Occurrence and Biosynthesis of β-Glucuronidic Linkages in Heparin*

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SUMMARY

Heparin was degraded with nitrous acid to yield carbohydrate-serine compounds with uronic acid located at the terminal, nonreducing position. In addition, a tetrasaccharide with the proposed structure, uronosyl-N-acetylglucosaminyluronosyl-anhydromannose, was isolated from heparin by a similar procedure. On digestion with liver β-glucuronidase, between one-third and all of the uronic acid located at the terminal position of these fragments was liberated as glucuronic acid. The results indicate that a large portion of the glucuronidic linkages of heparin have the β configuration.

Furthermore, one of the β-glucuronidase-treated fragments served as acceptor for glucuronic acid when incubated with UDP-[14C]glucuronic acid and a particulate enzyme preparation derived from a heparin-producing mouse mastocytoma. Previous treatment of the heparin fragment with β-glucuronidase was mandatory for acceptor activity. On digestion of the radioactive product with β-glucuronidase, all of the radioactivity was released as [14C]glucuronic acid.

Heparin, a high molecular weight polymer, is thought to consist of alternating residues of uronic acid and glucosamine, joined by α-1 → 4 glycosidic linkages (1). The regularity is interrupted at the reducing end of the molecule, which is linked to a polypeptide via a sequence of 3 neutral sugar molecules:

\[
\text{Polysaccharide} \rightarrow \text{GlcUA} \rightarrow \text{Gal} \rightarrow \text{Gal} \rightarrow \text{Xyl} \rightarrow \text{Ser} \rightarrow \text{Polypeptide}
\]

Although the glucosamine moieties are largely N-sulfated, the amino sugar constituents in the proximity of the carbohydrate-protein linkage region have been shown to be N-acetylated (2). Also, the more peripheral portions of the polysaccharide chain appear to contain a limited number of N-acetylgalactosamine residues (3).

Previous work has established that the neutral sugar units as well as the glucuronosyl-galactose linkage are all of the β-anomeric configuration (4). However, the possibility of some glucuronidic linkages other than the GlcUA-Gal unit having the β configuration has not been discounted. The optical molecular rotation of heparin and derivatives thereof (1) is lower than expected for an exclusive presence of α-D-glycosidic linkages. It has recently been suggested (5) that the low value may be due to the presence of L-iduronic acid, which constitutes a considerable portion of the uronic acid in heparin (5, 6). It is uncertain, however, whether the iduronic acid content could account quantitatively for the optical rotation data.

In the present study, fragments obtained after degradation of heparin with nitrous acid (2, 7) have been investigated as substrates for liver β-glucuronidase. Evidence is presented demonstrating that the enzyme released glucuronic acid from several heparin fragments, in amounts varying between one-third and all of the uronic acid residues present at the terminal, nonreducing position of the molecule.

Furthermore, glucuronosyl transfer has been studied in a cell-free system derived from a heparin-producing mouse mastocytoma, and the formation of glucuronidic linkages susceptible to cleavage by β-glucuronidase has been demonstrated.

EXPERIMENTAL PROCEDURE

Materials—Heparin was obtained from Wilson Laboratories, Chicago, Illinois, and purified as described (8). Hyaluronic acid was a gift from Professor T. C. Laurent of this institute. p-Nitrophenyl-α-D-glucoside and p-nitrophenyl-β-D-glucuronide were purchased from Sigma. 3-O-β-D-Glucuronosyl-D-galactose was prepared from chondroitin 6-sulfate as described (9). UDP-[14C]glucuronic acid (33 μCi per μmole) was obtained from The Radiochemical Centre, Amersham, England.

Liver β-glucuronidase (type B-1; 220 Fishman units per mg) and limpet β-glucuronidase (type L-1; 500 Fishman units per mg) were products of Sigma. Testicular hyaluronidase (14,000 units per mg) was obtained from Leo, Helsingborg, Sweden.

A mast cell tumor originally described by Furtner, Hagen, and Hirsch (10) was maintained as a solid tumor in (A/Sri X Leaden) F1 mice by subcutaneous and intramuscular transplantation in the hind legs every 10 to 14 days.

Analytical Methods—Uronic acid was determined as described...
by Bitter and Muir (11). Hexosamine was estimated after hydrolysis in 4 M HCl for 14 hours by a modification of the method of Bos (12) with omission of the resin treatment. Anhydromannose was quantitated by the indole reaction (13). Protein was determined by the method of Lowry et al. (14).

Paper chromatography was performed in Solvent A, ethyl acetate-acetic acid-water (3:1:1) or Solvent B, n-butyl alcohol-acetic acid-pyridine-water (15:3:10:12). Paper electrophoresis was carried out in Buffer C, 0.046 M acetic acid-0.08 M pyridine, pH 5.3, or Buffer D, 6% formic acid, pH 1.70. The papers were stained with a silver dip reagent (15).

**Determination of Radioactivity**—Strips of paper chromatograms or electropherograms were analyzed for radioactivity with a Packard model 7201 strip scanner. For quantitative purposes, areas containing the product were eluted and counted in a Beckman LS 250 liquid scintillation spectrometer.

**Synthesis of p-Nitrophenyl-α-D-Glucuronide**—Standard p-nitrophenyl-α-D-glucuronide was synthesized in a procedure similar to that described (16). A sample (0.45 g) of p-nitrophenyl-α-D-glucuronide was mixed with a suspension of freshly reduced platinum (10%) activated carbon catalyst (0.2 g) in 25 ml of water. The reaction mixture was kept at 90°, with an air flow of approximately 180 liters per hour. The pH was maintained at about 9 by intermittent addition of 5 ml of 0.5 M NaHCO₃. After 3 hours, the reaction mixture was filtered, acidified to pH 3.0, and passed through a column (2 × 5 cm) of Dowex 50-X2 (H⁺ form, 200 to 400 mesh). Upon concentration in the rotary evaporator, the product crystallized. The material was twice recrystallized from aqueous ethanol and stored over P₂O₅ (yield, 35 mg; m.p. 210–213°). The product reacted properly in the carbazole reaction for uronic acids and migrated at an Rᵢ value of 0.74 on paper electrophoresis in Buffer C.

**Isolation of Heparin Fragments after Treatment with Nitrous Acid**—Degradation of heparin with nitrous acid and the isolation of Fractions B¹ and B₂ has been described (2). The tetrasccharide, UA-GlcNAc-UA-AM, was isolated by a similar procedure, involving treatment of heparin with nitrous acid and gel chromatography. The tetrasccharide fraction from the Sephadex column (cf. Fraction II, Fig. 2 in Reference 2) was separated from contaminating carbohydrate-serine compounds by passage through a column (2 × 10 cm) of Dowex 50-X8 (200 to 400 mesh), equilibrated with 0.5 M formic acid-1.30 M acetic acid (pH 1.85). The effluent was concentrated with additions of methanol, and lyophilized. The dried material was suspended in methanolic HCl and desulfated according to Kantor and Schubert (17). After removal of methanol on a rotary evaporator, the solution was adjusted to pH 4.5 by addition of Dowex 3 (OH⁻ form, 20 to 50 mesh) and lyophilized. The desulfation procedure was repeated once. To check the completeness of the reaction, the product was passed through a column (2 × 10 cm) of Dowex 1-X2 (Cl⁻ form, 200 to 400 mesh). Approximately 80% of the carboxyl-positive material appeared with the water wash, indicating that desulfation had occurred, with concomitant esterification of the carboxyl groups on the uronic acid residues. The material was de-esterified by treatment with 0.1 M NaOH for 15 hours at room temperature. After neutralization with HCl and desalting on a column (2 × 40 cm) of Sephadex G-10, eluted with 10% aqueous ethanol, the material was concentrated and further purified by paper electrophoresis (Buffer C; 80 volts per cm for 70 min) and paper chromatography (Solvent A; 24 hours). Analytical data, essentially consistent with those expected for a compound with the structure indicated, are given in Table I. The yield of the pure compound was 0.5 mg per g of heparin.

**Isolation of Pentasccharide from Hyaluron Acid**—Hyaluron (0.3 g) was dissolved in 900 ml of 0.1 M acetic buffer, pH 5.0, containing 0.15 M NaCl and digested at 37° with 2 mg of testicular hyaluronidase for a period of 24 hours. After being kept in a boiling water bath for 10 min, the digestion mixture was concentrated, clarified by centrifugation and applied to a column (2 × 180 cm) of Sephadex G-50 (superfine), which was eluted with 0.2 M NaCl. The hexasaccharide fraction, appearing at an eluent volume of 405 to 430 ml, was pooled, concentrated, and desalted on a column (4.5 × 25 cm) of Sephadex G-15. Further purification was achieved by preparative paper chromatography for 96 hours in Solvent B on washed Whatman No. 3MM papers. The main spot had an Rᵢ value of 0.30 in this solvent and was homogeneous when analyzed by paper electrophoresis (Buffer C; Rᵢ = 0.85). As expected for a hexasaccharide, one-third of the glucuronic acid was present at the nonreducing terminal position, as seen by gel chromatography following digestion with β-glucuronidase. The corresponding pentasccharide, GlcNAc-GlcUA-GlcNAc-GlcUA-GlcNAc, was isolated by gel chromatography on Sephadex G-25 after digestion of the hexasaccharide with liver β-glucuronidase as described below.

**Digestion with β-Glucuronidase**—Unless indicated otherwise, samples were dissolved in 0.05 M acetic buffer, pH 5.0, to give a 0.1% solution of oligosaccharide and digested with liver β-glucuronidase (type R-1; 10 mg of enzyme per ml of digestion mixture). After 12 hours of incubation at 37° a second portion of the enzyme was added and the digestion was continued for another 12 to 60 hours. Limpet β-glucuronidase was incubated together with substrate in 0.05 M acetic buffer, pH 3.6, in a similar manner. The digests were analyzed by gel chromatography on a column (1 × 96 cm) of Sephadex G-25, which was eluted with 1.0 M KCl. Portions of the digests were also subjected to paper electrophoresis (Buffer C), or paper chromatography (Solvent A).

Control incubations with boiled enzyme or lacking substrate were carried out simultaneously and analyzed by a similar procedure.

**Transfer of Glucuronic Acid from UDP-glucuronic Acid to Exogenous Acceptors in Cell-free System from Mouse Mastocytoma**—Tumors from 20 mice (approximately 20 g, wet weight) were homogenized in a Virtis 45 homogenizer with two volumes of a
Substrate specificity of liver and limpet glucuronidases

Incubations with liver β-glucuronidase were carried out in 0.3 ml of 0.05 M acetate buffer, pH 5.0, at 37° for 24 hours. With the limpet enzyme, conditions were similar except that the pH was 3.6. To determine the product, aliquots (0.10-ml) were mixed with 4 ml of 0.2 M glycine buffer, pH 10.0. After centrifugation, the absorbance at 435 mp was measured with a Beckman B spectrophotometer.

Table II

| Substrate                  | Enzymea     | O.D. 435 nm |
|----------------------------|-------------|-------------|
| p-Nitrophenyl-α-D-glucuronideb | Bovine liver (boiled) | 0.015       |
|                            | Limpet (boiled)      | 0.010       |
| p-Nitrophenyl-β-D-glucuronidesc | Bovine liver (boiled) | 0.09        |
|                            | Limpet (boiled)      | 0.00         |

a 2 mg per incubation.

b 0.57 μmole, based on uronic acid contents.
c 0.44 μmole, based on uronic acid contents.

Buffer containing 50 mM Tris-acetate, pH 7.4, 70 mM KCl, 1 mM EDTA, and 20 mM MnCl₂. After centrifugation at 10,000 × g for 10 min, the supernatant fluid was further centrifuged at 100,000 × g for 60 min. The resulting pellet, suspended in 15 ml of the buffer indicated above, was used as glucuronosyltransferase preparation. Enzyme assays were carried out by incubating UDP-[14C]glucuronic acid with appropriate acceptors, as described in the legend to Table II. The reaction mixtures were spotted on Whatman No. 3MM paper and subjected to electrophoresis in Buffer C (80 volts per cm, 75 min). The distribution of radioactivity was determined with a strip scanner.

Preparation of Particulate Enzyme from Chick Embryo Cartilage—A particulate glucuronosyltransferase preparation was obtained from epiphyseal cartilage of 13-day-old chick embryos essentially as described (18). The 100,000 × g pellet was suspended in Tris-acetate buffer with the composition given above and incubated with UDP-[14C]glucuronic acid and exogenous acceptors as outlined above.

RESULTS

Specificity of Liver and Limpet Glucuronidases In order to confirm the specificities of the enzyme preparations employed, p-nitrophenyl α- and β-D-glucuronides were incubated with enzyme from bovine liver and from limpet. Table II shows that the liver β-glucuronidase did not hydrolyze p-nitrophenyl-α-D-glucuronide whereas the corresponding β-D-glucuronide was cleaved quantitatively under similar conditions. With the limpet enzyme, complete hydrolysis of both glucuronides was obtained (Table II).²

² Similar results were obtained with methyl α- and β-D-glucuronides, respectively. These were synthesized from the corresponding methyl glucosides by a procedure similar to that described under "Materials and Methods" for p-nitrophenyl-α-D-glucuronide with the exception that the temperature was kept at 60°. Although the methyl α-D-glucuronide was completely resistant to cleavage by liver β-glucuronidase, only 18% of the methyl-β-D-glucuronide was hydrolyzed under the same conditions, rendering these substrates less suitable for specificity studies.

These results are in agreement with earlier reports that mammalian glucuronidases do not hydrolyze α-glucuronidic linkages. In contrast, the limpet glucuronidase has been stated to cleave glucuronides of both anomeric configurations (for a review, see Reference 19).

Digestion of Fragments B₁ and B₂ from Heparin with Liver β-Glucuronidase—Fig. 2a shows the pattern obtained on gel chromatography of Fraction B₁ after treatment with β-glucuronidase. Of the total uronic acid in the digest, 15% emerged retarded on the Sephadex column and at the position expected for glucuronic acid. This value corresponds to 45% of the uronic acid units in nonreducing terminal position of Fraction B₁ (cf. the schematic structure, Fig. 1). The presence of glucuronic acid in the digest was confirmed by paper chromatography (Solvent A) and paper electrophoresis (Buffer C). On redigestion of Peak B₁-β (Fig. 2a) with liver or limpet β-glucuronidase, no further material was released. This result suggests that the remaining uronic acid units located at the terminal position of this fraction may be iduronic
acid since the latter has been shown previously to be an integral part of this fragment (2).

Fragment B2, which contains no iduronic acid (Fig. 1) was subjected to a similar treatment and, as is seen from Fig. 2b, half of the uronic acid was released by the enzyme, corresponding to quantitative release of the glucuronic acid at the terminal position. The molar ratio, uronic acid to hexosamine, for Fraction B2-β (Fig. 2b) was 1.5 as compared with a ratio of 2.8 for Compound B2, again indicating that almost half of the glucuronic acid present in Compound B2 had been removed by β-glucuronidase.*

Treatment of Tetrasaccharide, UA-GlcNAc-UA-AM, with β-Glucuronidase—To determine whether β-glucuronidic linkages were a unique feature of the glucuronic acid residues in the immediate vicinity of the linkage region, the tetrasaccharide, UA-GlcNAc-UA-AM, was also treated with β-glucuronidase. This fraction represents a fragment from that portion of the polysaccharide chain which is distal to the first N-sulfated glucosamine moiety (7). Treatment of this material with liver β-glucuronidase released 14% of the total uronic acid (Fig. 2c) corresponding to 26% of the uronic acid located at the terminal, nonreducing position of the molecule. Paper chromatography (Solvent A) of a portion of the digest showed the presence of glucuronic acid as well as material migrating at the rate of the undigested tetrasaccharide. In addition, a third, somewhat elongated spot with intermediate mobility was observed, presumably representing the trisaccharide product of the digestion. Insufficient material precluded a more thorough, structural study of these fragments.

Transfer of Glucuronic Acid from UDP-glucuronic Acid to Fraction B1-β—Incubation of the particulate enzyme from mouse mastocytoma with UDP-[14C]glucuronic acid and Fraction B1-β (Fig. 2a) resulted in the formation of a product (1013 cpm; Table III) which migrated similarly to the undigested Fragment B1 on paper electrophoresis in Buffers C (Fig. 3) and D. This radioactive peak was absent in control experiments without added acceptor. Furthermore, on gel chromatography (Sephadex G-50 column (2 × 180 cm)) the radioactive product and Fragment B1 emerged with the same effluent volume (Fig. 4). Preliminary treatment of Fragment B1 with β-glucuronidase appeared to be mandatory for acceptor activity; the undigested fragment was essentially inactive as substrate (Table III).

Specificity of Glucuronyltransferase Reaction—In order to investigate the specificity of the reaction, a pentasaccharide from hyaluronic acid, GlcNAc-GlcUA-GlcNAc-GlcUA-GlcNAc, was also incubated with the mastocytoma preparation and UDP-[14C]glucuronic acid (Table III). However, no evidence for hexasaccharide synthesis was obtained. It should be noted that whereas glucosamine is believed to occur in heparin linked via α-1 → 4 bonds, the glucosaminidic linkage in hyaluronic acid is β-1 → 4 (1).

Furthermore, in view of recent evidence concerning limited synthesis of chondroitin sulfate in the mouse mastocytoma preparation,† it was important to establish whether transfer of glucuronic acid to Fraction B1-β was due to the presence of a glucuronosyltransferase involved in the polymerization of chondroitin sulfate chains. For this reason, Fraction B1-β was incubated with a particulate enzyme preparation previously utilized in studies on the biosynthesis of chondroitin sulfate (18, 20). However, no significant transfer of glucuronic acid from UDP-glucuronic acid to Fraction B1-β was observed under conditions where transfer of glucuronic acid to the endogenous acceptor present in the cartilage homogenate occurred (Table III).

These results suggest that the mastocytoma preparation contained a specific enzyme, presumably involved in the biosynthesis of heparin and capable of transferring [14C]glucuronic acid from UDP-[14C]glucuronic acid to Fraction B1-β, to form Fraction B1.

Digestion of [14C]Glucuronic Acid-labeled Fraction B1 with β-Glucuronidase—Treatment of the radioactive product obtained as de-
scribed above, with liver \( \beta \)-glucuronidase, resulted in quantitative release of the labeled glucuronic acid, as evidenced by paper electrophoresis (Buffer C; Fig. 5) and paper chromatography (Solvent A).

Similarly, the product was unstable in the mastocytoma preparation utilized for its synthesis, indicating the presence of a \( \beta \)-glucuronidase in the particulate enzyme. This finding was substantiated by control experiments with \( p \)-nitrophenyl-\( \beta \)-D-glucuronide which was hydrolyzed by a 10,000 \( \times \) g supernatant solution derived from mouse mastocytoma. Under the conditions tested, no hydrolysis of the corresponding \( \alpha \)-glucuronide was observed.

**Table III**

**Substrate specificity of glucurono-syltransferase in mouse mastocytoma and in embryonic chick cartilage**

| Substrate                | Enzyme source | Incorporation into exogenous acceptor | Incorporation into endogenous acceptor |
|--------------------------|---------------|---------------------------------------|----------------------------------------|
| Fragment B1              | \( \mu \)moles | Mouse mastocytoma 52                  | 103                                    |
| B1-\( \beta \)           | 0.015         | Mouse mastocytoma 1013                | 116                                    |
| Hyaluronic acid pentasaccharide | 0.15           | Chick cartilage 110                  | 310                                    |

* Based on the amount of glucuronic acid liberated from Fraction B1 after treatment with \( \beta \)-glucuronidase.

* Determined in a similar but separate experiment.

The linkage of glucuronic acid to the nonreducing terminal galactose moiety of the neutral trisaccharide in the heparin-protein linkage region (4) represents yet another type of glucuronidic linkage in heparin. It is conceivable that the incorporation into heparin of this particular sugar unit is catalyzed by an enzyme which is different from the glucuronosyltransferase or transferase described above, with liver \( \beta \)-glucuronidase, resulted in quantitative release of the labeled glucuronic acid, as evidenced by paper electrophoresis (Buffer C; Fig. 5) and paper chromatography (Solvent A).

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**Fig. 5.** Digestion of material from Peak A, Fig. 3 with liver \( \beta \)-glucuronidase. The radioactive product (330 cpm) was dissolved in 0.2 ml of buffer, pH 5.0, and digested with 1 mg of enzyme for 24 hours. The reaction was stopped and the digest was analyzed by paper electrophoresis in Buffer C. The guide strip inserted below the tracing shows: (I) glucuronic acid; (II) Fraction B1.

**Fig. 6.** Proposed structure of heparin tetrasaccharide containing both iduronic and glucuronic acid residues.
ases involved in the formation of the more peripheral portions of the polysaccharide chain (cf. Reference 20).

The results of the present investigation are pertinent to the finding of Perlin et al. (24) that L-iduronic acid residues of heparin have the α-anomeric configuration. Although the biosynthesis of the iduronic acid moieties in glycosaminoglycans such as heparin has not been studied with cell-free systems, it is generally believed that UDP-α-D-glucuronic acid is the precursor of the D-glucuronic acid as well as the L-iduronic acid residues in these polysaccharides (25). Assuming that the mechanisms of incorporation into heparin of glucuronic acid and iduronic acid differ merely by an epimerization process at C-5, the presence in this polymer of α-L-iduronic acid residues would require the D-glucuronic acid units to have the β-anomeric configuration (see Fig. 6).

The problem of the number of enzymes involved in the biosynthesis of the various types of uronic linkage in heparin remains and should attract considerable interest.

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