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BIOSYNTHESIS OF HEPARIN. A NEW SUBSTRATE FOR HEPAROSAN-*N*-SULFATE-D-GLUCOPYRANOSYLURONATE 5-EPIMERASE*

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ABSTRACT

New substrates have been prepared for heparosan-*N*-sulfate-D-glucopyranosyluronate 5-epimerase, which catalyzes formation of L-iduronic acid residues in the course of heparin biosynthesis. Heparin and heparan sulfate were chemically modified by desulfation in aqueous dimethyl sulfoxide, deacetylation by hydrazinolysis, and *N*-sulfation with sulfur trioxide–trimethylamine complex. The modified polysaccharides were incubated with partially purified epimerase from bovine liver in the presence of tritium oxide to incorporate tritium into both D-gluco- and L-ido-pyranosyluronate residues. Incubation of the labeled polysaccharides with liver epimerase released tritium. The complete release of radioactivity after exhaustive incubation indicated that the tritium atom was located at C-5 of the uronate residues. Under appropriate conditions, the release was linear with time and enzyme concentration; K_m values of ~0.2mM (expressed as uronic acid concentration) were determined for both the heparin- and the heparan sulfate-derived substrates. In contrast to the modified polysaccharides, unmodified heparin did not incorporate significant amounts of radioactivity when exposed to tritium oxide in the presence of epimerase.

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INTRODUCTION

The L-iduronic acid units of heparin and heparan sulfate are formed by 5-epimerization of D-glucuronic acid residues in a polymeric intermediate, heparosan *N*-sulfate, which is composed of alternating D-glucuronic acid and *N*-sulfated 2-amino-2-deoxy-D-glucose residues[2–4]. Exchange of H-5 atoms of uronate residues with protons from the medium accompanies the reaction[5] and is the basis of the only assay of epimerase activity presently available[3]. The substrate in this assay, heparosan *N*-sulfate containing D-[5-^{3}H]glucopyranosyluronate residues, is prepared biosynthetically by incubation of mastocytoma microsomes with UDP-D-[5-^{3}H]glucuronic acid, UDP-*N*-acetylglucosamine, and (adenylyl 3′-phosphate) sulfate (PAPS), followed by proteolysis and fractionation of the reaction products on DEAE-cellulose[6]. When [^{3}H]heparosan *N*-sulfate is incubated with the 5-epimerase, tritium is released. It equilibrates with the water of the medium, and is determined after distillation of the reaction mixture. Whereas the assay itself is simple and rapid, the synthesis of [^{3}H]heparosan *N*-sulfate is complicated and time-consuming, and we have, therefore, investigated other methods for substrate preparation. We describe, herein, a simple procedure for enzymic incorporation of tritium from tritium oxide into heparin or heparan sulfate that have been chemically modified to resemble the natural substrate. The labeled products are substrates for the 5-epimerase, as determined by the tritium-release assay, and their properties and ready availability make them well suited as alternatives to the currently used substrate.

EXPERIMENTAL

Materials. — The following chemicals were obtained: Tritiated water (1 Ci/g) from New England Nuclear (Boston, MA 02118), anhydrous hydrazine and hydrazine sulfate from Pierce Chemical Co. (Rockford, IL 61105), dimethyl sulfoxide and 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid from Sigma Chemical Co. (St. Louis, MO 63178), and sulfur trioxide–trimethylamine complex from Aldrich Chemical Co., Inc. (Milwaukee, WI 53201). Other chemicals were reagent-grade quality.

Heparin from hog mucosa (U.S.P. grade, Cohelfred Laboratories, Inc., Chicago, IL 60606; or Inolex Pharmaceutical Division, Park Forest South, IL 60466) was purified by repeated precipitation with cetylpyridinium chloride from 1.4M sodium chloride, essentially as described previously[7].

Anal. Found: uronic acid, 30.5; hexosamine, 15.6; sulfate, 26.4.

Heparan sulfate was prepared as follows (see also refs. 7–10). Heparin by-products (20 g, Inolex, Lot #04058039) were dissolved in de-ionized water (2 L), saturated sodium hydroxide (200 mL) and Benedict's solution[11] (1.6 L) were added to the continuously stirred solution, and, after 10 min, the precipitated dermatan sulfate was removed by centrifugation. The supernatant solution was degassed and

concentrated to 800 mL in an Amicon DC-2 concentrator, equipped with an HDP>5 hollow fiber-cartridge. A reservoir of distilled water (6 L) was connected, and the instrument was operated in the "Diaflo" mode until the solution was almost colorless. After further concentration to 500 mL, the retentate was passed through a column (4.6 × 60 cm) of Dowex 50W-X8 (H^+, 200–400 mesh) cation-exchange resin, followed by distilled water (500 mL), and heparan sulfate was precipitated by addition of ethanol (4 vol.) and sodium acetate. The mixture was stirred for 1 h to ensure complete precipitation and, when the precipitate had settled, the clear supernatant solution was siphoned off. The precipitate was collected by centrifugation, washed twice with ethanol and once with ether, and then dried *in vacuo* in the presence of phosphorus pentaoxide. Yields ranged between 17 and 20% of the starting material.

Anal. Found: uronic acid 42.5, sulfate 17.2, 2-amino-2-deoxy-D-glucose 21.2, 2-amino-2-deoxy-D-galactose 0.6.

Further fractionation with cetylpyridinium chloride was carried out as follows: A solution of heparan sulfate (20 g) in 2M sodium chloride (800 mL) was mixed with 10% cetylpyridinium chloride (600 mL), and then distilled water (600 mL) was added slowly with stirring, yielding a final sodium chloride concentration of 0.8M. After stirring for another 30 min, the turbid solution was kept for 1 h in a water bath at 47° and then filtered through a1.5-cm thick pad of Celite filter-aid in a Büchner funnel (diam. 26.5 cm). Before filtering the polysaccharide, the Celite had been rinsed with water (500 mL) and 0.8M sodium chloride in 0.05% cetylpyridinium chloride (500 mL). The filtrate was diluted to 0.4M sodium chloride by addition of distilled water (1 vol.), and the precipitate was again collected by filtration through Celite (prerinsed with water and 0.4M sodium chloride in 0.05% cetylpyridinium chloride). The two Celite pads were processed as described[7] to yield the sodium salts of the polysaccharides. Average yields from 20 g of copper-fractionated heparan sulfate were 12.3 g of the 0.8M fraction and 3.9 g of the 0.4M sodium chloride fraction*.

Anal. Found: (0.8M fraction): uronic acid 42.1, sulfate 17.2, 2-amino-2-deoxy-D-glucose 18.7, 2-amino-2-deoxy-D-galactose 0.4; (0.4M fraction): uronic acid 48.3, sulfate 11.5, 2-amino-2-deoxy-D-glucose 27.5, 2-amino-2-deoxy-D-galactose 0.2.

Determination of the uronic acid composition of the 0.4M fraction showed the presence of 70% D-glucuronic and 30% L-iduronic acid**. It was further observed that the 0.4M fraction could not be precipitated by cetylpyridinium chloride from a 0.6M sodium chloride solution.

Chemical modification of heparin and heparan sulfate. — Polysaccharide preparations were desulfated as described by Nagasawa *et al.*[12]. The pyridinium salt of the polysaccharide (3 g) was dissolved in water (30 mL), 270 mL of dimethyl

*Hereafter called 0.8M and 0.4M fractions.

**The authors are grateful to Dr. L.-Å. Fransson for this analysis.

sulfoxide was added, and the solution was kept for 7 h at 100°. Recovery of the desulfated product after dialysis and lyophilization averaged 2 g of material that contained ~3% of sulfate.

The desulfated preparations were *N*-deacetylated by the method of Dmitriev *et al.*[13]. Anhydrous hydrazine (100 mL) was added to a 250-mL flask containing the lyophilized polysaccharide (3 g) and hydrazine sulfate (1 g), and the mixture was heated for 4 h at 100° with occasional stirring. Hydrazine was removed by codistillation with toluene (100 mL) at 40° under reduced pressure. This procedure was repeated 5 times. The residue was dissolved in 2M sodium chloride (100 mL), and the polysaccharide was precipitated by addition of ethanol (4 vol.). After centrifugation, the precipitate was repeatedly dissolved in water (100 mL) and precipitated with ethanol, until addition of sodium acetate was necessary to cause precipitation. The final precipitate was washed twice with ethanol and once with ether, and was dried in the presence of phosphorus pentaoxide under reduced pressure. The average yield of three preparations was 99% on a weight basis (a fourth preparation gave a recovery of only 33%).

N-Sulfation was carried out by the procedure of Levy and Petracek[14]. The polysaccharide, sodium carbonate, and sulfur trioxide–trimethylamine complex (3 g each) were dissolved in distilled water (60 mL), and the mixture was kept for 24 h at 55° with constant stirring. After being cooled to room temperature, the mixture was diluted with water to 600 mL, and 10% cetylpyridinium chloride (66 mL) was added. The resulting precipitate was collected by centrifugation and dissolved in 5:1 (v/v) 2M sodium chloride–ethanol. Following precipitation with ethanol (3 vol.), the polysaccharide was twice redissolved in water and precipitated with ethanol, and was finally dissolved in water, dialyzed overnight against distilled water, and lyophilized. The average yield was 73%, and the sulfate content of the modified polysaccharides ranged from 14 to 16%.

Analytical methods. — Uronic acid was determined either manually by the method of Dische[15] or by the automated procedure of Ford and Baker[16] (for column eluates only), hexosamine by the method of Ford and Baker [16], and sulfate by the method of Terho and Hartiala[17]. The methods for the determination of protein and radioactivity have been described[18]. When polysaccharides (0.05–0.2 mL) were counted, 0.5M sulfuric acid (0.5 mL) was added to the scintillation fluid (10 mL of Scintiverse, Fisher Scientific Co., Pittsburgh, PA 15219) to prevent precipitation.

Deaminative cleavage of the various polysaccharide preparations by nitrous acid was carried out at pH 1.5 as described by Shively and Conrad[19]. Gel chromatography of the product was carried out on a column (1.5 × 190 cm) of Sephadex G-25, superfine, which was eluted with 0.2M ammonium hydrogencarbonate at a flow rate of 12 mL/h. Ammonium hydrogencarbonate was removed by repeated evaporation to dryness under reduced pressure at 37°. In some cases, the isolated disaccharide fraction was analyzed by chromatography on Dowex 1

resin in 0.3M formic acid[20] or on Aminex A-25 resin after reduction with sodium borohydride or tritide[21,22].

Enzyme preparation and assay. — Heparosan-*N*-sulfate-D-glucopyranosyluronate 5-epimerase was partially purified from beef liver as previously described[23,24]. The preparation used for the present work was purified ~30-fold over the initial homogenate.

Assay of epimerase activity in the course of enzyme purification was carried out, as described by Jacobsson *et al.*[3], with an unfractionated mixture of D-[5-^{3}H]glucosyluronate-labeled polysaccharides as substrate. Assay with the new substrates described herein was carried out as follows. Reaction mixtures contained an aqueous solution of substrate (25 μL, ~30 μg of polysaccharide, 2000–12 000 c.p.m.) and epimerase (275 μL, 0.1 mg protein or less) in 50mM Hepes–50mM potassium chloride–15mM EDTA, pH 7.4. After incubation at 37° for various periods of time (1 h in the standard assay), the reaction mixtures were heated for 1 min at 100° and transferred to round-bottom tubes for distillation as previously described[3]. A 0.2-mL sample of the distillate was withdrawn for liquid-scintillation counting.

Incorporation of tritium from tritium oxide into modified heparin and heparan sulfate. — Reaction mixtures had a final volume of 300 μL and contained enzyme (250 μL, 0.7 mg protein/mL) in 50mM Hepes–50mM potassium chloride–15mM EDTA, pH 7.4, tritiated water (25 μL, 1 Ci/g), and substrate (25 μL, 40 mg/mL) in 0.24% sodium azide. After being incubated in a hood at room temperature for various periods of time, each reaction mixture was heated for 3 min at 100°, diluted 3-fold with 0.3M sodium chloride and applied to a column (QS-A Quik-Sep; Isolab Inc., Akron, OH 44321) of AG 1-X4 (Cl^-; 200–400 mesh; bed volume, 2 mL) resin. The column was eluted with 0.3M sodium chloride (60 mL), followed by 2M sodium chloride (15 mL). The latter eluate was dialyzed and lyophilized, and the incorporated radioactivity was measured by liquid-scintillation spectrometry.

Large-scale preparation of ^{3}H-labeled substrate. — A solution of polysaccharide (160 mg) was lyophilized in a 25-mL Erlenmeyer flask. All succeeding operations were carried out in a well-ventilated hood, approved for use with 1 Ci of tritium. Enzyme (100–140 mg protein) in 50mM Hepes–50mM potassium chloride–15mM EDTA–0.02% sodium azide, pH 7.4 (10 mL) was added with slow stirring, followed by tritiated water (1 mL, 1 Ci/g). The flask was sealed with parafilm, and the mixture was stirred slowly for 1 week at room temperature. After dilution with 0.3M sodium chloride (11 mL), the solution was heated for 3 min at 100°, and applied to a column (4.5 × 40 cm) of AG 1-X4 (Cl^-, 200–400 mesh) ion-exchange resin together with two 10-mL washes of 0.3M sodium chloride. The column was washed with 0.3M sodium chloride at 60 mL/h until the effluent contained less than 2000 c.p.m./mL (~4 L), and then the ^{3}H-labeled polysaccharide was eluted with 2M sodium chloride (1 L). The eluate was removed from the hood, evaporated to dryness under reduced pressure at 40°, dissolved in water, and dialyzed for 3 days against 3 changes of de-ionized water (40 L). The retentate was evaporated to dry-

ness under reduced pressure at 40°, dissolved in water, and lyophilized. The residue was dissolved in water (20 mL), and the solution filtered through a Unichem filter sampler (Unichem, Fairburn, Georgia 30213) and stored frozen at −20° after aliquots had been taken for uronic acid analysis and determination of incorporated radioactivity. Recovery was 80% of the original polysaccharide, based on uronic acid analyses. The substrate solution was occasionally viscous and difficult to pipet. This problem could be obviated by centrifugation at 100 000*g* for 1 h or by precipitation with an equal volume of 40% trichloroacetic acid, followed by dialysis of the supernatant against 0.2M sodium chloride and de-ionized water*.

RESULTS AND DISCUSSION

Tritium incorporation into modified heparin and heparan sulfate. — Modification of heparin by desulfation, *N*-deacetylation, and *N*-sulfation, followed by incubation with 5-epimerase in tritiated water resulted in incorporation of tritium, as shown in Table I. Without added substrate, incorporated radioactivity was only 0.4% of that observed for the complete system, indicating that significant amounts of endogenous substrates were not present in the enzyme preparation. Similarly, the product obtained in the absence of enzyme had <1% of the radioactivity obtained in the complete system. Unmodified heparin did not incorporate significant amounts of tritium, and this observation is of interest in view of our previous finding that heparin is a potent inhibitor of tritium release from the biosynthetic substrate[3,18]. It seemed plausible at the time that the inhibitory effect was due to

TABLE I

INCORPORATION OF TRITIUM FROM TRITIATED WATER INTO MODIFIED HEPARIN

Composition of reaction mixtures[a]	*Radioactivity incorporated (c.p.m.)*	*Specific activity of product (d.p.m./μmol of uronic acid)*
Modified heparin + enzyme	259 100	168 400
Enzyme alone	930	
Modified heparin alone	2310	1310
Boiled control (modified heparin + enzyme)[b]	6240	2950
Heparin + enzyme[c]	1230	1100

[a]For each set of conditions, three separate analytical-scale, incubation mixtures were prepared, as described in the Experimental section. After incubation for 45 h, aliquots were chromatographed separately on AG 1-X4 resin, and the 2M sodium chloride eluates were pooled and processed as described [b]Heated to 100° for 3 min prior to incubation. [c]The incubation period was 42 h

*The contaminating material was not characterized but may have consisted of proteins or nucleic acids, or both. Although removal prior to the fractionation-column step would have been preferable, containment of the large amounts of radioactivity in the reaction mixtures was of major concern at this point in the procedure.

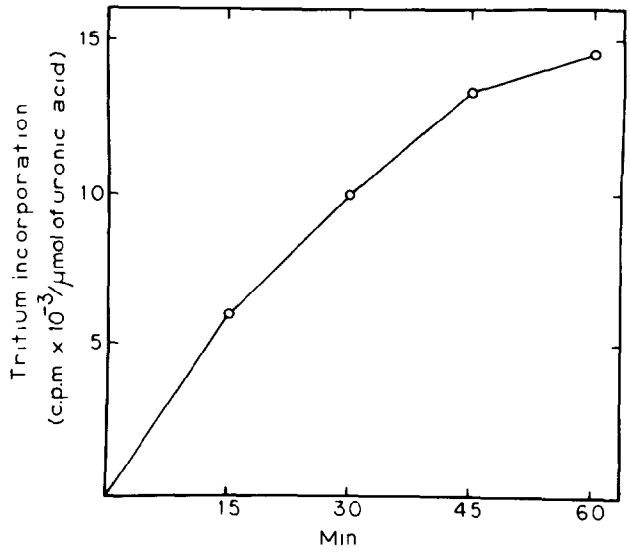

Fig. 1. Incorporation of tritium into modified heparin as a function of time. Each point represents the average of three separate incubations, and a zero-time control (1700–1800 c.p.m.) has been subtracted.

the presence, in the unlabeled heparin, of substrate sites that compete with the ^{3}H-labeled substrate for the enzyme, but the experiment shown in Table I demonstrates clearly that this was not the case.

The time course of tritium incorporation into modified heparin is shown in Fig. 1. The reaction proceeded at an almost constant rate for 45 min, and continued at a decreasing rate for several hours thereafter.

Results of several preparative incubations show (Table II) that, under identical experimental conditions, the modified 0.4M heparan sulfate incorporated the

TABLE II

LARGE-SCALE PREPARATION OF ^{3}H-LABELED SUBSTRATES

Substrate	*Specific activity of product (d.p.m. · 10^{-5}/μmol of uronic acid*
Modified heparin	
Preparation No. 1	1.22
Preparation No. 2	2.31
Modified heparan sulfate	
0.8M sodium chloride fraction	3.87
0.4M sodium chloride fraction	4.24
Modified heparin	
Preparation No. 1 (80 mg)	2.43
Preparation No. 1 (160 mg)	1.19

highest amount of radioactivity, closely followed by the 0.8M fraction, whereas the modified heparin (Preparation No. 2) incorporated 56% as much radioactivity as the 0.4M fraction. Variation in tritium levels was also observed from one preparation to another (*cf.* Preparations No. 1 and 2). When the amount of substrate was increased from 80 to 160 mg, the specific activity decreased to half, while the total radioactivity remained constant. This suggests that, at least in the mixture having the higher amount of substrate, equilibrium had not been attained.

Characterization of substrates and products. — Deaminative cleavage of the unmodified heparin (Fig. 2) yielded 79% of disaccharides and 21% of tetrasaccharides and higher oligosaccharides, indicating that ~10% of the 2-amino-2-deoxy-D-glucose residues were *N*-acetylated. The chemical modifications (desulfation, deacetylation, and *N*-sulfation) caused only a relatively small shift in the distribution of the products of nitrous acid cleavage, with an increase in disaccharides to 83% and a corresponding decrease in the larger fragments. In contrast to the unmodified heparin, the 0.4M heparan sulfate fraction yielded only 16% of disaccharides upon deaminative cleavage, but this value rose to 60% after the chemical

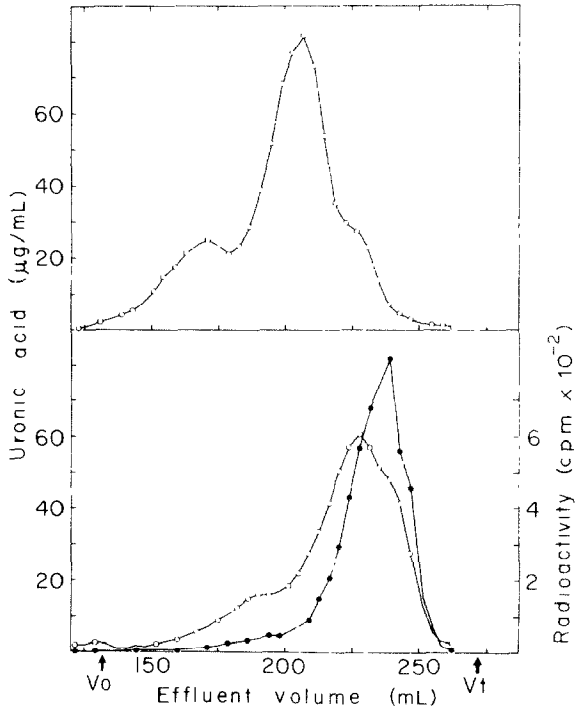

Fig. 2. Gel chromatography on Sephadex G-25 of deamination products from: (A) heparin (10 mg), and (B) ^{3}H-labeled, modified heparin. The column (1.5 × 190 cm) was eluted at a rate of 12 mL/h, fractions (4 mL) were collected, and aliquots were analyzed for uronic acid (○—○) and radioactivity (●—●). Note that partial resolution of disulfated, monosulfated, and nonsulfated disaccharides occurred (Effluent volumes of peak fractions were approximately 200, 225, and 235 mL, respectively.) V_o, void volume; V_t, volume where salts emerge.

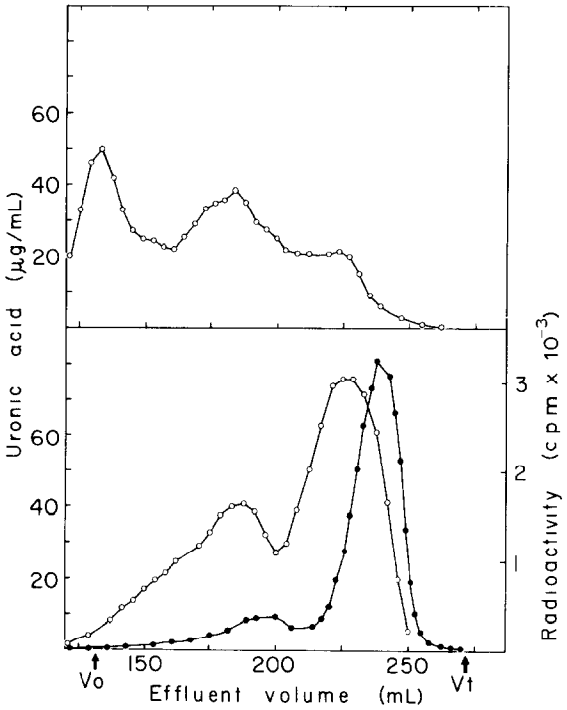

Fig. 3. Gel chromatography on Sephadex G-25 of deamination products from: (A) heparan sulfate (0.4M fraction, 10 mg), and (B) ^{3}H-labeled, modified heparan sulfate. The column (1.5 × 190 cm) was eluted at a rate of 12 mL/h; fractions (4 mL) were collected, and aliquots were analyzed for uronic acid (○—○) and radioactivity (●—●). V_o, V_t, see legend to Fig. 2.

modifications (Fig. 3). It was estimated that more than half of the 2-amino-2-deoxy-D-glucose residues were *N*-acetylated initially, and that close to 25% were still acetylated after the modifications.

If it is assumed that the best epimerase substrate is that where all 2-amino-2-deoxy-D-glucose residues are *N*-sulfated*, then the *N*-acetyl groups of heparin and heparan sulfate should, ideally, be removed completely and replaced with *N*-sulfate groups. In practice, this could not be accomplished as the hydrazinolysis used for *N*-deacetylation also causes depolymerization of the polysaccharides (a 4-h reaction time was therefore chosen as a compromise to avoid excessive degradation). From a practical standpoint, the projected gain in substrate activity should also be weighed against the additional effort involved in *N*-deacetylation. Thus, in view of the already low *N*-acetyl content of heparin, it is unlikely that the substrate obtained after chemical deacetylation would be significantly better than the analogous material without this treatment. (However such preparations were not directly

*The 2-amino-2-deoxy-α-D-glucopyranosyl residue linked to O-4 of the uronic acid residue that undergoes epimerization must be *N*-sulfated, but the 2-amino-2-deoxy-α-D-glucopyranosyl linked at O-4 by the pyranosyluronate residue may be either *N*-sulfated or -acetylated[26].

compared.) On the other hand, it is probable that the removal of more than half of the *N*-acetyl groups from the 0.4M heparan sulfate fraction (initially acetylated to the extent of >50%) resulted in a substantial increase in substrate activity.

Six different disaccharides, two nonsulfated, three monosulfated, and one disulfated were formed on deaminative cleavage of heparin: G-M, I-M, G-M(S), I-M(S), I(S)-M, and I(S)-M(S)*. Analysis of the disaccharide fraction obtained from modified heparin by chromatography on Aminex A-25 resin[21,22] after reduction with sodium borotritide showed the following distribution among the six components: 16% G-M, 32% I-M, 42% I(S)-M, 2% G-M(S), 6% I-M(S), and 3% I(S)-M(S). Apparently, the desulfation was not complete, and, in particular, the high content of I(S)-M was surprising**. However, since this work was completed, Kosakai and Yosizawa[25] have reported that the 2-sulfate group on the L-iduronic acid residue is comparatively resistant to treatment with dimethyl sulfoxide under the conditions used. Since virtually all sulfate groups can be removed by prolonged exposure to dimethyl sulfoxide[26], it should be possible to prepare a modified heparin in which all but a few uronic acid residues have been converted into substrates for the epimerase.

The labeling pattern in the tritiated modified heparin was examined by nitrous acid degradation followed by gel chromatography. As seen in Fig. 2B, the bulk of the radioactivity emerged in the disaccharide region, but was shifted *vis-à-vis* the uronic acid profile towards the most retarded portion of the peak where the nonsulfated disaccharides are found (notice the shoulder in the uronic acid peak). This pattern is to be expected in view of the presence of a substantial proportion of 2-sulfated L-iduronic acid residues in the substrate, which cannot incorporate tritium and appear earlier within the disaccharide peak. Further, the distribution of ^{3}H-label between D-glucuronic and L-iduronic acid was determined by chromatography of the disaccharide fraction on Dowex 1 or Aminex A-25 resin (Figs. 4 and 5). Although D-glucuronic acid was the minor uronic acid component in the starting material (18% D-glucuronic acid *vs.* 82% L-iduronic acid in the disaccharide fraction), it contained approximately twice as much tritium as the L-iduronic acid in the product. This finding is in reasonable agreement with the ratio of 4:1 that was observed earlier by Prihar *et al.*[24] after degradation of the uronic acids to D-glucose and L-idosan, respectively.

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*Abbreviations: G-M, 2,5-anhydro-4-*O*-(β-D-glucopyranosyluronic acid)-D-mannose, I-M, 2,5-anhydro-4-*O*-(α-L-idopyranosyluronic acid)-D-mannose, G-M(S), 2,5-anhydro-4-*O*-(β-D-glucopyranosyluronic acid)-D-mannose 6-sulfate, I-M(S), 2,5-anhydro-4-*O*-(α-L-idopyranosyluronic acid)-D-mannose 6-sulfate; I(S)-M, 2,5-anhydro-4-*O*-(α-L-idopyranosyluronic acid 2-sulfate)-D-mannose, and I(S)-M(S), 2,5-anhydro-4-*O*-(α-L-idopyranosyluronic acid 2-sulfate)-D-mannose 6-sulfate

**The high proportion of sulfated disaccharides was unexpected in view of the presence of only 3% of sulfate in the parent preparation before *N*-sulfation. However, at the time of the determination of disaccharide composition, the parent preparation was no longer available for repeated sulfate analysis. For the following reasons, we believe that the indicated sulfate content is in error rather than the disaccharide composition: (a) the uronic acid profile in Fig. 2B is asymmetric and indicates the presence of at least 50% of sulfated disaccharides, and (b) in another experiment[26], desulfation for 12 h was required to lower the sulfate content to about 3%

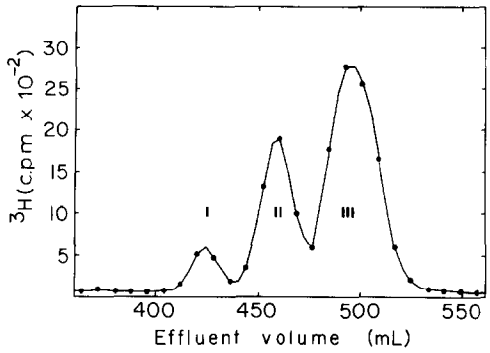


Fig. 4. Ion-exchange chromatography on AG 1-X4 resin of the disaccharide fraction from ^{3}H-labeled, modified heparin. Labeled polysaccharide (1.3 × 10^6 c.p.m.) was degraded with nitrous acid as described in the Experimental section, and the products were chromatographed on a column (1.5 × 190 cm) of Sephadex G-25. The pooled disaccharide fraction (1.25 × 10^6 c.p.m.) was applied to a column (1 × 140 cm) of AG 1-X4 (>400 mesh) resin, which was eluted with 0.3M formic acid at a rate of 12 mL/h. Fractions (4 mL) were collected, and aliquots were analyzed for radioactivity. Radioactivity present in the three peaks accounted for 65% of the radioactivity applied to the column: (I) L-iduronic acid, (II) 2,5-anhydro-4-*O*-(α-L-idopyranosyluronic acid)-D-mannose, and (III) 2,5-anhydro-4-*O*-(β-D-glucopyranosyluronic acid)-D-mannose. In this system, D-glucuronic acid is eluted in the same position as peak II but was not present, as determined by paper chromatography.

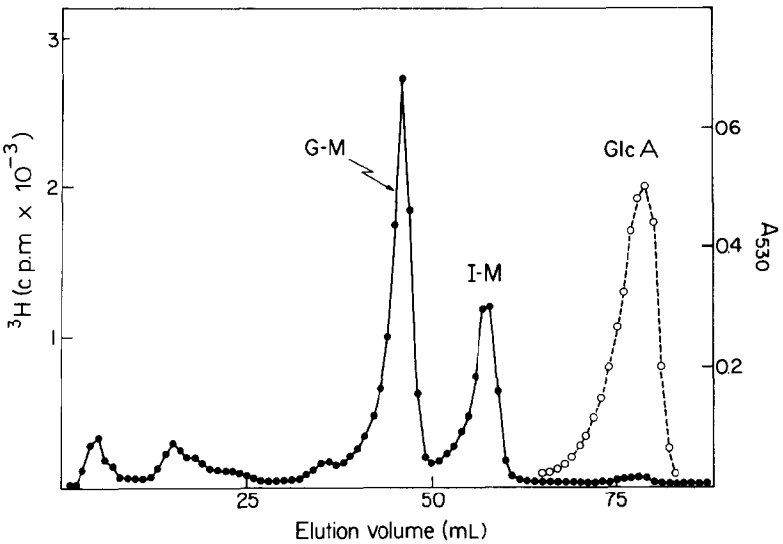
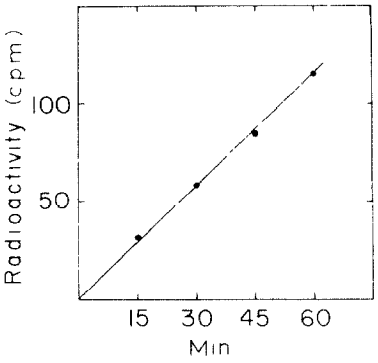


Fig. 5. Chromatography, on Aminex A-25 resin, of reduced disaccharides obtained from ^{3}H-labeled, modified heparin. After deaminative cleavage and reduction with unlabeled sodium borohydride, the disaccharide fraction was isolated by gel chromatography on Sephadex G-15 and applied to a column (0.6 × 26 cm) of Aminex A-25 resin, together with D-glucuronic acid (100 μg) as a marker. The column was eluted with 0.1M Tris acetate, pH 7.4, at a flow rate of 30 mL/h. Fractions (1 mL) were collected and analyzed for radioactivity and uronic acid content.



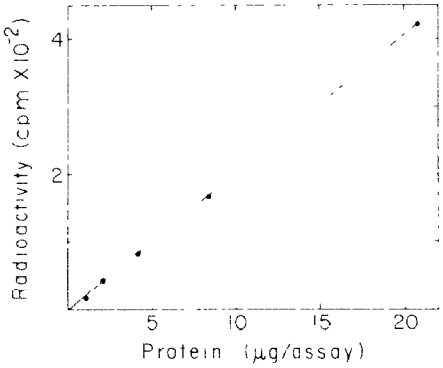


Fig. 6. Tritium release from ^{3}H-labeled, modified heparin as a function of time. Assay mixtures contained 3400 c.p.m. of substrate and 7 μg of protein.

Fig. 7. Tritium release from ^{3}H-labeled, modified heparin as a function of protein concentration. Assay mixtures contained 3000 c.p.m. of substrate and the indicated quantities of protein.

Tritium release from ^{3}H-labeled polysaccharides. — Incubation of the tritiated polysaccharides with 5-epimerase resulted in release of the radioactivity as tritium oxide, and the reaction was linear with time for at least 1 h (Fig. 6). All radioactivity was released on prolonged incubation with the enzyme, supporting our previous conclusion[24] that the label was located exclusively at C-5 of the glycosyluronate residues. Under standard assay conditions, the reaction was proportional to enzyme concentration up to at least 70 μg/mL of protein (Fig. 7). Apparent K_m values were 0.17 and 0.2mM, respectively, for the heparin- and heparan sulfate-derived substrates (Fig. 8), calculated with respect to the uronic acid concentration.

In conclusion, we describe herein a method for the preparation of specific [5-

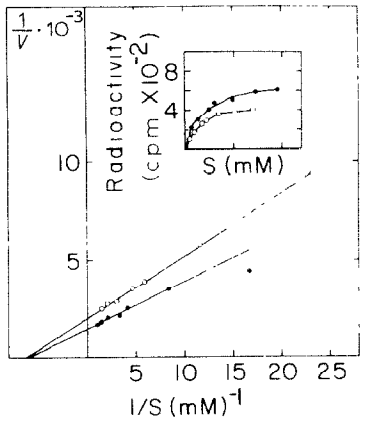


Fig. 8. Tritium release from ^{3}H-labeled polysaccharides as a function of substrate concentration (expressed as concentration of uronic acid). The standard assay was used, except that the concentration of the ^{3}H-labeled substrates was varied as indicated. (●—●) modified 0.4M heparan sulfate fraction, (○—○) modified heparin.

^{3}H]glucosyluronate-labeled substrates for heparosan-*N*-sulfate-D-glucopyranosyluronate 5-epimerase, which involves simple chemical modification of heparin or heparan sulfate, followed by incubation with crude liver-epimerase in the presence of tritiated water. Furthermore, the kinetic data demonstrate that a valid quantitative assay of epimerase activity may be carried out with these substrates under appropriately chosen conditions. The new method is much simpler than the previously published procedure[3] for preparation of a "biosynthetic substrate", and it is anticipated that the ready availability of substrates will facilitate studies of the properties of the epimerase.

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