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- Jones, T. A. (1978) *J. Appl. Crystallogr.* 11, 268-272.
- Jung, C., Bendzko, P., Ristau, O., & Gunsalus, I. C. (1985) in *Cytochrome P450, Biochemistry, Biophysics, and Induction* (Vereczkey, L., & Magyar, K., Eds.) pp 19-22, Akademiai Kiado, Budapest.
- Karplus, M., & McCammon, J. A. (1983) *Annu. Rev. Biochem.* 53, 263-300.
- Kassner, R. J. (1972) *J. Am. Chem. Soc.* 95, 2674-2677.
- Lewis, B. A., & Sligar, S. G. (1983) *J. Biol. Chem.* 258, 3591-3601.
- Luzzati, V. (1952) *Acta Crystallogr.* 5, 802-810.
- Mitani, F., Tetsutaro, I., Shimada, H., Ueno, R., & Ishimura, Y. (1985) *J. Biol. Chem.* 260, 12042-12048.
- Philson, S. B. (1976) Ph.D. Thesis, University of Illinois, Urbana, IL.
- Philson, S. B., Debrunner, P. G., Schmidt, P. G., & Gunsalus, I. C. (1979) *J. Biol. Chem.* 254, 10173-10179.
- Poulos, T. L. (1986) in *Cytochrome P450: Structure, Mechanism, and Biochemistry* (Ortiz de Montellano, P. R., Ed.) pp 505-523, Plenum, New York.
- Poulos, T. L., & Finzel, B. C. (1984) *Pept. Protein Rev.* 4, 115-171.
- Poulos, T. L., Perez, M., & Wagner, G. C. (1982) *J. Biol. Chem.* 257, 10427-10429.
- Poulos, T. L., Finzel, B. C., Gunsalus, I. C., Wagner, G. C., & Kraut, J. (1985) *J. Biol. Chem.* 260, 16122-16130.
- Salemme, F. R. (1977) *Annu. Rev. Biochem.* 46, 299-329.
- Sharrock, M., Debrunner, P. G., Schulz, C., Lipscomb, J. D., Marshall, V., & Gunsalus, I. C. (1976) *Biochim. Biophys. Acta* 420, 8-26.
- Sligar, S. G. (1976) *Biochemistry* 15, 5399-5406.
- Sligar, S. G., & Gunsalus, I. C. (1976) *Proc. Natl. Acad. Sci. U.S.A.* 73, 1078-1082.
- Sligar, S. G., & Gunsalus, I. C. (1979) *Biochemistry* 18, 2290-2295.
- Sligar, S. G., & Murray, R. I. (1986) in *Cytochrome P450: Structure, Mechanism, and Biochemistry* (Ortiz de Montellano, P. R., Ed.) pp 429-503, Plenum, New York.
- Sono, M., Anderson, L. A., & Dawson, J. H. (1982) *J. Biol. Chem.* 257, 8308-8320.
- Takano, T., & Dickerson, R. E. (1980) *Proc. Natl. Acad. Sci. U.S.A.* 77, 6371-6375.
- Tsai, R., Yu, C. A., Gunsalus, I. C., Peisach, J., Blumberg, W., Orme-Johnson, W. H., & Beinart, H. (1970) *Proc. Natl. Acad. Sci. U.S.A.* 66, 1157-1163.
- Van Gunsteren, W. F., & Karplus, M. (1981) *Nature (London)* 293, 677-678.
- Wagner, G. C., & Gunsalus, I. E. (1982) in *The Biological Chemistry of Iron* (Dunford, H. B., Dolphin, D., Raymond, K. N., & Sieker, L., Eds.) pp 405-412, Reidel, Boston.

## Chemically Modified Heparins as Inhibitors of Heparan Sulfate Specific Endo- $\beta$ -glucuronidase (Heparanase) of Metastatic Melanoma Cells<sup>†</sup>

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Received February 11, 1986; Revised Manuscript Received April 14, 1986

**ABSTRACT:** To determine the significance of the heparan sulfate (HS) degradative endo- $\beta$ -glucuronidase (heparanase) in tumor invasion and metastasis and to develop possible antimetastatic agents, we synthesized specific inhibitors of this enzyme. We previously found that heparanase activity correlates with the lung colonization abilities of murine B16 melanoma cells and is inhibited by heparin [Nakajima, M., Irimura, T., Di Ferrante, N., & Nicolson, G. L. (1984) *J. Biol. Chem.* 259, 2283-2290]. In this study, heparin was chemically modified in order to determine which portions of its structure are responsible for heparanase inhibitory activity and to obtain heparanase inhibitors that have minimal additional biological effects, such as anticoagulation. N-Sulfate groups and O-sulfate in heparin were removed separately, and the resultant free amino groups were acetylated or resulfated. Heparin was also reduced at the carboxyl groups of uronic acid. The heparanase inhibitory activities of these heparin derivatives were examined by high-speed gel-permeation chromatography and by the use of radioactive HS immobilized on agarose beads. The results indicated that although N-sulfate and O-sulfate groups on glucosamine residues, and carboxyl groups on uronic acid residues, are important for heparanase inhibition, they are not essential for full activity. When highly metastatic B16-BL6 melanoma cells were incubated with N-acetylated N-desulfated heparin, N-resulfated N- and O-desulfated heparin, or carboxyl-reduced heparin and injected intravenously to syngenic C57BL/6 mice, significant reductions in the numbers of experimental melanoma lung metastases occurred.

The malignancy of solid tumors can be explained, in part, by their abilities to invade and destroy normal tissues, including extracellular matrix and basement membranes (Nicolson, 1982, 1984; Liotta et al., 1983; Irimura et al., 1983b). Gly-

cosaminoglycans, such as heparan sulfate (HS),<sup>1</sup> are important constituents in these structures. Recently, we found that HS degradative activities of murine B16 melanoma sublines correlated with their metastatic lung colonization and invasive

<sup>†</sup>This study was supported by U.S. Department of Health and Human Services Grants BRSG RR-5511-23 and R01-CA39319 to T.I. and Grant R01-CA42346 to G.L.N. and a grant from the National Foundation for Cancer Research to G.L.N.

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<sup>1</sup> Abbreviations: DPBS, Dulbecco's phosphate-buffered saline; HS, heparan sulfate;  $M_r$ , relative molecular weight; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

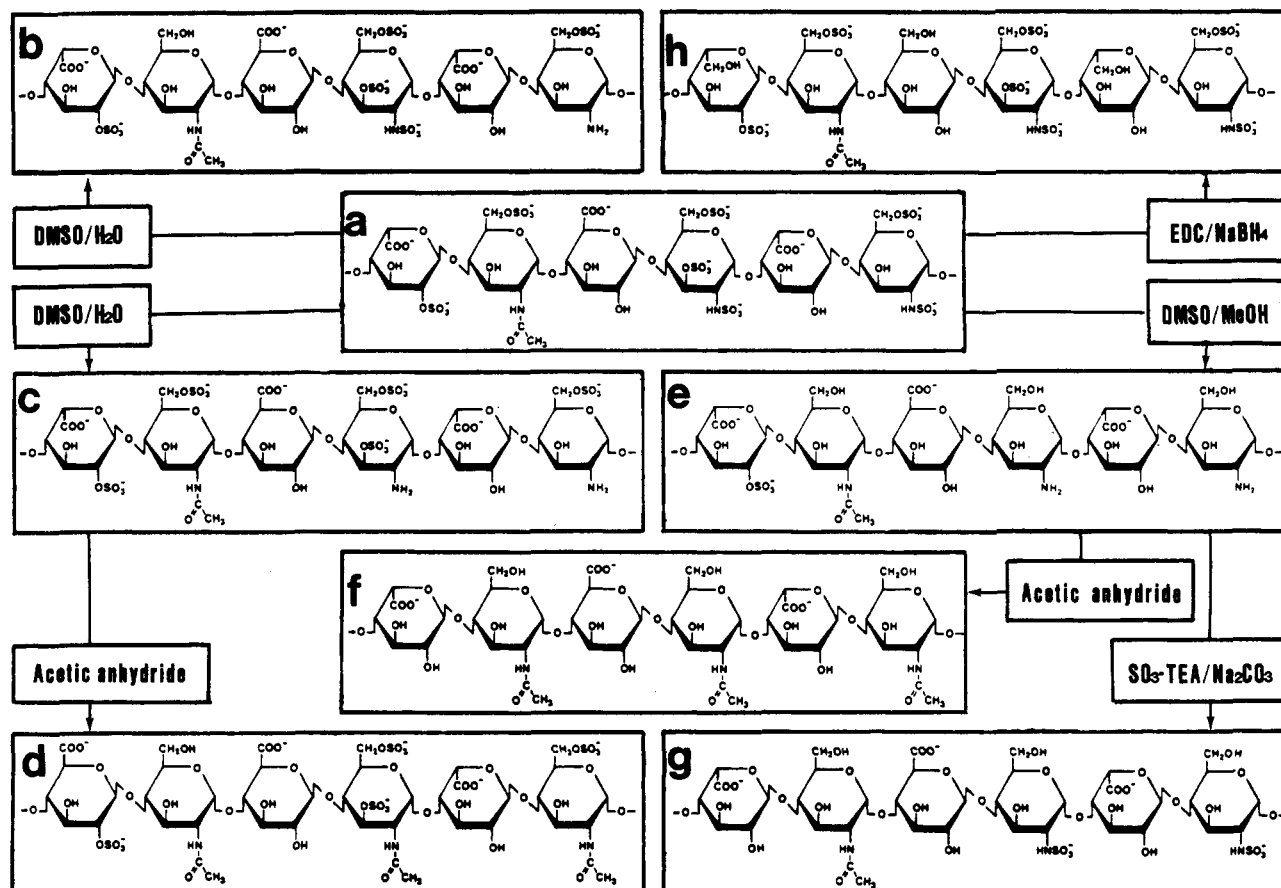


FIGURE 1: Hypothetical representative structures of the heparin derivatives used in this study: (a) intact heparin; (b) partially N-desulfated heparin; (c) completely N-desulfated heparin; (d) N-acetylated N-desulfated heparin; (e) N- and O-desulfated heparin; (f) N-acetylated N- and O-desulfated heparin; (g) N-resulfated N- and O-desulfated heparin; (h) carboxyl-reduced heparin.

abilities (Nakajima et al., 1984).

Using high-speed gel-permeation chromatography to analyze HS degradation products, we demonstrated that the degradative activity is caused by an endo- $\beta$ -glucuronidase (Irimura et al., 1983a; Nakajima et al., 1984). We also found that heparin was a poor substrate but a potent inhibitor of this enzyme (Nakajima et al., 1984). Heparin preparations are known to be heterogeneous in molecular structure and biological activities (Jaques, 1980), and the molecular basis for this heterogeneity is the anionic groups on the polysaccharide backbone. Therefore, we have attempted to determine the structure required for heparin's inhibitory activity against heparanase of metastatic melanoma cells.

Heparin and related compounds have been used experimentally as antimetastatic agents (Tsubura et al., 1977; Hilgard, 1984). The basis for this use was the assumption that platelet aggregation, together with activation of the coagulation cascade, enhanced the formation of tumor emboli and increased blood-borne implantation and metastatic colonization of blood-borne tumor cells. However, other studies on the effects of heparin on metastasis have shown that heparin administration increased, decreased, or had no effect on tumor cell dissemination and organ colonization, depending on the experimental system (Tsubura et al., 1977; Hilgard, 1984). Mechanisms other than the anticoagulation effects of heparin on tumor metastasis were suggested by these results, but the possible involvement of tumor heparanase had not been considered. We have found that heparin derivatives without anticoagulant properties inhibit the heparanase of metastatic mouse melanoma cells. These substances have proven to be useful as experimental tools for in vitro and in vivo studies of

the role of heparanase in tumor invasion and metastasis.

#### MATERIALS AND METHODS

**Chemical Modification of Heparin.** Chemical modifications and the resultant compounds used in this study are schematically shown in Figure 1. Heparin from porcine intestinal mucosa (sodium salt) was purchased from Sigma Chemical Co. (St. Louis, MO). One gram of heparin was dissolved in 30 mL of water and applied to a  $1.5 \times 8$  cm column of Dowex 50W  $\times 8$ , 50–100 mesh ( $H^+$  form) (Bio-Rad, Richmond, CA) at 4 °C. The pass-through fraction eluted with water was neutralized immediately with pyridine and the pH adjusted to between 6 and 7 (Nagasawa & Inoue, 1980a). The heparin pyridinium salt was collected by lyophilization after dialysis against running deionized water for 24 h and twice with 6 L of distilled water for 12 h each. Partial N-desulfation, complete N-desulfation, and complete N- and O-desulfation starting with 100 mg each of heparin pyridinium salt were achieved by solvolysis in 10 mL of dimethyl sulfoxide (ACS grade, Fisher Scientific, Fair Lawn, NJ) containing water or methanol as described by Nagasawa and Inoue (1980a,b). Reaction conditions were 10% water in dimethyl sulfoxide at 80 °C for 5 h for complete N-desulfation, and 10% anhydrous methanol in dimethyl sulfoxide at 80 °C for 18 h for complete N- and O-desulfation. After these reactions, the mixtures were cooled, and 1 M sodium hydroxide was added to adjust the pH to between 8.5 and 9.5; the mixtures were dialyzed against running deionized water for 24 h and then twice against 6 L of distilled water for 12 h each.

N-Acetylation of N-desulfated and N- and O-desulfated heparin was performed with acetic anhydride under alkaline

conditions as follows: 50 mg of modified heparin was dissolved in 5 mL of 4.5 M sodium acetate plus 1.0 mL of methanol and added to 5 portions of 1.0 mL of acetic anhydride at 10-min intervals. After 1-h incubation with occasional mixing, the reaction mixture was dialyzed against running deionized water for 24 h and then twice against 6 L of distilled water for 12 h each and was lyophilized.

The N-resulfation reaction was performed by sulfation with a triethylamine-sulfur trioxide complex prepared according to the method of Cherniak and Davidson (1964). Complete N- and O-desulfated heparin (50 mg) was dissolved in 2 mL of 1.0 M sodium carbonate and added to 50 mg of triethylamine-sulfur trioxide. The atmosphere was replaced with nitrogen, and the mixture was heated at 50 °C for 24 h with occasional agitation. The resulfated heparin was dialyzed against running deionized water for 24 h and then twice with 6 L of distilled water for 12 h and finally lyophilized. Carboxyl-reduced heparin was prepared from the sodium salt of porcine intestinal mucosa heparin as described by Taylor et al. (1976) by use of sodium borohydride (Aldrich Chemical Co., Milwaukee, WI) and *N*-ethyl-*N'*-[3-(dimethylamino)-propyl]carbodiimide hydrochloride (Fluka Chemical Co., Hauppauge, NY).

**Analytical Procedures.** Sulfate contents of chemically modified heparins were determined according to Dodgson (1961) and Dodgson and Price (1962). Hydrolysis of sulfate esters was achieved by heating 2 mg each of samples in 0.2 mL of 1 M HCl at 110 °C for 2 h. The homogeneity of the chemically modified heparins was assessed by cellulose acetate electrophoresis and high-speed gel-permeation chromatography. Electrophoresis was performed on a 7.6 × 7.6 cm Titan III zip zone cellulose acetate sheet (Helena Laboratories, Beaumont, TX) in 0.5 M/0.5 M pyridine/acetate buffer, pH 5.0 (Hata & Nagai, 1979). Each 1-μL sample dissolved in water (5 mg/mL) was applied to a 1.5 cm wide zone which was blotted with water before the sample application, as described by Cappeletti et al. (1979). Electrophoresis was carried out under constant voltage (75 V) for 45 min. During the run, the sheet was dipped in petroleum ether cooled under ice (Cappeletti et al., 1979). The cellulose acetate sheet was then stained with 0.1% Toluidine blue O in 1% acetic acid and destained with 1% acetic acid.

High-speed gel-permeation chromatography was performed as previously described (Irimura et al., 1983a) by using a Constametric III (LDC-Milton Roy, Riviera Beach, FL) with a single 0.7 × 75 cm stainless steel column packed with Fractogel TSK HW-55(S). Elution was accomplished with 0.2 M sodium chloride at flow rates of 1.0 or 0.75 mL/min. Absorption at 210 nm was monitored for the analogues of chemically modified heparin. For the qualitative examination of radiolabeled HS degradation products, each fraction corresponding to 30-s elution was collected into plastic scintillation vials, and radioactivity in each vial was determined after the fraction was mixed with 3.0 mL of Hydrofluor (National Diagnostics, Somerville, NJ).

**Source of Heparanase.** Highly invasive mouse B16 melanoma (B16-BL6) cells were provided by Dr. I. J. Fidler (M. D. Anderson Hospital, Houston, TX) and were cultured as previously described (Irimura et al., 1983; Irimura & Nicolson, 1984). Cell extracts were prepared in 5 mM Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose, 50 μL of calcium chloride, 10 μL of phenylmethanesulfonyl fluoride, and 0.2% Nonidet P-40 (Irimura et al., 1983a). The melanoma extracts were stored frozen at -80 °C and used as crude heparanase.

**Radiolabeling of HS.** [<sup>14</sup>C]- or [<sup>3</sup>H]HS was prepared by chemical deacetylation and radioactive reacetylation. Nine milligrams of bovine lung HS (provided by Dr. N. Di Ferrante, Baylor College of Medicine, Houston, TX) was dried with 0.1 mg of hydrazine sulfate over phosphorus pentoxide under vacuum at 50 °C for 48 h. Anhydrous hydrazine (0.5 mg, Pierce Chemical Co., Rockford, IL) was added to the dried HS and the mixture heated in a tightly sealed tube under nitrogen atmosphere at 100 °C for 1 h. After the reaction, the hydrazine was removed by repeated evaporation with toluene over sulfuric acid desiccant under vacuum conditions. To separate deacetylated HS from residual reagents and partial degradation products, the completely dried residue was dissolved in 0.5 mL of water and subjected to gel filtration on a 0.8 × 30 cm column of Bio-Gel P-10 (400 mesh) and elution with distilled water. The void volume fraction was collected and lyophilized, and the yield by weight was about 60%. N-Deacetylated HS was then N-acetylated with 50 μCi of [<sup>14</sup>C]acetic anhydride (10 mCi/mmol; New England Nuclear, Boston, MA) or 5 mCi of [<sup>3</sup>H]acetic anhydride (400 mCi/mmol; New England Nuclear) in 0.5 mL of 4 M sodium acetate for 18 h. N-Acetylation was completed by addition of 0.1 mL of unlabeled acetic anhydride to the reaction mixture and incubation for 1 h. [<sup>14</sup>C]- or [<sup>3</sup>H]HS was purified on the same Bio-Gel P-10 column as described above.

**High-Speed Gel-Permeation Chromatography Assay for Heparanase.** Fifty microliters of melanoma extract (equivalent to 10<sup>6</sup> cells) was mixed with chemically modified heparin (5 mg/mL in water), 50 μL of 4× heparanase assay buffer (0.4 M sodium phosphate buffer, pH 5.8, containing 0.4% Triton X-100, 0.6 M sodium chloride, and 0.4% sodium azide), and about 3000 cpm of [<sup>14</sup>C]HS. Incubation was performed at 37 °C with continuous gentle mixing for 6 h. The reaction mixture was placed on ice, 20 μL of 50% trichloroacetic acid was added, and incubation was continued on ice for 10 min. After centrifugation at 9000g for 5 min in a Microfuge B (Beckman Instruments, Irvine, CA), the supernatant was injected into the gel-permeation chromatography system and analyzed as described above (Irimura et al., 1983a).

**Preparation of Solid-Phase Substrates for Heparanase and Inhibitor Assays.** For the solid-phase heparanase assay, [<sup>3</sup>H]HS was aminated at the reducing terminal with 2 M ammonium acetate in the presence of 0.4 M sodium cyanoborohydride in 50% methanol at 50 °C for 6 days. Aminated [<sup>3</sup>H]HS was purified gel filtration as above, and the resulting solution was diluted to 0.1 M in sodium carbonate. To 10<sup>6</sup> cpm of aminated [<sup>3</sup>H]HS was added 1.0 mL of Affi-Gel 15 (Bio-Rad) after the gel beads were washed with 2-propanol and chilled distilled water according to the manufacturer's recommendations. The coupling reaction was continued at 4 °C for 48 h with continuous agitation. The agarose beads were reacted with 0.1 M glycine monomethyl ester dissolved in 0.1 M sodium carbonate for 1 h at room temperature and then washed with 4 M sodium chloride repeatedly to remove noncovalently attached [<sup>3</sup>H]HS from the beads.

**Solid-Phase Assays for Heparanase and Heparanase Inhibitors.** [<sup>3</sup>H]HS-agarose was suspended in Dulbecco's phosphate-buffered saline (DPBS) at about 20% (v/v). The incubation conditions for the solid-phase assay were identical with those of the high-speed gel-permeation chromatography assay, except that 75 μL of the [<sup>3</sup>H]HS-agarose suspension was used instead of the HS so that the incubation mixture consisted of B16 melanoma extract, chemically modified heparins, 4× heparanase assay buffer, and [<sup>3</sup>H]HS-agarose suspension. After incubation, the reaction mixture was placed

on ice, chilled 5% trichloroacetic acid (50  $\mu$ L) was added, and the mixture was incubated for 10 min and centrifuged. Radioactivity in the supernatant and the pellet was determined separately after mixing with Hydrofluor.

**Experimental Metastatic Lung Colonization.** Male C57BL/6 mice, 4–6 weeks old, were obtained from Charles River, Inc. (Kingston, MD) and quarantined for 2 weeks. Animals were fed normal rodent chow and unchlorinated spring water. B16–BL6 cells were grown to subconfluency, detached from plastic dishes by incubating in 2 mM EDTA, 0.14 M NaCl, and 10 mM sodium phosphate buffer, pH 7.4, for 5–10 min, and suspended in a 1:1 mixture of Dulbecco's modified minimum essential medium and Ham's F12 medium as described previously (Irimura et al., 1981a, 1983a). The cells were incubated with heparin, N-acetylated N-desulfated heparin, N-resulfated N- and O-desulfated heparin, or carboxyl-reduced heparin (each 0.5 mg/mL in the media described above) at 4 °C for 2 h. Viability of B16–BL6 cells at the end of the incubation was >95%. Treated or untreated cells ( $5 \times 10^4/0.1$  mL) were injected intravenously into the tail vein of each mouse. Mice were sacrificed 20 days later and autopsied. The numbers of pulmonary tumor nodules were counted after the lungs were perfused via the trachea with 4% formalin in DPBS. Extrapulmonary tumor formation was assessed in each animal and recorded.

## RESULTS AND DISCUSSION

**Chemical Modifications of Heparin.** Heparanase from metastatic melanoma cells is an endo- $\beta$ -glucuronidase specific for HS (Irimura et al., 1983b; Nakajima et al., 1984). Although heparin is structurally and biosynthetically related to HS, it is a poor substrate for heparanase, and it interferes with HS degradation (Nakajima et al., 1984). Structural differences between heparin and HS are based primarily on the degrees of sulfation of glucosamine residues and the relative contents of iduronic acid. The heparanase-inhibitory activity of heparin was expected to be determined, therefore, by its sulfate as well as carboxyl groups. If one of these groups were responsible for heparanase inhibition, this information could be useful in developing specific heparanase inhibitors. Furthermore, since N-sulfate and O-sulfate groups of glucosamine, O-sulfate groups of iduronic acid, and carboxyl groups of uronic acid are essential for heparin's anticoagulation activities, some of the chemical modifications could produce heparin derivatives that inhibit heparanase but are not anticoagulants.

Porcine intestinal mucosal heparin was chemically modified as schematically shown in Figure 1. When the electrophoretic mobilities of these substances in pyridine/acetate buffer on cellulose acetate sheets were compared (Figure 2), all of the chemically modified heparins migrated more slowly than intact heparin under the electrophoretic conditions used. N- and O-desulfated heparins remained at the top of the zone blotted with water and did not stain intensely with Toluidine blue O. The migration distances of the modified heparins were the following, in order from smallest to longest: (e) N- and O-desulfated heparin, (f) N-acetylated N- and O-desulfated heparin, (c) N-desulfated heparin, (g) N-resulfated N- and O-desulfated heparin, (d) N-acetylated N-desulfated heparin, (b) partially N-desulfated heparin, and (h) carboxyl-reduced heparin. High-speed gel-permeation chromatography of these substances was performed as described under Materials and Methods (Figure 3). A slight change in the apparent molecular size of compounds d, e, f, g, and h was observed, probably as a result of detachment and reattachment of sulfate or acetyl groups. Degradation products were not observed in any of the modified heparin preparations. Sulfate contents

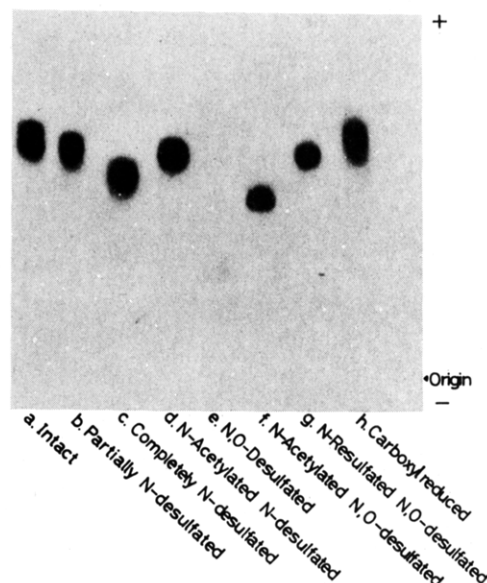


FIGURE 2: Cellulose acetate sheet electrophoresis of chemically modified heparins. The sheet is of 7.6 cm in total length, with a 1.5-cm zone blotted with water and a 6.1-cm zone blotted with 0.5 M pyridine/0.5 M acetic acid, pH 5.0. The electrodes are in the same buffer. Electrophoresis is performed under constant voltage (12 V/cm) at 4 °C for 45 min. After electrophoresis, the sheet is stained with Toluidine blue O (0.1% in 1% acetic acid) and destained with 1% acetic acid.

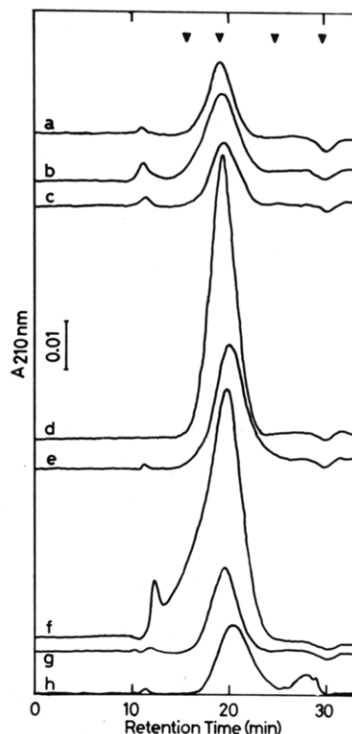


FIGURE 3: High-speed gel-permeation chromatography of chemically modified heparin on a single  $0.7 \times 75$  cm column of Fractogel TSK HW-55(S). Elution is accomplished with 0.2 M sodium chloride at a flow rate of 0.75 mL/min. During the separation the column is kept at 55 °C by a water jacket. Each sample is dissolved in water (5  $\mu$ g/mL), and 25  $\mu$ L is injected. Patterns a–h indicate the analytical results of the derivatized heparins shown in Figure 1.

of the modified heparins were measured by turbidimetry of inorganic sulfate liberated after treatment of the samples with hydrochloric acid. Sulfate contents of (a) intact heparin, (b) partially N-desulfated heparin, (c) N-desulfated heparin, (d) N-acetylated N-desulfated heparin, (e) N- and O-desulfated heparin, (f) N-acetylated N- and O-desulfated heparin, (g)

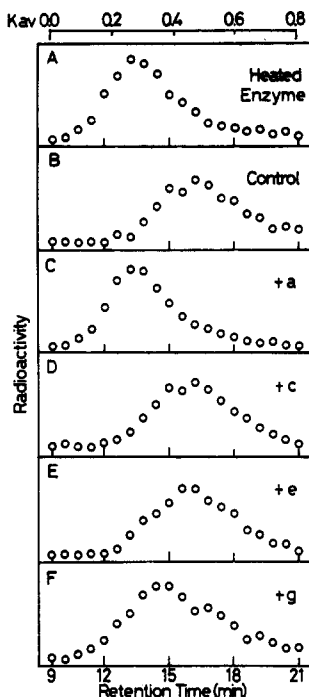


FIGURE 4: Results of high-speed gel-permeation chromatography assay of B16 melanoma heparanase activity in the presence of chemically modified heparin derivatives using a system equipped with a  $0.70 \times 75$  cm column of Fractogel TSK HW55(S). Elution is performed with 0.2 M NaCl at a flow rate of 1.0 mL/min at 55 °C. Each 0.5-mL fraction corresponding to 30-s elution is mixed with 3 mL of Hydrofluor and its radioactivity determined. (Panel A) [ $^{14}\text{C}$ ]HS was incubated with heat-inactivated crude heparanase ( $10^6$  B16-BL6 melanoma cell equiv) at 37 °C for 6 h and analyzed. (Panel B) Same incubation as panel A except that the enzyme was not heat-inactivated. (Panel C) Same incubation and analysis as panel B, but with 1 mg/mL heparin added to the incubation mixture. (Panel D) Same incubation and analysis as panel B, but with 1 mg/mL completely N-desulfated heparin added to the incubation mixture. (Panel E) Same incubation and analysis as panel B, but with 1 mg/mL N- and O-desulfated heparin added to the mixture. (Panel F) Same as panel B, but with 1 mg/mL N-resulfated N- and O-desulfated heparin added.

N-resulfated N- and O-desulfated heparin, and (h) carboxyl-reduced heparin as  $\text{SO}_4^{2-}$  were as follows: (a) 33.55%, (b) 31.18%, (c) 23.74%, (d) 23.82%, (e) <5%, (f) <5%, (g) 16.16%, and (h) 29.26%. These results indicated that removal or addition of sulfate groups was nearly complete.

**High-Speed Gel-Permeation Chromatography Assay of Heparanase.** Intact heparin, N-desulfated heparin, N- and O-desulfated heparin, and N-sulfated N- and O-desulfated heparin (5 mg/mL dissolved in water) were mixed with 50  $\mu\text{L}$  of crude heparanase and [ $^{14}\text{C}$ ]HS as described under Materials and Methods. The elution profiles of the radioactivity on high-speed gel-permeation chromatography are shown in Figure 4. The elution profile of [ $^{14}\text{C}$ ]HS incubated with heat-inactivated (100 °C for 5 min) heparanase was identical with that of untreated [ $^{14}\text{C}$ ]HS, which eluted at the position corresponding to an approximate  $M_r$  of 34 000. After incubation with heparanase, the average apparent  $M_r$  decreased to 6000. In the presence of heparin, no degradation was observed. N-Desulfated heparin or N- and O-desulfated heparin failed to inhibit degradation of HS by heparanase. After N-resulfation of N- and O-desulfated heparin, the heparanase inhibitory activity was partially restored. These results suggested that the N-sulfate groups are essential for heparins' inhibition of heparanase. O-Sulfate groups on the 3- or 6-position of glucosamine, as well as on the 2-position of iduronic acid, seem less essential to heparanase inhibition, provided that the amino groups are completely resulfated.

**Solid-Phase Assay for Heparanase.** Since heparanase is an endoglycosidase that produces relatively large fragments of HS, rapid isolation of the fragmented HS from intact HS is necessary for quantitative assay. Therefore, we immobilized HS chains at their ends to a solid-phase support, such as small beads. First, we immobilized [ $^{35}\text{S}$ ]HS [purified from the subendothelial matrix in cell culture (Wang et al., 1985)] onto Affi-Gel 15, since this HS preparation contains amino acid residues at its reducing terminal end. With use of this substrate, however, the proportion of released  $^{35}\text{S}$ -labeled materials from the beads by crude heparanase was negligible, and this method is not likely to be useful. Next, we attempted to immobilize chemically labeled and modified HS to beads. Deacetylation of HS was achieved by hydrazinolysis. We chose lower temperatures and shorter reaction times than the usual hydrazinolysis reactions for glycoproteins (Fukuda et al., 1976; Irimura et al., 1981b) because of possible cleavage of glucopyranosyl (uronic acid) 2-acetamido-2-deoxyglucopyranoside linkages. The resultant N-deacetylated heparin was labeled by N-acetylation with [ $^{14}\text{C}$ ]- or [ $^3\text{H}$ ]acetyl groups. In the next step, radiolabeled HS was aminated exclusively at its reducing terminal by reductive amination and coupled to Affi-Gel 15 under alkaline conditions. The proportion of labeled HS coupled to agarose beads fluctuated between 50% and 80%, calculated from the amount of material used for the amination reaction. We found later that, as a heparanase substrate, partially N-desulfated and  $^3\text{H}$ - or  $^{14}\text{C}$ -N-acetylated HS was as useful as deacetylated and reacylated HS. Partial N-desulfation of HS was achieved by the same reaction conditions used for partial N-desulfation of heparin described under Materials and Methods. As an alternate method of coupling, the reducing terminal saccharide of radioactive HS was reduced with sodium borohydride, and it was mildly oxidized with periodate to introduce aldehyde groups. The resultant substance was intended to be coupled to the amino derivative of agarose (Affi-Gel 102, Bio-Rad) in the presence of sodium cyanoborohydride, but the overall yield of immobilized, radioactive HS was lower than that of the method described above.

**Heparanase Inhibition by Chemically Modified Heparins.** The dose-response curves of the inhibitory effects of chemically modified heparins obtained by using a solid-phase heparanase assay are shown in Figure 5. The use of intact, N-desulfated N- and O-desulfated, and N-resulfated N- and O-desulfated heparin produced results consistent with those of the high-speed gel-permeation chromatography assay. N-Desulfated and N- and O-desulfated heparin failed to show any inhibitory activity, whereas partial restoration of activity was obtained by the addition of N-sulfate groups (Figure 5g). Interestingly, when the exposed amino groups formed by N-desulfation were acetylated, heparanase inhibitory activity was partially restored (Figure 5d). N-Acetylation of N- and O-desulfated heparin did not, however, restore the inhibitory activity (Figure 5f). These results indicated that N-sulfate groups affect, but are not essential for, heparanase inhibition. Removal of N-sulfate groups with intact O-sulfate groups of heparin results in inhibitory activity provided that the exposed amino groups are blocked by acetylation. Carboxyl-reduced heparin was shown to possess weaker inhibitory activity than heparin, which indicated that heparin carboxyl groups are necessary, but not essential, for the full inhibitory activity. Similar observations have been described concerning heparin's stimulatory activity on the growth of smooth muscle cells (Castellot et al., 1984). Our results suggested that the heparanase inhibitory activity of heparin cannot be explained by any single moiety in the

Table I: Effects of Chemically Modified Heparins on Blood-Borne Lung Colonization of B16-BL6 Melanoma Cells in Mice

treatment <sup>a</sup>	experiment 1		experiment 2	
	no. of lung tumor colonies	median	no. of lung tumor colonies	median
none	8, 20, 28, 33, 75, 85, 106, 116, 193	75	0, 1, 26, 48, 75, 163, 193, 200+, 200+	75
heparin	0, 1, 8, 9, 16, 18, 21, 82, 174	16	0,0,0,0,1,1,2,12	0
N-acetylated N-desulfated heparin	11, 19, 22, 23, 43, 89, 109, 199	43	0, 1, 2, 3, 5, 5, 25, 37, 200+	5
N-resulfated N- and O-desulfated heparin			0, 0, 2, 5, 8, 13, 20, 90, 200+	8
carboxyl-reduced heparin	0, 15, 25, 25, 29, 36, 45, 46, 53	29	7, 13, 42, 49, 51, 55, 58, 89, 120	51

<sup>a</sup> The cells were incubated with chemically modified heparins (500 µg/mL) at 4 °C for 2 h before intravenous injection of  $5 \times 10^4$  cells/0.1 mL into C57BL/6 mice (nine per group). Experimental blood-borne metastasis to lung and other organs was determined after 20 days.

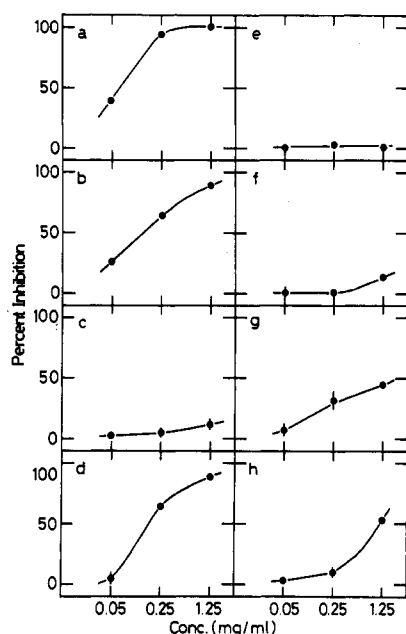


FIGURE 5: Inhibitory activities of chemically modified heparins on release of <sup>3</sup>H-acetylated HS fragments by metastatic B16 melanoma heparanase. The measurements are performed by incubating B16-BL6 melanoma cell lysates equivalent to  $10^6$  cells with a suspension of [<sup>3</sup>H]HS-agarose beads prepared by partial N-desulfation and <sup>3</sup>H-acetylation (approximately 20% suspension) and various concentrations of heparanase inhibitors at 37 °C for 24 h with gentle mixing. Panels a-h indicate the results using eight different chemically modified heparins as indicated in Figures 1 and 2. The inhibitory activities are indicated by the percent release of HS fragments compared to the release without added inhibitors.

heterogeneous heparin molecule. We measured anticoagulation activities of N-acetylated N-desulfated heparin, N-resulfated N- and O-desulfated heparin, and carboxyl-reduced heparin by the activated factor Xa neutralization assay (Sigma). The activities of all of these compounds accounted for less than 1% of the unmodified starting material. Therefore, these three modified heparins could be useful for in vitro and in vivo studies on the role of heparanase in tumor invasion and metastasis.

**Effects of Modified Heparins on Experimental Blood-Borne Metastasis of B16 Melanoma Cells.** As shown in Table I, the number of visible melanoma lung colonies 20 days after intravenous injection of the tumor cells was significantly reduced by preincubation of the cells with intact or chemically modified heparin. The effects of intact heparin were greater, probably because multiple factors such as inhibition of melanoma-induced platelet aggregation are involved. Since the three chemically modified heparins do not possess anticoagulation activity, the inhibition of melanoma lung colonization is likely to be due to inhibition of melanoma heparanase. These three modified heparins were not cytotoxic to B16-BL6 cells. Viability of the cells after 2-h incubation with these substances was higher than 95%, and the growth rate

of B16-BL6 cells was not affected by the presence of these substances (0.5 mg/mL) for at least 4 days. Lung colonization was not completely prevented in our in vivo experiments, presumably because the inhibitors were diluted at the time of administration, or because heparanase is important but not essential for melanoma cells to colonize lung. We are using systemic administration of these non-anticoagulant heparin derivatives to prevent metastasis. Since heparanase appears to be an enzyme associated with invasive cells, even normal cells, such as platelets, activated lymphocytes, and macrophages, systemic administration of these inhibitors could affect some normal cell functions. Such possibilities are under investigation.

**Registry No.** HS, 9050-30-0; heparanase, 89800-66-8; heparin, 9005-49-6.

#### REFERENCES

- Cappeletti, R., Del Rosso, M., & Chiarugi, V. P. (1979) *Anal. Biochem.* 99, 311-315.
- Castellot, J. J., Beeler, D. L., Rosenberg, R. D., & Karnovsky, M. J. (1984) *J. Cell. Physiol.* 120, 315-320.
- Cherniak, R., & Davidson, E. A. (1964) *J. Biol. Chem.* 239, 2986-2990.
- Dodgson, K. S. (1961) *Biochem. J.* 78, 312-319.
- Dodgson, K. S., & Price, R. G. (1962) *Biochem. J.* 84, 106-110.
- Fukuda, M., Kondo, T., & Osawa, T. (1976) *J. Biochem. (Tokyo)* 80, 1223-1232.
- Hata, R., & Nagai, Y. (1979) *Anal. Biochem.* 45, 462-468.
- Hilgard, P. (1984) in *Cancer Invasion and Metastasis: Biologic and Therapeutic Aspects* (Nicolson, G. L., & Milas, L., Eds.) pp 353-360, Raven Press, New York.
- Irimura, T., & Nicolson, G. L. (1984) *Cancer Res.* 44, 791-798.
- Irimura, T., Gonzalez, R., & Nicolson, G. L. (1981a) *Cancer Res.* 41, 3411-3418.
- Irimura, T., Tsuji, T., Tagami, S., Yamamoto, K., & Osawa, T. (1981b) *Biochemistry* 20, 560-566.
- Irimura, T., Nakajima, M., Di Ferrante, N., & Nicolson, G. L. (1983a) *Anal. Biochem.* 130, 461-468.
- Irimura, T., Nakajima, M., & Nicolson, G. L. (1983b) *Gann Monogr. Cancer Res.* 29, 35-46.
- Jaques, L. B. (1980) *Pharmacol. Rev.* 31, 99-166.
- Liotta, L., Rao, C. N., & Barsky, S. H. (1983) *Lab. Invest.* 49, 636-649.
- Nagasawa, K., & Inoue, Y. (1980a) *Methods Carbohydr. Chem.* 8, 287-289.
- Nagasawa, K., & Inoue, Y. (1980b) *Methods Carbohydr. Chem.* 8, 291-294.
- Nakajima, M., Irimura, T., Di Ferrante, D. T., Di Ferrante, N., & Nicolson, G. L. (1983) *Science (Washington, D.C.)* 220, 611-613.
- Nakajima, M., Irimura, T., Di Ferrante, N., & Nicolson, G. L. (1984) *J. Biol. Chem.* 259, 2283-2290.



Nicolson, G. L. (1982) *Biochim. Biophys. Acta* 695, 113-176.  
 Nicolson, G. L. (1984) *Exp. Cell Res.* 150, 3-22.  
 Taylor, R. L., Shively, J. E., & Conrad, H. E. (1976) *Methods Carbohydr. Chem.* 7, 149-151.

Tsubura, E., Yamashita, T., Kobayashi, M., Higuchi, Y., & Isobe, J. (1977) *Gann Monogr. Cancer Res.* 20, 147-153.  
 Wang, Z.-W., Irimura, T., Nakajima, M., Belloni, P. N., & Nicolson, G. L. (1985) *Eur. J. Biochem.* 153, 125-130.

## Isolation and Identification of 1,24,25-Trihydroxyvitamin D<sub>2</sub>, 1,24,25,28-Tetrahydroxyvitamin D<sub>2</sub>, and 1,24,25,26-Tetrahydroxyvitamin D<sub>2</sub>: New Metabolites of 1,25-Dihydroxyvitamin D<sub>2</sub> Produced in Rat Kidney<sup>†</sup>

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Received February 18, 1986; Revised Manuscript Received April 16, 1986

**ABSTRACT:** Three new metabolites of vitamin D<sub>2</sub> were produced in vitro by perfusing isolated rat kidneys with 1,25-dihydroxyvitamin D<sub>2</sub>. They were isolated and purified from the kidney perfusate by the techniques of methanol-methylene chloride lipid extraction and high-performance liquid chromatography. By means of ultraviolet absorption spectrophotometry, mass spectrometry, and specific chemical reactions, the metabolites were identified as 1,24,25-trihydroxyvitamin D<sub>2</sub>, 1,24,25,28-tetrahydroxyvitamin D<sub>2</sub>, and 1,24,25,26-tetrahydroxyvitamin D<sub>2</sub>. Both 1,24,25,28-tetrahydroxyvitamin D<sub>2</sub> and 1,24,25,26-tetrahydroxyvitamin D<sub>2</sub> were also produced when a kidney was perfused with 1,24,25-trihydroxyvitamin D<sub>2</sub>. Thus, it becomes clear that 1,25-dihydroxyvitamin D<sub>2</sub> is first hydroxylated at C-24 to form 1,24,25-trihydroxyvitamin D<sub>2</sub>, which is then further hydroxylated at C-28 and C-26 to form 1,24,25,28-tetrahydroxyvitamin D<sub>2</sub> and 1,24,25,26-tetrahydroxyvitamin D<sub>2</sub>, respectively. From several recent studies, it has been well established that 1,25-dihydroxyvitamin D<sub>3</sub> is converted into various further metabolites in the kidney as a result of chemical reactions such as C-23, C-24, and C-26 hydroxylations, C-24 ketonization, and C-23:C-26 lactonization. From our study it is obvious that 1,25-dihydroxyvitamin D<sub>2</sub> does not undergo all of the aforementioned chemical reactions except C-24 and C-26 hydroxylations. Also, our study indicates that C-28 hydroxylation plays a significant role in the further metabolism of 1,25-dihydroxyvitamin D<sub>2</sub>. Thus, for the first time, we describe a novel further metabolic pathway for 1,25-dihydroxyvitamin D<sub>2</sub> in a mammalian kidney.

It has been known that there are species differences in the biological activity of ergocalciferol (vitamin D<sub>2</sub>) and cholecalciferol (vitamin D<sub>3</sub>). For example, vitamin D<sub>2</sub> is about 10 times less biologically active in avian species when compared to vitamin D<sub>3</sub> (Chen & Bosmann, 1964). On the other hand, it is being assumed that both vitamins have similar biological activity in mammals. Recent studies, however, demonstrated that mammals also show differences in the biological activity of the two vitamins (Hunt et al., 1972; Sjoden et al., 1985; Tjellesen et al., 1985a,b). There may be several reasons for the above observations. One of the reasons may be the differences in the further metabolism of these vitamins (Horst et al., 1982; Napoli & Horst, 1985). It is already well established that both vitamins undergo hydroxylations at C-25 (in liver) and C-1 (in kidney) to form 1,25-dihydroxyvitamin D<sub>2</sub> [1,25(OH)<sub>2</sub>D<sub>2</sub>]<sup>1</sup> and 1,25-dihydroxyvitamin D<sub>3</sub> [1,25(OH)<sub>2</sub>D<sub>3</sub>], respectively (Jones et al., 1975; Norman et al., 1982).

Also, it became obvious that the further metabolism of 1,25-(OH)<sub>2</sub>D<sub>3</sub> in both kidney and intestine is mainly due to the chemical alterations of its side chain (Napoli et al., 1983; Napoli & Horst, 1983; Mayer et al., 1983; Horst et al., 1984; Ishizuka et al., 1984). Because of the structural differences between the side chains of the two vitamins (the side chain of vitamin D<sub>2</sub> has an extra methyl group at C-24 and a double bond between C-22 and C-23 when compared to the side chain of vitamin D<sub>3</sub>), we hypothesized that the side-chain metabolism of 1,25(OH)<sub>2</sub>D<sub>2</sub> differs from that of 1,25(OH)<sub>2</sub>D<sub>3</sub>. In this investigation, we studied the further metabolism of 1,25-

<sup>†</sup> This work was supported by grants from the Cuyahoga County Hospital Foundation and the Kidney Foundation of Ohio to G.S.R. and the Veteran Administration Research Service to K.-Y.T.

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<sup>1</sup> Abbreviations: 24(OH)D<sub>2</sub>, 24-hydroxyvitamin D<sub>2</sub>; 25(OH)D<sub>2</sub>, 25-hydroxyvitamin D<sub>2</sub>; 24,25(OH)<sub>2</sub>D<sub>2</sub>, 24(R),25-dihydroxyvitamin D<sub>2</sub>; 1,24,25(OH)<sub>2</sub>D<sub>2</sub>, 1,24,25-trihydroxyvitamin D<sub>2</sub>; 1,24,25,28(OH)<sub>4</sub>D<sub>2</sub>, 1,24,25,28-tetrahydroxyvitamin D<sub>2</sub>; 1,24,25,26(OH)<sub>4</sub>D<sub>2</sub>, 1,24,25,26-tetrahydroxyvitamin D<sub>2</sub>; 1(OH)-24-keto-25,26,27-trinor-D<sub>2</sub>, 1-hydroxy-24-keto-25,26,27-trinorvitamin D<sub>2</sub>; 1,25(OH)-24-keto-28-nor-D<sub>2</sub>, 1,25-dihydroxy-24-keto-28-norvitamin D<sub>2</sub>; 1,24(OH)-25-keto-26-nor-D<sub>2</sub>, 1,24-dihydroxy-25-keto-26-norvitamin D<sub>2</sub>; 1,25(OH)<sub>2</sub>D<sub>3</sub>, 1,25-dihydroxyvitamin D<sub>3</sub>; HPLC, high-performance liquid chromatography; Me<sub>3</sub>Si, trimethylsilyl; NaIO<sub>4</sub>, sodium metaperiodate.