorbance at 405 nm is linear in the range of 0–5 µg of heparin and 0–50 µg of heparin fragments/ml sample. In order to compare the different samples at a standard curve was made for both the injected fragments and the urine samples collected after 6 or 24 h. All samples were measured in duplicate.

RESULTS

[$^3$H]Heparin Fragments Are Rapidly Excreted into the Urine of Rats—Intravenously administered [$^3$H]heparin-derived octa-, hexa-, tetra-, or disaccharides 2 mg/kg body weight) were rapidly excreted into the urine. By 24 h, 63–69% of the injected radioactivity was recovered in the urine with about two-thirds of this being excreted within the first 6 h. In comparison, 45% of injected [$^3$H]heparin was recovered in the urine of rats 24 h after the injection of a similar dose.

Characterization of Urinary Metabolites by Gel Permeation Chromatography—The elution profiles of radioactive material excreted into the urine during the first 6 h and the subsequent 18 h after administration of [$^3$H]heparin fragments are shown in Figs. 2 through 5. For comparison, the elution profile of the injected material is also shown. The radioactive material present in the urine of rats injected with [$^3$H]heparin disaccharide eluted in the same position as the disaccharide marker (Fig. 2). No material of lower molecular weight was detected. Most of the radioactive material present in the urine of rats injected with [32S]tetrasaccharides eluted in the same position as the tetrasaccharide marker (Fig. 3). In addition some material eluted between the hexa- and tetrasaccharide marker (Fig. 3B and C). A new peak, corresponding to material of disaccharide size, was observed in the urine excreted 6–24 h after dosing. Additional experiments showed that this low molecular weight compound first appeared in the urine 20 h after dosing (results not shown). Fig. 4 shows that the radioactive material excreted in the urine of rats dosed with [34S]hexaxosaccharides eluted in the same position as the injected

![Graph](image-url)
Biotransformation of Heparin Fragments

Fig. 3. Gel permeation chromatography of the radioactive material excreted into the urine of rats during the first 6 h (B) or the subsequent 18 h (C) after an intravenous dose of 2 mg/kg $[^{35}S]$heparin tetrasaccharides (A). Each sample was analyzed as described (legend of Fig. 2). Void, octa, hexa, tetra, di, sulfate, and mono denote the respective elution volumes of blue dextran, heparin octa-, hexa-, tetra-, and disaccharides, $[^{35}S]$sulfate and glucosamine.

Fig. 5. Gel permeation chromatography of the radioactive material excreted into the urine of rats during the first 6 h (B) or the subsequent 18 h (C) after an intravenous dose of 2 mg/kg $[^{35}S]$heparin octasaccharides (A). Each sample was analyzed as described (legend of Fig. 2). Void, octa, hexa, tetra, di, sulfate, and mono denote the respective elution volumes of blue dextran, heparin octa-, hexa-, tetra-, and disaccharides, $[^{35}S]$sulfate and glucosamine.

Hexasaccharide. Even by 24 h, no low molecular weight material could be detected (Fig. 4C). Radioactive material present in the urine of rats 6 h after dosing with $[^{35}S]$-octasaccharide eluted in the same position as the octasaccharide marker (Fig. 5). By 24 h, about 15% of the radioactivity eluted as di- and tetrasaccharide size material. For comparison, the urine of rats dosed with $[^{35}S]$heparin was examined in a similar manner (Fig. 6). By 6 h, 5% of the excreted activity eluted in the same position as inorganic sulfate; this had increased to 68% by 24 h. Taken together, these observations suggest that little, if any, degradation, had taken place in rats dosed with $[^{35}S]$heparin di- and hexasaccharides. In animals dosed with $[^{35}S]$-tetrasaccharides, a total of 96% of the radioactivity excreted into the urine the first 24 h after dosing still eluted in the same position as the injected tetrasaccharide while 94% eluted as the injected octasaccharide in animals dosed with $[^{35}S]$-octasaccharides. In comparison, only 68% of the total radioactivity present in the urine by 24 h of rats receiving $[^{35}S]$heparin eluted in the same position as the injected material.

The Heparin Fragments Present in the Urine Have Anti-factor Xa Activity—The anti-factor Xa activity appearing in the urine of rats after an intravenous dose of $[^{35}S]$heparin, heparin octasaccharides, or heparin hexasaccharides is shown in Table I. The heparin metabolites excreted into the urine the first 24 h after dosing only exhibit a total of 34% of the anti-factor Xa activity of the injected material when the same amount of radioactivity is compared. In contrast, a total of about 96% of the anti-factor Xa activity is retained for the octasaccharide. Since about 95% of the radioactivity of the material excreted into the urine during the first 24 h after dosing can be recovered as octasaccharide size material (Fig. 5), this means that loss of anti-factor Xa activity of the octasaccharide was due to degradation to smaller fragments.
with less anti-factor Xa activity whereas the urinary material eluting in the octasaccharide position was the same biological activity as the original material. The hexasaccharide metabolite, which is not depolymerized, retained all its anti-factor Xa activity even 24 h after dosing.

Characterization of Unlabeled Urinary Metabolites—Six potential metabolites, D, T1, T1a, T2, T2a, and T3 were recovered from the urine after cation exchange chromatography followed by gel permeation chromatography as shown in Fig. 7. After desalting, the total absorbance at 232 nm present in each sample was calculated (Table II) and a spectra from 200 to 300 nm was obtained. Only D, T1, and T1a showed absorbance maxima at 232 nm characteristic of a Δ-4.5 unsaturated uronic acid residue. The other samples possessed absorbance maxima around 200 nm which extend across the 232 nm region. A carbazole assay was performed on each of the six samples and the total milligrams of uronic acid in each is also given in Table II. Each sample was then analyzed by gel-permeation chromatography-HPLC using UV detection at 232 nm. Both D and T1 gave a single symmetrical peak having a K_{av} corresponding to the trisulfated disaccharide and the hexasulfated tetrasaccharide, respectively. All the remaining samples, except for T3, contained some of the corresponding unmetabolized trisulfated disaccharide or hexasulfated tetrasaccharide (Table II). Each sample was also analyzed by SAX-HPLC using UV detection at 232 nm. Both D and T1 gave single peaks corresponding to the retention times of trisulfated disaccharide and hexasulfated tetrasaccharide, respectively (Table I). All the remaining samples, except for T3, showed some of the corresponding unmetabolized trisulfated disaccharide and hexasulfated tetrasaccharide. Both D and T1 coeluted with disaccharide or tetrasaccharide markers after spiking with a known amount of either trisulfated disaccharide or hexasulfated tetrasaccharide (Fig. 8) confirming the peak assignments made on the SAX-HPLC chromatograms.

Metabolized samples which had been demonstrated by chromatographic analysis to contain single components, D and T1, were analyzed by 80 MHz proton NMR (Merchant et al., 1985), FT-IR (Linhardt et al., 1986), FAB, and California plasma desorption mass spectra (Merchant et al., 1985; McNeal et al., 1986). The disaccharide (D) collected in the urine gave a proton NMR spectra which was consistent with the trisulfated disaccharide (Merchant et al., 1985). It also gave a FAB mass spectra with a molecular ion at m/1 642 [M−Na]^+ (Merchant et al., 1985) and a FT-IR identical to the trisulfated disaccharide. The tetrasaccharide, T1, also gave a consistent proton NMR spectra (Merchant et al., 1985), a California plasma desorption mass spectra with a molecular ion m/1 5972 [(TDMA)_2] (tetrasaccharide)^+ and a FT-IR identical to the hexasulfated tetrasaccharide. The lack of sufficient sample did not permit the spectral analysis of the minor metabolites recovered in urine after hexasulfated tetrasaccharide administration.

**DISCUSSION**

To study the metabolism of heparin fragments, we have used a combined approach using ^35S-labeled heparin frag-
Biotransformation of Heparin Fragments

Table II

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<th>Chemical and chromatographic properties of urinary metabolites of a tri sulfated heparin disaccharide and a hexa sulfated heparin tetrasaccharide</th>
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<td><strong>Units of absorbance at 232 nm</strong></td>
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<td><strong>Absorbance</strong></td>
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<td>Samples administered†</td>
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<td>Hexa sulfated tetrasaccharide</td>
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- Uronic acid as determined by carbazole assay (Bitter and Muir, 1962).
- The underlined K<sub>R</sub> and retention time values correspond to those associated with the tri sulfated disaccharide.
- The percent recovery is calculated by dividing the total sample administered by the total sample recovered as measured by either absorbance at 232 nm or by uronic acid content.
- The percent of total absorbance recovered corresponding to unmetabolized sample is calculated from the percent of unmetabolized sample in each fraction as estimated by SAX-HPLC.

...ments as well as unlabeled chemically defined substances. [3H]Heparin is, in our opinion, not suited for metabolic studies of small heparin fragments since the labeling procedure results in chemical alteration of the reducing end (Hatton et al., 1980). This structural change is recognized by the bacterial heparinase since treatment of [3H]heparin with heparinase results in a product distribution different from that observed with unlabeled heparin (results not shown). In contrast, heparinase cleavage of [35S]heparin results in a product distribution identical to that observed with unlabeled heparin (Larsen et al., 1984). Gel permeation chromatography of [35S]-labeled material enabled us to detect whether depolymerization or O-desulfation has taken place. In order to detect O-desulfation or other structural alterations, heparin fragments with a chemically defined structure were required. The isolation of urinary metabolites was based on cation exchange chromatography at pH 3.0 (Larsen et al., 1986); at this pH only the strongly negatively charged heparin fragments and other sulfated compounds will pass through the column and elute into the void volume. This means that totally desulfated metabolites may be lost, while other sulfated components may be included. The heparin fragments were detected at 232 nm, which is their absorbance maximum (Hoving and Linker, 1970). This allowed detection of fragments as small as disaccharides; smaller metabolites containing this chromophore might be overlooked due to background absorbance at 232 nm.

The tri sulfated disaccharide was not metabolized, as shown by anion exchange chromatography (Fig. 8), infrared spectrometry, proton nuclear magnetic resonance spectroscopy, mass spectrometry, and gel permeation chromatography of [35S]-disaccharide (Fig. 2). In contrast, a total of ~5% of the mixed [35S]-tetrasaccharides are degraded to disaccharide-size material within 24 h after dosing (Fig. 3). Five potential metabolites were recovered in the urine of rats dosed with a chemically defined hexa sulfated tetrasaccharide (Fig. 7). The major metabolite (T1) consisting of at least 65% of the total was characterized as described for the disaccharide and shown to be identical to the injected hexa sulfated tetrasaccharide. A minor metabolite (T1a) eluted as material larger than tetrasaccharide (Fig. 7). This substance contains uronic acid, has an absorbance maximum at 232 nm, and is highly sulfated (Table II). Most likely, this fraction is a mixture of the administered hexa sulfated tetrasaccharide and a tetrasaccharide conjugate. Two minor metabolites, T2 and T2a (Fig. 7), eluted as disaccharide size material. Both fractions contained some tri sulfated disaccharide (Table II), in addition to some material with low sulfation. This suggests the action of a glycosidase cleaving the tetrasaccharide into two tri sulfated disaccharides of which one has an unsaturated Δ-4,5 disulfonate bond. A heparin-degrading endoglycosidase capable of depolymerization of heparin into oligosaccharides as small as disaccharides has been isolated from rat spleen (Hook et al., 1977). The last peak, T3 (Fig. 7), is probably not a tetrasaccharide metabolite since it did not contain uronic acid (Table II). Also, no radiolabeled material was detected in this position in the urine of rats dosed with [35S]-tetrasaccharides (Fig. 3). If the recovery is based on the presence of uronic acid, the original hexa sulfated tetrasaccharide amounts to about 80% of the total material recovered in the urine.

The resistance of the heparinase-derived heparin fragments to biotransformation was unexpected. We have previously shown (Larsen et al., 1986a) that heparin metabolites present in the plasma of rats dosed with oral heparin are extensively degraded and excreted into the urine as mono- and disaccharides. Free sulfate was also demonstrated indicating extensive N-desulfation. In this study [35S]heparin was extensively N-desulfated as shown by the presence of free sulfate (Fig. 6). This was accompanied by a loss of anti-factor Xa activity, which agrees with previous studies linking loss of anticoagulant activity with O- and, in particular, N-desulfation (Erlrich and Stivala, 1973). In contrast, very little depolymerization and no N-desulfation was demonstrated for the heparin fragments. The resistance of the heparinase-derived heparin frag-
Biotransformation of Heparin Fragments

Fig. 8. Analytical SAX-HPLC of standards and recovered samples D and T1. A, standard trisulfated disaccharide (a), penta-sulfated tetrasaccharide (Linhart et al., 1986) (b), and hexasulfated tetrasaccharide were prepared in a mixture, and 15 μl was analyzed by SAX-HPLC. B, standard mixture (15 μl) and D (2 μl) were combined and analyzed by SAX-HPLC. The arrow indicates the peak with enhanced intensity. C, standard mixture 15 μl and T1 (2 μl) were combined and analyzed by SAX-HPLC. The arrow indicates the peak with enhanced intensity.

ments to biotransformation may be due to the Δ-4,5 unsaturated double bond generated in the nonreducing end by the heparinase reaction (Hovingh and Lineker, 1970) since heparin generally is believed to be metabolized in a stepwise manner starting from the non-reducing end (see Rodén, 1980). A second explanation of the small extent to which heparin fragments were metabolized is that only a very small part of the dose (~0.05%) may have reached the spleen (Larsen et al., 1984), which is believed to be the major site of heparin metabolism (Lloyd et al., 1966; Dietrich, 1970; Friedman and Arsenis, 1974). The di (D)-, and tetrasaccharide (T1) contains no internal glucuronic acid. Thus, no degradation by endogluconidase is expected (Nakajima et al., 1986). However, both the hexa- and the octasaccharide mixtures contain internal glucuronic acid residues. Indeed, in the case of the hexasaccharides, the major component representing >80% of the mixture contains an internal glucuronic acid (Lindhart et al., 1986). Further studies will be required to understand why the endogluconidase does not act on these glucuronic acid containing fragments.

Our results are important for two reasons. First, the heparinase reaction results in the formation of heparin fragments having biological activity but which are resistant to biotransformation. These fragments could be useful in defining structure-activity relationships in vivo. Second, these fragments are the products of the enzyme, heparinase, which may be used to remove heparin in a variety of clinical situations, such as dialysis, where heparin can cause harm and even death (Bernstein et al., 1987). Since the fragments are not biotransformed in vivo their toxicological evaluation is simplified.

In conclusion, our experiments demonstrate the following: (i) heparinase-derived heparin fragments were extensively excreted into the urine for at least 24 h after a single intravenous dose, (ii) None of the heparin fragments appeared to be N-desulfated. The tetra- and octasaccharides underwent minor (~5%) depolymerization, whereas no change was observed for the di- and hexasaccharides. (iii) The hexa- and octasaccharide metabolites present in the urine 24 h after dosing exhibited the same anti-factor Xa activity as that of the injected material. (iv) One-hundred % of a chemically defined trisulfated disaccharide and up to 80% of a hexasulfated tetrasaccharide was recovered unchanged in the urine. The remainder of the tetrasaccharide was recovered as disaccharide-size material and, possibly, as a tetrasaccharide conjugate. Taken together, these results show that heparinase-derived heparin fragments are very resistant to biotransformation compared with heparin and endogenous heparin fragments.

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Biotransformation of Heparin Fragments
