THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 264, No. 3, Issue of January 25, pp. 1570-1577, 1989
Printed in U.S.A.

Resistance of Heparinase-derived Heparin Fragments to Biotransformation*

(Received for publication, August 9, 1988)

Annette K. Larsen,[a,b] Kevin G. Rice,[c] Robert J. Linhardt,[c] Gerald Wogan,[d,e] and Robert Langer[a,d,f,g,h]

From the [a]Department of Chemical Engineering, the [d]Whitaker College of Health Sciences, and the [e]Department of Chemistry, Massachusetts Institute of Technology, and the [g]Harvard-Massachusetts Institute of Technology Division of Health Sciences and Technology, Cambridge, Massachusetts 02139, the [b]Laboratoire de Pharmocologie Moleculaire, Centre National de la Recherche Scientifique UA 147, and Groupe de Recherches sur la Pharmacologie de Medicaments Anticancereux, Institut National de la Santé et de la Recherche Médicale U 140, Institute Gustave-Roussy, Villejuif 94800, France, the [c]Division of Medicinal and Natural Products Chemistry, College of Pharmacy, The University of Iowa, Iowa City, Iowa, 52242, and the [f]Department of Surgery, Children's Hospital Medical Center, Boston, Massachusetts 02115

The biotransformation of heparinase-derived heparin fragments was examined via a combined approach using ^{35}S-labeled heparin fragments as well as unlabeled chemically defined heparin fragments. Rats dosed with either [^{35}S]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 urinary material shows 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 antifactor *Xa* 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 Δ-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 antifactor *Xa* activity (Linhardt *et al.*, 1982; Larsen *et al.*, 1984) and by anticomplement activity (Linhardt *et al.*, 1988). Heparinase-derived heparin fragments of hexasaccharide size, when combined with cortisone, prevent tumor formation 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 vivo* 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-

* This work was supported by National Institutes of Health Grant GM 25810 (to R. L.), National Institutes of Health Grant HL 29797-04 (to R. J. L.), Association pour le Developpement de la Recherche sur le Cancer, Villejuif, France (to A. K. L.), and Continental Pharma Cryosan (to R. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[h] To whom correspondence and reprint requests should be addressed: Rm E25-342, Massachusetts Institute of Technology, Cambridge, MA 02139.

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 fragments 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 bile (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*-sulfonate-^{35}S]heparin (specific activity 21 mCi/g; 1 Ci = 37 GBq) and [^{35}S]sulfate (carrier-free) were purchased from Amersham Corp. while [^{3}H]glucosamine was from Du Pont-New England Nuclear. The manufacturer states that sulfate-labeled heparin is prepared by *N*-resulfation of *N*-desulfated material and that the resulfated heparin shows no difference in optical rotation, viscosity, sulfur content, infrared data as well as *in vitro* and *in vivo* anticoagulant potency as compared with the starting material. However, ≈1% of the *O*-sulfate is irreversibly removed during the *N*-desulfation procedure (Levy and Petracek, 1962). Carbazole, $^{2}H_2O$ (99.996 atom %), 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt, and potassium bromide were from Aldrich. Tridodecyl methylammonium 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 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 degraded with immobilized heparinase at 37 °C until the degradation was complete (Linhardt *et al.*, 1982). Heparinase is an eliminase that cleaves certain α-glycosidic linkages in heparin, resulting in the generation of approximately 50 mol % disaccharides, 25% tetrasaccharides, 15% hexasaccharides, and 10% oligosaccharides (Dietrich, 1968; Linhardt *et al.*, 1982). These fragments contain a Δ-4,5 site of unsaturation in their nonreducing end which can be detected by its UV absorbance at 232 nm (Hovingh 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, Gibbstown, 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 heparin di-, tetra-, hexa-, and octasaccharide markers (Folkman *et al.*, 1983; Larsen *et al.*, 1984), [^{3}H]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 YC05 (300 mW, cut off) membrane. The sample, 1 ml at 30 mg/ml, was then subjected to strong anion exchange-high pressure liquid chromatography (SAX-HPLC).[1] The system consists of a Rheodyne 7125 injector connected to dual LDC Constametric III pumps and gradient mixer with microprocessor gradient control and data collection. The reaction mixture was injected onto a 1.0 × 25.0-cm SAX preparative column containing Spherisorb 5-μm particles (Phase Separations, Norwalk, 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 corresponding to the trisulfated disaccharide (34 min) and the hexasulfated tetrasaccharide (72 min) were collected (Merchant *et al.*, 1985; Rice *et al.*, 1987). An authentic trisulfated disaccharide (4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid-2-sulfate(1→4)-α-D-2-sulfamino-2-deoxy-glucopyranose-6-sulfate) and an authentic hexasulfated tetrasaccharide (4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid-2-sulfate(1→4)-α-D-2-sulfamino-2-deoxy-glucopyranose-6-sulfate(1→4)-α-L-idopyronosyluronic acid-2-sulfate(1→4)-α-D-2-sulfamino-2-deoxy-glucopyranose-6-sulfate) (Fig. 1), were prepared and fully characterized as previously described (Merchant *et al.*, 1985; Rice *et al.*, 1987). The trisulfated disaccharide (Fig. 1) is the only disaccharide present in the product mixture comprising about 50 mol % of the total (Merchant *et al.*, 1985). The hexasulfated tetrasaccharide comprises about 5 mol % of the total and is the major tetrasaccharide present (Merchant *et al.*, 1985). It is also the only tetrasaccharide which exhibits significant anticomplement activity (Linhardt *et al.*, 1988). These two fractions were each freeze dried, desalted on a Sephadex G-10 column, freeze dried, and rechromatographed and desalted under the same conditions. Chromatographic analysis on analytical SAX-HPLC showed the trisulfated disaccharide to be >99% pure and the hexasulfated tetrasaccharide to be >95% pure.

Drug Administration and Sample Collection—Male Wistar rats (300–350 g) were anesthetized by intraperitoneal injection of sodium pentobarbital (Nembutal, 50 mg/kg body weight) and the right jugular vein cannulated as previously described (Larsen *et al.*, 1984). This shunt was used for sample injection. The 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 (Nalge 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.

Analysis of ^{35}S-Labeled Heparin Fragments—The radioactivity present in the urine was determined by liquid scintillation spectrometry. The samples were filtered on a 0.22-μm Millex filter (Millipore Corporation, Bedford, MA) and subjected to gel permeation chromatography on a Fractogel column as described above. The elution profiles were then compared to that of injected material and of markers.

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.) equilibrated with diluted HCl, pH 3.0, at 0.5 ml/min as previously described (Larsen *et al.*, 1986a). At this pH, only the strongly negatively charged heparin fragments and other sulfated compounds pass through the cation exchange column and elute in the void volume. The recovery, as judged by radioactivity, was 70–75%. After cation exchange chromatography, the heparin fragment-containing fractions were pooled, neutralized with ammonium bicarbonate, and freeze dried. The samples were then applied to the Fractogel column and the elution profile followed by measuring the absorption of each

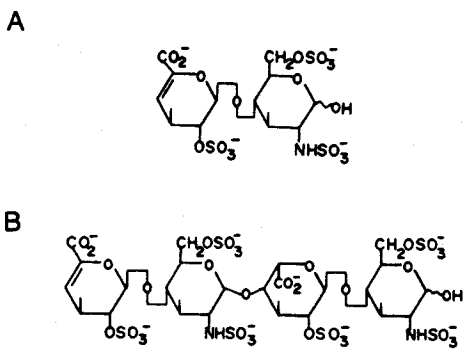

FIG. 1. **Structure of the trisulfated disaccharide (*A*) and the hexa-sulfated tetrasaccharide (*B*).**

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

fraction at 232 nm. Major peaks were pooled and freeze dried. Each sample was redissolved 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 0.03 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-HPLC—Each of the desalted samples were analyzed by gel permeation chromatography-HPLC using a SI 100 Polyol 3-μm particle column of 0.95 × 50-cm as described (Rice *et al.*, 1985). A sample was dissolved up to 100 μl with 0.2 M NaCl, pH 3.5, injected onto the column and eluted at a 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_{av} of each sample was determined using blue dextran and sodium azide to measure the void and total volume. Authentic disaccharide, tetrasaccharide, and hexasaccharides 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 hexasulfated 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 hexasulfated tetrasaccharide were analyzed by 80 MHz proton NMR on an IBM-NR 80 spectrometer. The samples, 0.8 mg of trisulfated disaccharide metabolite and 3 mg of hexasulfated tetrasaccharide metabolite, were prepared separately. Each desalted sample was freeze dried and dissolved in 1 ml of ^2H_2O (99.996 atom %), frozen, and freeze dried. This ^2H_2O exchange was performed two times on each sample before finally dissolving each in 0.3 ml of ^2H_2O 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 hexasulfated tetrasaccharide were each analyzed by FT-IR on a Nicolet Fourier transform infrared spectrometer. Each desalted freeze dried sample (100 μg) was mixed thoroughly with 20 mg 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 xenon gas with 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 hexasulfated tetrasaccharide (50 μg) was bound to an aluminized Mylar foil impregnated with tridodecyl methylammonium chloride. The 252 Californium 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 absorption at 450 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, 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

[^{35}S]Heparin Fragments Are Rapidly Excreted into the Urine of Rats—Intravenously administered [^{35}S]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 [^{35}S]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 [^{35}S]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 [^{35}S]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 ^{35}S-tetrasaccharides eluted in the same position as the tetrasaccharide marker (Fig. 3). In addition some material eluted between the hexa- and tetrasaccharide marker (Fig. 3*B* 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 ^{35}S-hexasaccharides eluted in the same position as the injected

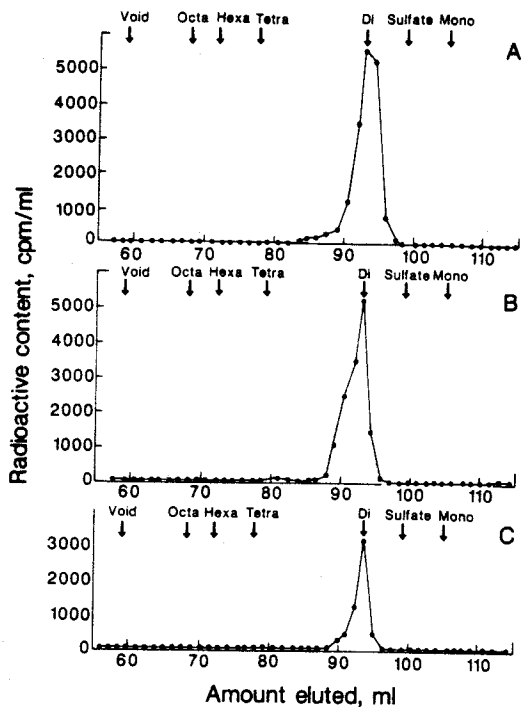

FIG. 2. **Gel permeation chromatography of the radioactive material excreted into the urine of rats during the first 6 h (*B*) or the subsequent 18 h (*C*) after an intravenous dose of 2 mg/kg [^{35}S]heparin disaccharides (*A*).** A portion of each sample was applied to a Fractogel-TSK HW-40 (S) column (1.5 × 110 cm) equilibrated with 0.5 M ammonium bicarbonate. The elution was performed at a flow rate of 0.1 ml/min. Fractions (≈1 ml) were collected, and the radioactive content of each fraction was determined by liquid scintillation spectrometry. *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.

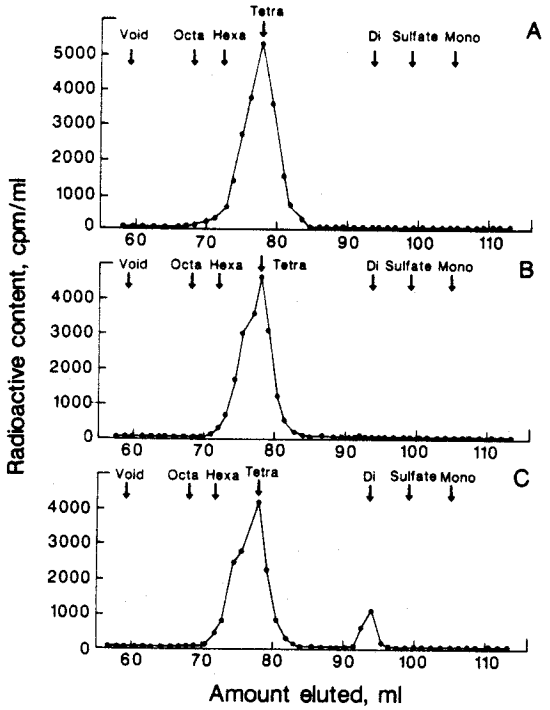

FIG. 3. **Gel permeation chromatography of the radioactive material excreted into the urine of rats during the first 6 h (*B*) or the subsequent 18 h (*C*) after an intravenous dose of 2 mg/kg [^{35}S]heparin tetrasaccharides (*A*).** Each sample was analyzed as described (legend of Fig. 2). *Void, octa, 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.

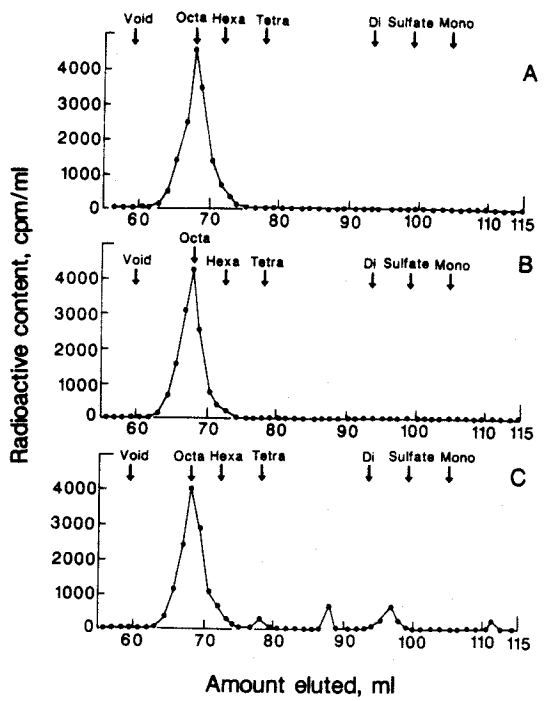

FIG. 5. **Gel permeation chromatography of the radioactive material excreted into the urine of rats during the first 6 h (*B*) or the subsequent 18 h (*C*) after an intravenous dose of 2 mg/kg [^{35}S]heparin octasaccharides (*A*).** Each sample was analyzed as described (legend of Fig. 2). *Void, octa, 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.

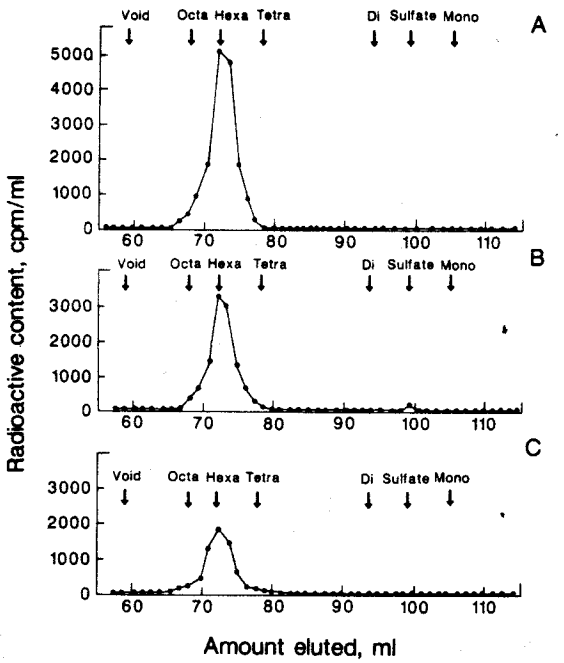

FIG. 4. **Gel permeation chromatography of the radioactive material excreted into the urine of rats during the first 6 h (*B*) or the subsequent 18 h (*C*) after an intravenous dose of 2 mg/kg [^{35}S]heparin hexasaccharides (*A*).** Each sample was analyzed as described (legend of Fig. 2). *Void, octa, 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. 4*C*). 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 58% 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 95% 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

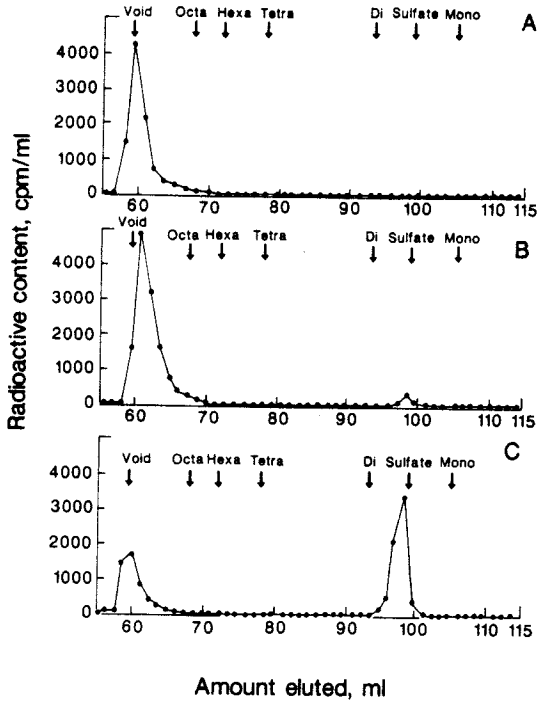

FIG. 6. **Gel permeation chromatography of the radioactive material excreted into the urine of rats during the first 6 h (*B*) or the subsequent 18 h (*C*) after an intravenous dose of 2 mg/kg [^{35}S]heparin (*A*).** Each sample was analyzed as described (legend of Fig. 2). *Void, octa, hexa, tetra,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

TABLE I

Presence of anti-factor Xa activity in the urine of rats dosed with [^{35}S]heparin, heparin octasaccharides, or hexasaccharides (2 mg/kg)

The numbers indicate the % of anti-factor Xa activity detected in the urine at the indicated time compared to the activity of the injected material.

	Urine collected 0–6 h after dosing	Urine collected 6–24 h after dosing
	%	%
[^{35}S]Heparin	53	14
[^{35}S]Octasaccharides	≈100	82
[^{35}S]Hexasaccharides	≈100	≈100

with less anti-factor *X*a activity whereas the urinary material eluting in the octasaccharide position exhibited the same biological activity as the original material. The hexasaccharide metabolite, which is not depolymerized, retained all its anti-factor *X*a 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 extended 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_{avg} corresponding to the trisulfated disaccharide and the

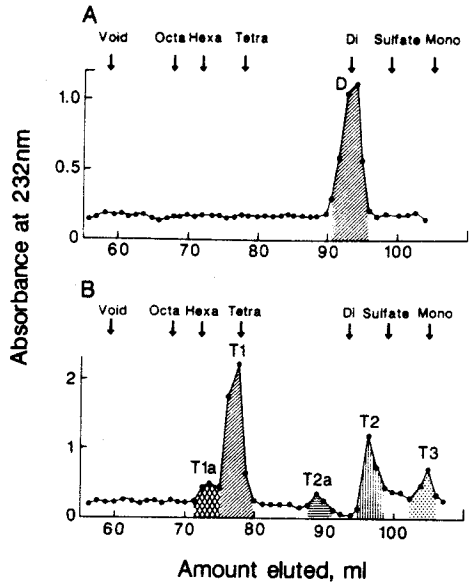

FIG. 7. **Gel permeation chromatography of material excreted into the urine of rats during 24 h after an intravenous dose of 2 mg/kg trisulfated heparin disaccharide (*A*) or hexasulfated heparin tetrasaccharide (*B*).** Each sample was analyzed as described (legend of Fig. 2) and the absorbance at 232 nm determined for each sample. *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.

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 Californium 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/I 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 Californium plasma desorption mass spectra with a molecular ion m/I 5972 [$(TDMA^+)_9$ (tetrasaccharide)$^{8-}$]$^+$ 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 ^{35}S-labeled heparin frag-

TABLE II

Chemical and chromatographic properties of urinary metabolites of a trisulfated heparin disaccharide and a hexasulfated heparin tetrasaccharide

	Units of absorbance at 232 nm	Total uronic[a] acid	GPC-HPLC peaks[b] K_{avg}	SAX-HPLC peaks[b] Rt in min
	absorbance	*mg*	*% total absorbance*	*% total absorbance*
Samples administered[c]				
Trisulfated disaccharide	47	2.0	0.494 (100)	14 (100)
Hexasulfated tetrasaccharide	24	2.0	0.434 (100)	24 (100)
Samples recovered				
D	5.0	0.28	0.494 (100)	14 (100)
Total	5.0 (12%)[d]	0.28 (14%)[d]		
Unmetabolized	5.0 (100)[e]			
T1	6.4	0.69	0.434 (100)	24 (100)
T1a	1.2	0.12	0.406 (70), 0.436 (30)	24 (50), 24.5 (50)
T2	1.8	0.05	>0.560 (100)	<10 (84), 14 (7), 24 (7)
T2a	0.6	0.09	>0.560 (100)	<10 (95), 14 (5), 24 (<1)
T3	1.5	0	>0.560 (100)	<10 (100) 24 (0)
Total recovered	11.5 (49%)[d]	0.95 (48%)[d]		
Unmetabolized	7.1 (62%)[e]			

[a] Uronic acid as determined by carbazole assay (Bitter and Muir, 1962).

[b] The underlined K_{avg} and retention time values correspond to those associated with the trisulfated disaccharide.

[c] Amount of sample administered based on $\epsilon = 5700\ M^{-1}$ at 232 nm in 0.03 n HCl with M_r for trisulfated disaccharide of 665 and M_r of hexasulfated tetrasaccharide of 1330 was 6.0 mg of each.

[d] The percent recovery is calcualted by dividing the total sample administered by the total sample recovered as measured by either absorbance at 232 nm or by uronic acid content.

[e] 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. [^{3}H]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 [^{3}H]heparin with heparinase results in a product distribution different from that observed with unlabeled heparin (results not shown). In contrast, heparinase cleavage of [^{35}S]heparin results in a product distribution identical to that obtained with nonlabeled heparin (Larsen *et al.*, 1984). Gel permeation chromatography of ^{35}S-labeled material enabled us to detect whether depolymerization or *N*-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 (Hovingh 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 trisulfated 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 ^{35}S-disaccharide (Fig. 2). In contrast, a total of ~5% of the mixed ^{35}S-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 hexasulfated 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 hexasulfated 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 hexasulfated tetrasaccharide and a tetrasaccharide conjugate. Two minor metabolites, T2 and T2a (Fig. 7), eluted as disaccharide size material. Both fractions contained some trisulfated disaccharide (Table II), in addition to some material with low sulfation. This suggests the action of a glycosidase cleaving the tetrasaccharide into two trisulfated disaccharides of which one has an unsaturated Δ-4,5 double 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 ^{35}S-tetrasaccharides (Fig. 3). If the recovery is based on the presence of uronic acid, the original hexasulfated 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 [^{35}S]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 (Erlich 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-

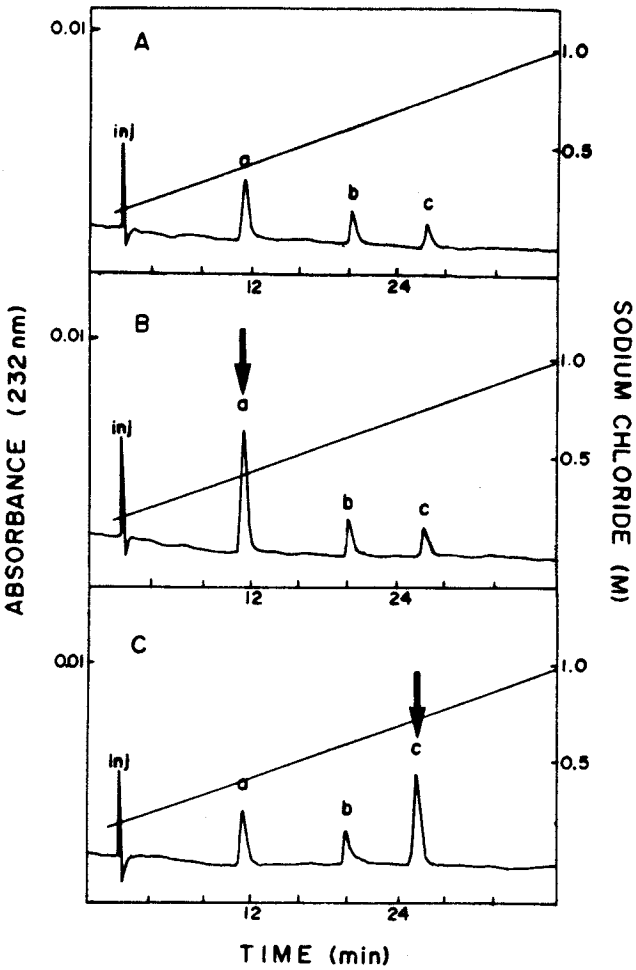

FIG. 8. **Analytical SAX-HPLC of standards and recovered samples D and T1.** *A*, standard trisulfated disaccharide (*a*), pentasulfated tetrasaccharide (Linhardt *et al.*, 1986) (*b*), and hexasulfated tetrasaccharide were prepared in a mixture, and 15 μl was analyzed by SAX-HPLC. *B*, standard mixture (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 Linker, 1970) since heparin generally is believed to be metabolized in a stepwise manner starting from the non-reducing end (for review, see Roden, 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 endoglucuronidase 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 >60% of the mixture contains an internal glucuronic acid (Linhardt *et al.*, 1986). Further studies will be required to understand why the endoglucuronidase 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.

Acknowledgments—We wish to thank Professor Ian Jardine of the Mayo Clinic, Rochester, MN for obtaining the mass spectra of the disaccharide and tetrasaccharide metabolites and Micheline Re, Institute Gustave-Roussy for expert technical assistance with the animal experiments. We also thank Dr. Alain Gouyette for critical review of the manuscript. We thank Pamela Brown for clerical assistance.

REFERENCES

Bernstein, H., Yang, V. C., Lund, D., Randhawa, M., Harmon, W., and Langer, R. (1987) *Kidney Int.* **32,** 452–463
Bitter, T., and Muir, H. M. (1962) *Anal. Biochem.* **109,** 901–905
Casu, B. (1985) *Adv. Carbohydr. Chem. Biochem.* **43,** 51–134
Comper, W. D. (1981) in *Heparin and Related Polysaccharides. Polymer Monographs* (Huglin, M. B., ed) pp. 33–63, Gordon and Breach Science Publishers, New York
Dietrich, C. P. (1968) *Biochem. J.* **108,** 647–654
Dietrich, C. P. (1970) *Can. J. Biochem.* **48,** 725–733
Ehrlich, J., and Stivala, S. S. (1973) *J. Pharmacol. Sci.* **62,** 517–544
Folkman, J., Langer, R., Linhardt, R. J., Haudenschild, C., and Taylor, S. (1983) *Science* **221,** 719–725
Friedman, Y., and Arsenis, C. (1974) *Biochem. J.* **139,** 699–708
Hatton, M. W. C., Berry, L. R., Machovich, R., and Regoecli, E. (1980) *Anal. Biochem.* **106,** 417–426
Hook, M., Pettersson, I., and Ogren, S. (1977) *Thromb. Res.* **10,** 857–861
Hovingh, P., and Linker, A. (1970) *J. Biol. Chem.* **245,** 6170–6175
Langer, R., Linhardt, R. J., Hoffberg, S., Larsen, A. K., Cooney, C. L., Tapper, D., and Klein, M. (1982) *Science* **217,** 261–263
Larsen, A. K., Hetelekidis, S., and Langer, R. (1984) *J. Pharmacol. Exp. Ther.* **231,** 373–378
Larsen, A. K., Lund, D. P., Langer, R., and Folkman, J. (1986a) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 2964–2968
Larsen, A. K., Newberne, P. M., and Langer, R. (1986b) *Fundam. Appl. Toxicol.* **7,** 86–93
Laurent, T. C., Tengblad, A., Thunberg, L., Hook, M., and Lindahl, U. (1978) *Biochem. J.* **175,** 691–701
Levy, L., and Petracek, F. J. (1962) *Proc. Soc. Exp. Bio. Med.* **101,** 901–904
Linhardt, R. J., Grant, A., Cooney, C. L., and Langer, R. (1982) *J. Biol. Chem.* **257,** 7310–7313
Linhardt, R. J., Rice, K. G., Merchant, Z. M., Kim, Y. S., and Lohse, D. L. (1986) *J. Biol. Chem.* **261,** 14448–14454
Linhardt, R. J., Rice, K. G., Kim, Y. S., Engelkin, J., and Weiler, J. (1988) *J. Biol. Chem.* **263,** 13090–13096
Linker, A., and Hovingh, P. (1972) *Biochemistry* **11,** 563–568
Linker, A., and Hovingh, P. (1984) *Carbohydr. Res.* **127,** 75–94
Lloyd, A. G., Embery, G., Powell, G. M., Curtis, C. G., and Dodgson,

K. S. (1966) *Biochem. J.* **98,** 33–34
March, S. C., Parikh, I., and Cuatrecasas, P. (1974) *Anal. Biochem.* **60,** 149–152
McNeal, C. J., Macfarlane, R. D., and Jardine, I. (1986) *Biochem. Biophys. Res. Commun.* **139,** 18–24
Merchant, Z. M., Kim, Y. S., Rice, K. G., and Linhardt, R. J. (1985) *Biochem. J.* **229,** 369–377
Nakajima, M., Irimura, T., and Nicolson, G. L. (1986) *Anal. Biochem.* **157,** 162–171
Rice, K. G., Kim, Y. S., Grant, A. C., Merchant, Z. M., and Linhardt, R. J. (1985) *Anal. Biochem.* **150,** 325–331
Rice, K. G., Rottink, M. K., and Linhardt, R. J. (1987) *Biochem. J.* **244,** 515–522
Roden, L. (1980) in *The Biochemistry of Glycoproteins and Proteoglycans* (Cennarz, W. J., ed) pp. 343–371, Plenum Publishing Co., New York
Yang, V. C., Linhardt, R. J., Bernstein, H., Cooney, C. L., and Langer, R. (1985) *J. Biol. Chem.* **260,** 1849–1857