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Biosynthesis of Heparin. Hydrogen Exchange at Carbon 5 of the Glycuronosyl Residues†

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ABSTRACT: O-Desulfated heparin is known to incorporate tritium into its glycosyluronic acid moieties when incubated in ^3H_2O with heparosan-*N*-sulfate D-glucuronosyl 5-epimerase. We have now established the location of the incorporated tritium as follows. L-[^{3}H]Idosan and a mixture of D-[^{3}H]-glucose and L-[^{3}H]idose were isolated from enzymatically tritiated O-desulfated heparin by esterification of the carboxyl groups with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide, reduction with sodium borohydride, acid hydrolysis, and deaminative cleavage. When the mixture of the two hexoses was converted to methyl [^{3}H]glycosides and subsequently degraded by successive periodate oxidation, hypobromite oxidation, and acid hydrolysis, the only ^{3}H-labeled fragment was glyceric acid. The same result was obtained upon degradation of the L-[^{3}H]idosan, indicating that in both the D-glucuronic acid and L-iduronic acid residues, the tritium was located at C-5. In another approach, D-[^{3}H]glucuronic acid was isolated from the ^{3}H-labeled polysaccharide and was then converted to methyl (methyl D-[^{3}H]glucopyranoside)uronate. On reduction with sodium borohydride in anhydrous methanol, this compound lost its radioactivity and yielded unlabeled methyl α-D-glucopyranoside. This finding indicates that the label was located at C-5, since it was previously shown that the hydrogen in this position is completely exchanged under the reaction conditions used and that hydrogen atoms in other positions are not affected. It is concluded that incubation of O-desulfated heparin in ^3H_2O with heparosan-*N*-sulfate D-glucuronosyl 5-epimerase introduces tritium at C-5 of the glycuronosyl moieties of the substrate and that no exchange of hydrogen atoms at C-2, C-3, or C-4 with protons of the medium or with the C-5 hydrogen occurs during the reaction.

Heparin biosynthesis is initiated by the assembly of monosaccharide units into a polymer composed of alternating D-glucuronosyl and 2-acetamido-2-deoxy-D-glucosyl moieties. This polysaccharide subsequently is modified by successive N-deacetylation, N-sulfation, C-5 D-glucuronosyl epimerization, and finally O-sulfation of most of the L-iduronosyl and 2-deoxy-2-sulfamido-D-glucosyl moieties. The epimerization, catalyzed by heparosan-*N*-sulfate D-glucuronosyl 5-epimerase, is accompanied by an exchange of the C-5 hydrogen atoms with protons of the aqueous medium, as indicated by the loss of radioactivity from heparosan *N*-sulfate specifically labeled with tritium in the C-5 position of the D-glucuronosyl moieties (Lindahl et al., 1976, 1977; Jacobsson et al., 1979). Some direct evidence is available for the reversibility of this process, inasmuch as the enzyme is known to catalyze incorporation of tritium from ^3H_2O into the uronosyl residues of heparan sulfate and a heparin derivative from which the *O*-sulfate groups have been removed (Jensen et al., 1979; I. Jacobsson, U. Lindahl, J. Jensen, L. Rodén, D. S. Feingold, and H. Prihar, unpublished results). However, the exact location of the incorporated tritium has not been established.

In the present investigation, a procedure has been developed for the determination of the position of incorporated tritium, in D-glucuronosyl as well as in L-iduronosyl units. Application of this methodology to enzymatically tritiated O-desulfated heparin shows that radioactivity is present in both D-glucuronosyl and L-iduronosyl groups and, furthermore, that the label is associated with the C-5 position only.

Materials and Methods

Materials. L-[6-^{14}C]Idose, a product of Centre d'études nucléaires de Saclay, France, was converted to L-[6-^{14}C]idosan by the method of Sorkin & Reichstein (1945). D-[U-^{14}C]-Glucose and D-[5-^{3}H]glucose were purchased from the Radiochemical Center, Amersham/Searle, Chicago, IL. Methyl α-D-[U-^{14}C]glucopyranoside and methyl α-D-[5-^{3}H]glucopyranoside were prepared from these compounds by the method of Fischer (1893, 1895). The position of the label in the

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tritiated glycoside was confirmed by periodate oxidation as described by Prihar et al. (1978). D-Glucuronic acid, D-glyceric acid, glyoxylic acid, and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide were obtained from Sigma Chemical Co., St. Louis, MO. L-Iduronic acid was isolated from a hydrolysate (2 M trifluoroacetic acid; 100 °C; 4 h) of pig skin dermatan sulfate by chromatography on Whatman No. 3MM paper in solvent A (see below). Methanol was dried by distillation from magnesium methoxide and stored over molecular sieves.

General Methods. Paper chromatography was performed on Whatman No. 1 paper in the following solvents: A, ethyl acetate–acetic acid–water (3:1:1); B, ethyl acetate–acetic acid–formic acid–water (18:3:1:4); C, ethyl acetate–acetic acid–water (2:1:1); D, 1-butanol–ethanol–water (4:1:5, upper phase). Electrophoresis was carried out on Whatman No. 1 paper in 0.2 M ammonium acetate, pH 5.8 (solvent E). Carbohydrates, glyceric acid, and glyoxylic acid were detected with alkaline silver nitrate (Trevelyan et al., 1950). Radioactive compounds on paper were located with a 4 π strip counter (Tracerlab, Waltham, MA) and eluted with water. Solutions were concentrated at 37 °C in vacuo, by using a rotary evaporator. Radioactivity was measured in a Mark III liquid scintillation system, Model 6880 (Searle Analytic Inc., Chicago, IL), in Aquasol (New England Nuclear, Boston, MA). $^3H/^{14}C$ ratios were determined by using a preset instrument program for two-channel analysis of unquenched ^3H and ^{14}C dual labeled samples. Uronic acid was quantitated by an automated carbazole method (Ford & Baker, 1978). Separation and quantitation of glucosamine and galactosamine in polysaccharide hydrolysates (6 M HCl; 105 °C; 7 h) were carried out as described by Ford & Baker (1978). Sulfate was determined by the method of Terho & Hartiala (1971). Protein was estimated by a fluorescamine method (Böhlen et al., 1973).

Preparation of O-Desulfated Heparin. Hog mucosa heparin (U.S.P. grade, Cohelfred Laboratories, Inc., Chicago, IL) was purified by precipitation with cetylpyridinium chloride from 1.4 M NaCl as described by Rodén et al. (1972). The product contained 51.2% uronic acid, 23.6% D-glucosamine, 0.7% D-galactosamine, and 26.4% sulfate. It was converted to the pyridinium salt and desulfated by heating in 90% dimethyl sulfoxide according to the procedure of Nagasawa et al. (1977). The product (3% residual sulfate) was deacetylated by treatment with anhydrous hydrazine (Dmitriev et al., 1973) for 4 h, and, after removal of the hydrazine by distillation, the desulfated, N-deacetylated heparin was recovered by repeated precipitation with ethanol from 2 M NaCl; finally, the polysaccharide was repeatedly precipitated from water solution until addition of sodium acetate was necessary to cause precipitation and was then washed with diethyl ether and dried in vacuo over P_2O_5. The desulfated polysaccharide was re-N-sulfated with sulfur trioxide–trimethylamine as described by Levy & Petracek (1962), and the reaction product was isolated by precipitation with cetylpyridinium chloride, followed by repeated precipitation with ethanol from 2 M NaCl, exhaustive dialysis against water, and lyophilization. Analysis of the product, O-desulfated heparin, showed the presence of 52.8% uronic acid, 23.2% D-glucosamine, 0.7% D-galactosamine, and 15.8% sulfate. The overall yield from the cetylpyridinium chloride purified heparin was 38% on a weight basis. Upon deaminative cleavage with nitrous acid at pH 1.5 (Shively & Conrad, 1976), the only products were disaccharides, indicating that most of the amino groups of the polymer were sulfated.

Preparation and Assay of Heparosan-N-Sulfate D-Glucuronosyl 5-Epimerase from Bovine Liver. The 5-epimerase was assayed as described by Jacobsson et al. (1979), except that tritiated O-desulfated heparin was used as the substrate. This material was prepared as described below [see also Jensen et al. (1979)] and had a specific activity of approximately 10^5 cpm/mg; each incubation mixture contained 32 μg of substrate ($\sim$3000 cpm).

Although the 5-epimerase has been extensively purified from bovine liver (Rodén et al., 1977), enzyme of lower purity was satisfactory for the purposes of the present investigation and was prepared by the following briefly described procedure. Bovine liver (1 kg) was homogenized in 2 L of 0.15 M KCl, and the soluble material was fractionated with ammonium sulfate. The fraction precipitating between 35 and 65% saturation was dissolved in 0.05 M 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid, pH 7.4, 0.05 M in KCl and 0.015 M in EDTA, and dialyzed exhaustively against the same buffer. The retentate (1500 mL, $\sim$100 g of protein) was applied to a column (5 × 50 cm) of heparin–Sepharose [prepared by coupling heparin to 1,6-diaminohexane–Sepharose (Shaltiel & Er-el, 1973) by using 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (Cuatrecasas, 1970)]. After the column was washed with the same buffer until no more protein emerged (as indicated by the absorbency at 280 nm), the enzyme was eluted with 0.05 M 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid, pH 7.4, 0.25 M in KCl and 0.015 M in EDTA. Active fractions were dialyzed against 0.05 M 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid, pH 7.4, 0.05 M in KCl and 0.015 M in EDTA, and were then concentrated in an Amicon filtration cell with a PM-10 membrane (Amicon Corp., Lexington, MA) to a protein concentration of 8 mg/mL. The epimerase activity of this preparation, expressed as release of tritium from the substrate, was 32 000 cpm/(mg of protein h). When held at 25 °C, the enzyme retained 70% of the original activity after 6 days in the presence of 0.02% sodium azide to inhibit bacterial growth. It was routinely stored frozen at −20 °C.

Preparation of Tritiated O-Desulfated Heparin. A reaction mixture containing 10 mL of heparosan-*N*-sulfate D-glucuronosyl 5-epimerase (8 mg of protein per mL), 160 mg of O-desulfated heparin in 2 mL of H_2O, 1 mL of ^3H_2O (1 Ci/g), and 0.13 mL of 2% sodium azide was incubated at 24 °C for 1 week. After being heated at 100 °C for 3 min, the mixture was cooled, and 20 mL of 0.3 M NaCl was added. Precipitated protein was dispersed with the aid of a Pasteur pipet, and the entire reaction mixture was applied to a column (4.5 × 40 cm) of Ag 1-X4 (chloride form; 200–400 mesh). The reaction vessel was washed with two 20-mL portions of 0.3 M NaCl, which were added to the column. After 4 L of 0.3 M NaCl was passed through the column at a rate of 60 mL/h, tritium-labeled polysaccharide was eluted with 1 L of 2 M NaCl, and the eluate was taken to dryness to remove remaining volatile radioactivity. The residue was dissolved in approximately 500 mL of water and dialyzed for a total of 48 h against two 40-L portions of distilled water, and the retentate was concentrated to a syrup, dissolved in a small volume of water, and lyophilized. Recovery was 83%, based on uronic acid content. The ratio of D-[^{3}H]glucuronic acid to L-[^{3}H]-iduronic acid, determined after degradation by the method of Höök et al. (1974), ranged between 1.4 and 1.6 for several preparations.

Degradation of ^3H-Labeled O-Desulfated Heparin. The carboxyl groups of tritium-labeled O-desulfated heparin were reduced by a modification of the procedure described by

Shively et al. (1976). To the labeled polysaccharide diluted with unlabeled O-desulfated heparin (8 mg; 100000 cpm) in 1 mL of water was added 20 mg of solid 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide, the pH was adjusted to 4.7 with 0.1 M hydrochloric acid, and the reaction mixture was maintained at this pH for 2 h at 20 °C. Solid sodium borohyride (70 mg) was added, and the mixture was held at 50 °C for 2 h. The mixture was then cooled to 0 °C, and excess borohydride was destroyed by acidification with 1.5 M sulfuric acid. Salts were removed by dialysis for 15 h at 4 °C against distilled water (2 × 500 mL), and the retentate was taken to dryness.

The carboxyl-reduced O-desulfated heparin was taken up in 500 μL of 1 M sulfuric acid and heated at 100 °C for 6 h in a tube closed with a Teflon-coated screw cap. The hydrolysate was cooled to 20 °C, 1.25 mL of 5.5 M sodium nitrite was added, and, after 10 min at 20 °C, the solution was loaded onto a column (2.2 × 15 cm) of Dowex 50 W-X8 (H^+ form; 20–50 mesh). The column was washed with 100 mL of distilled water, the combined effluent and washings were neutralized with solid barium carbonate, and, after filtration, the solution was concentrated to 20 mL. Some additonal precipitate was removed by a second filtration, and the clear filtrate was loaded onto a column (2.2 × 15 cm) of mixed-bed resin (Ag 501-X8, Bio-Rad Corp., Richmond, CA) which was then washed with 50 mL of distilled water. The pooled effluent and washing were concentrated to 200 μL and subjected to chromatography in solvent B. The only radioactive compounds present, which had chromatographic mobilities corresponding to those of D-glucose (or L-idose) and L-idosan, were eluted with water, and the solutions were evaporated to dryness. (Localization of L-idosan, which has a blocked reducing group and reacts only slowly with alkaline silver nitrate, was facilitated by the use of L-[6-^{14}C]idosan as a marker.)

Preparation of Methyl [^{3}H]Hexopyranosides from [^{3}H]-Hexoses. The radioactive component with the mobility of D-glucose isolated from the paper chromatogram was dried and quantitatively converted to methyl hexopyranoside by the method of Fischer (1893, 1895), using dry methanol and acetyl chloride. The reaction mixture contained a single radioactive component (methyl [^{3}H]hexopyranosides), with the chromatographic mobility of methyl α-D-glucopyranoside in solvent D. It was eluted from the paper with water and subjected to degradation with periodate.

Periodate Oxidation of Methyl [^{3}H]Hexopyranosides. The methyl hexopyranosides were degraded to glyoxylic acid, formic acid, and glyceric acid by a micromodification of the method reported by Bevill et al. (1965). Glyoxylic acid represents C-1 and C-2 in the glycuronosyl residues of the parent polysaccharide, formic acid originates from C-3, and the glyceric acid is derived from C-4, C-5, and C-6. Typically, a reaction mixture contained 1 mg of a mixture of methyl [^{3}H]hexopyranosides (47000 cpm) and methyl α-D-[U-^{14}C]-glucopyranoside (37000 cpm) and contained 5 mg of paraperiodic acid in a total volume of 200 μL. After 19 h at 20 °C in the dark, the reaction mixture was neutralized with 10% ammonium hydroxide and diluted with water to a total volume of 3.0 mL. A portion (0.1 mL) of the reaction mixture was distilled under reduced pressure in a closed system (Grant, 1946), and the ^{3}H/^{14}C ratio of the formic acid in the distillate was determined. Strontium carbonate (160 mg) and bromine (12 μL) were added to the remaining, undistilled reaction mixture, and the mixture was stirred for 30 min. It was then aerated for 30 min, salts were removed by filtration, and the clear filtrate was taken to dryness, dissolved in 1 mL of 1 M HCl, and heated at 100 °C for 2 h. The cooled solution was neutralized with solid silver carbonate and filtered. The filtrate was concentrated and treated with several small portions of Dowex 50 W-X8 (H^+ form; 20–50 mesh) to remove excess silver ions, and the components present were separated by chromatography in solvent C. Labeled glyceric acid was located by scanning the strip and eluted with water, and its ^{3}H/^{14}C ratio was determined.

Periodate Oxidation of L-[^{3}H]Idosan. To a mixture of L-[^{3}H]idosan (24300 cpm), isolated from the paper chromatograms, and L-[6-^{14}C]idosan (19000 cpm) was added paraperiodic acid (5 mg) in a final volume of 0.2 mL. After 56 h at 20 °C in the dark, the reaction mixture was neutralized with 10% ammonium hydroxide and diluted to 4.0 mL with water. Strontium carbonate (160 mg) and bromine (12 μL) were added, and the mixture was stirred for 30 min, aerated for 30 min, and filtered. The filtrate was concentrated to dryness, dissolved in 1 mL of 1 M hydrochloric acid, and held at 100 °C for 3 h. Following neutralization with solid silver carbonate, labeled glyceric acid was isolated as described, and its ^{3}H/^{14}C ratio was determined.

Isolation of Uronic Acids from ^{3}H-Labeled O-Desulfated Heparin. D-[^{3}H]Glucuronic acid was isolated from ^{3}H-labeled O-desulfated heparin as described by Höök et al. (1974). The O-desulfated heparin (~1 mg; 80000 cpm), dissolved in 1.0 mL of 2 M trifluoroacetic acid, was heated at 100 °C for 3 h in a tube closed with a Teflon-lined screw cap. The resulting solution was concentrated to dryness, and 200 μL of freshly prepared 3.9 M sodium nitrite in 0.28 M acetic acid was added. After 10 min at 22 °C, 2 mL of 1 M acetic acid was added, and the resulting solution was immediately loaded onto a column (1 × 6 cm) of Dowex 50 W-X8 (H^+ form; 20–50 mesh), equilibrated in 1 M acetic acid. The column was eluted with 50 mL of water, and the effluent was concentrated to 5 mL and subsequently repeatedly evaporated to dryness in the presence of methanol. The dry residue was taken up in 2 mL of 2 M trifluoroacetic acid, heated at 100 °C for 4 h, taken to dryness as described previously, and finally taken up in a small volume (50 μL) of water and subjected to paper chromatography in solvent A. The D-[^{3}H]glucuronic acid isolated by this procedure was converted to methyl α-D-glucopyranoside as described by Prihar et al. (1978).

Results

The position of tritium incorporated from ^3H_2O into O-desulfated heparin was investigated by two independent procedures, which are illustrated in Figures 1 and 2. In one approach, the polysaccharide was degraded as described by Höök et al. (1974), yielding radioactive D-glucuronic acid and L-iduronic acid and their lactones (Figure 1). The position of the label in the D-glucuronic acid was determined by taking advantage of the observation by Prihar et al. (1978) that reduction of methyl (methyl D-glucopyranoside)uronate with sodium borohydride in methanol results in loss of the C-5 hydrogen atom. The second approach was based on the procedure developed by Shively et al. (1976), in which the D-glucuronosyl and L-iduronosyl residues are carboxyl-reduced and, following hydrolysis and deaminative cleavage by nitrous acid, are obtained in high yield as D-glucose, L-idose, and L-idosan. The position of the label in the monosaccharides was determined by controlled oxidative degradation and hydrolysis, followed by separation and analysis of the fragments as shown in Figure 2. Details of these procedures are reported below.

Isolation and Conversion to Methyl α-D-Glucopyranoside of D-[^{3}H]Glucuronic Acid from O-Desulfated Heparin.

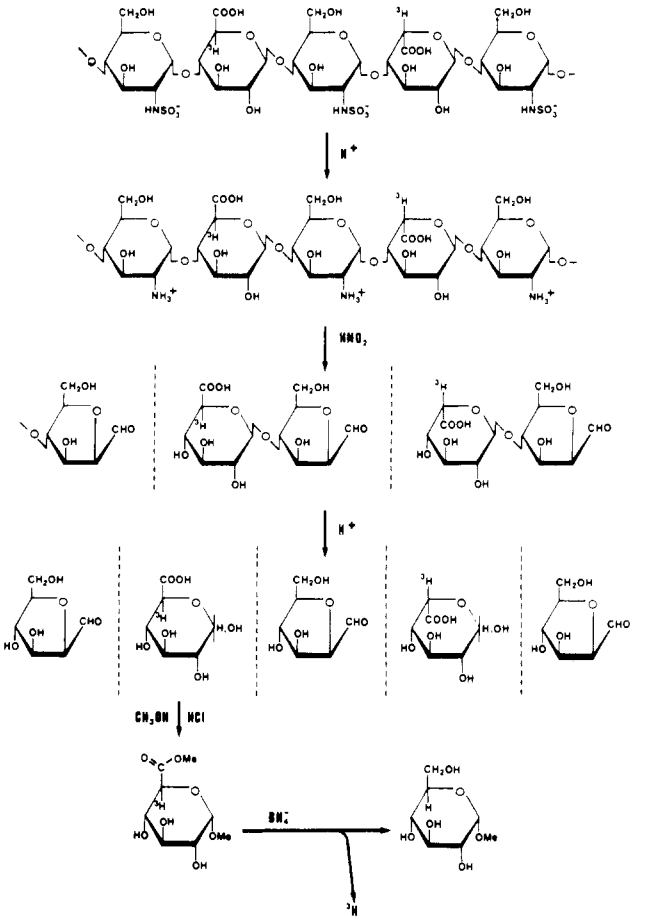

FIGURE 1: Degradation of tritiated O-desulfated heparin by the procedure of Höök et al. (1974).

Tritium-labeled O-desulfated heparin was degraded by the method of Höök et al. (1974), and the products were separated by paper chromatography in solvent A. In confirmation of findings described elsewhere (Jensen et al., 1979; I. Jacobsson, U. Lindahl, J. Jensen, L. Rodén, D. S. Feingold, and H. Prihar, unpublished results), both D-glucuronic and L-iduronic acid were labeled; 9% of the total radioactivity recovered was present in D-glucuronic acid, 20% was present in L-iduronic acid, 6% was present in a peak of unknown composition, 26% was present in D-glucuronolactone, and 3% was present in L-iduronolactone. A large radioactive peak near the origin of the chromatogram, presumably unhydrolyzed oligosaccharides, accounted for about 36% of the radioactivity recovered. (In four separate experiments, recovery was nearly complete up to the paper chromatography step; the recovery in this step ranged from 61 to 78%.)

Treatment of the D-[^{3}H]glucuronic acid (50000 cpm pooled from several preparations) with anhydrous methanol and acetyl chloride and subsequent reduction with sodium borohydride yielded unlabeled methyl α-D-glucopyranoside, demonstrated by paper chromatography in solvent D, strip scanning, and detection with alkaline silver nitrate. This shows that the D-glucuronosyl moiety of the O-desulfated heparin was labeled at C-5 only, since only the C-5 hydrogen atom is lost when D-glucuronic acid is converted to methyl α-D-glucopyranoside by the method described (Prihar et al., 1978).

Conversion of Tritium-Labeled, O-Desulfated Heparin to D-[^{3}*H*]*Glucose,* L-[^{3}*H*]*Idose, and* L-[^{3}*H*]*Idosan.* The carboxyl groups of O-desulfated heparin (8 mg; 100000 cpm) were converted to primary alcohol groups by using a scaled-up and slightly modified version of the procedure of Shively et al.

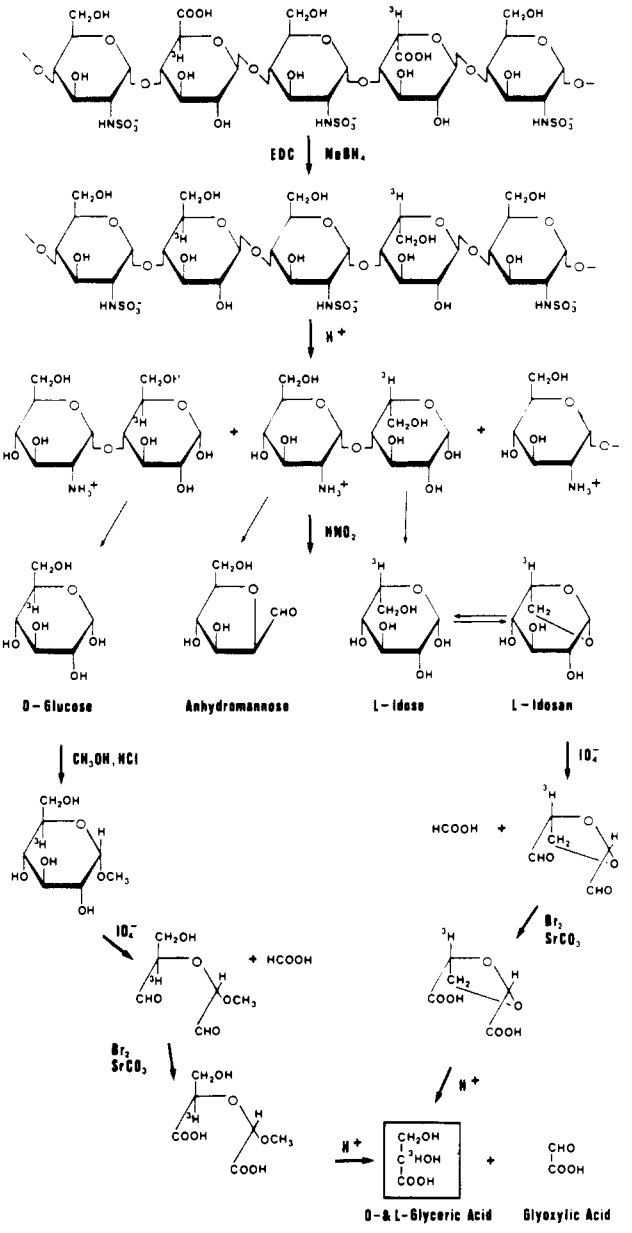

FIGURE 2: Degradation of tritiated O-desulfated heparin by the procedure of Shively et al. (1976).

(1976). During the reactions with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide and sodium borohydride, there was no loss of tritium from the polymer, as shown by absence of label in the dialysate of the reaction mixture and complete recovery of the initial radioactivity in the carboxyl-reduced O-desulfated heparin. Acid hydrolysis and deaminative depolymerization converted the carboxyl-reduced heparin to products migrating as D-glucose, L-idosan, and anhydromannose on paper chromatography in solvent B. No other compounds were detectable. The D-glucose area presumably contained also some L-idose, which is a primary product of the degradation but is largely converted to L-idosan in acid solution. No attempts were made to separate the presumptive L-idose component, since it was not necessary for the purposes of this investigation to analyze the two sugars separately. The anhydromannose was not radioactive, demonstrating that no incorporation of tritium had occurred into the glucosamine moieties of the polysaccharide. Over 80% of the radioactivity initially present in the carboxyl-reduced heparin could be accounted for by the ^{3}H label found in the hexose component

Table I: Degradation of L-Idosan and Methyl Hexopyranosides to Glyceric Acid

expt no.	compds degraded	ratio of ^3H to ^{14}C			% difference between obsd and calcd
		undegraded mixture	glyceric acid produced		
			obsd	calcd[a]	
1[b]	L-[^{3}H]idosan from tritiated O-desulfated heparin and L-[6-^{14}C]idosan	1.27	1.39	1.27	+9
2[c]	methyl [^{3}H]hexopyranosides from tritiated O-desulfated heparin and methyl α-D-[U-^{14}C]glucopyranoside	1.28	2.32	2.56	−8
3[d]	methyl α-D-[5-^{3}H]glucopyranoside and methyl α-D-[U-^{14}C]glucopyranoside	4.20	7.69	8.40	−9

[a] Calculated on the assumption that all tritium label is located at C-5. [b] Experiment 1: a mixture of L-[^{3}H]idosan (24 000 cpm), from enzymatically tritiated O-desulfated heparin, and L-[6-^{14}C]idosan (19 000 cpm; sp act. 45 mCi/mol) was treated successively with paraperiodic acid and hypobromite, followed by acid hydrolysis; [^{3}H,^{14}C]glyceric acid was isolated from the degradation products by paper electrophoresis and counted; details are given under Materials and Methods. [c] Experiment 2: a mixture of the methyl [^{3}H]hexopyranoside fraction (47 000 cpm), from enzymatically tritiated O-desulfated heparin, and methyl α-D-[U-^{14}C]glucopyranoside (37 000 cpm; sp act. 230 mCi/mmol) was degraded as described in experiment 1. [d] Experiment 3: methyl α-D-[5-^{3}H]glucopyranoside (84 000 cpm; sp act. 5 Ci/mmol) and methyl α-D-[U-^{14}C]glucopyranoside (20 000 cpm; sp act. 230 mCi/mmol) were mixed with unlabeled methyl α-D-glucopyranoside (1 mg), and the mixture was degraded as described in experiment 1.

and in L-idosan. The hexose/L-idosan ^{3}H ratio was 4:1. [This ratio is different from that determined for the starting material by the method of Höök et al. (1974), as will be discussed later.]

Periodate Degradation of L-[^{3}*H*]*Idosan and the* [^{3}*H*]*Hexose Fraction.* Degradation of the L-[^{3}H]idosan by periodate, as outlined in Figure 2, yielded radioactive glyceric acid, indicating that tritium label was present in the C-5 position of the parent L-iduronosyl residues. The extent of labeling in other positions was assessed by a double-labeling experiment, in which the L-[^{3}H]idosan was mixed with authentic L-[6-^{14}C]-idosan and the mixture was subjected to the same degradation procedure. As seen from Table I, experiment 1, the isolated glyceric acid had a ratio of ^{3}H to ^{14}C which did not differ significantly from that of the undergraded mixture. Therefore, the tritium label was located at C-5 only, since a lower ratio would otherwise have been observed.

Similar results were obtained on analysis of the [^{3}H]hexoses (D-glucose and L-idose). Glyceric acid (45 000 cpm) was the only labeled fragment produced on degradation of the [^{3}H]-hexose fraction (~1 mg; 50 000 cpm), and double-labeling experiments provided evidence that most, if not all, of the tritium was located at C-5. Initially, it appeared that a small proportion of the radioactivity was located elsewhere, since degradation of the methyl [^{3}H]hexopyranoside fraction in mixture with methyl α-D-[U-^{14}C]glucopyranoside yielded glyceric acid with a ^{3}H to ^{14}C ratio 8% lower than predicted (Table I, experiment 2). However, when authentic methyl α-D-[5-^{3}H]glucopyranoside was degraded together with methyl α-D-[U-^{14}C]glucopyranoside (Table I, experiment 3), the ratio of the product was 9% less than the theoretical value, in good agreement with the results observed in experiment 2. These findings suggest that a minor loss of tritium from the C-5 position occurred in the course of the degradation or, alternatively, that the labeling of the methyl α-D-[U-^{14}C]glucopyranoside was not uniform. In any event, the results clearly demonstrate that the [^{3}H]hexoses from tritiated, O-desulfated heparin contained no significant amount of tritium in positions other than C-5.

Discussion

The specific aim of the present investigation was to establish the location of tritium atoms incorporated from ^3H_2O into the glycuronosyl moieties of O-desulfated heparin by the catalytic activity of heparosan-*N*-sulfate D-glucuronosyl 5-epimerase. A major difficulty in accomplishing this aim was the lack of a convenient method for determining the position of the label in the polymer-bound uronic acid residues.

The elegant procedure described by Shively et al. (1976) made it possible to convert smoothly the glycuronosyl moieties of the parent polysaccharide to D-glucose, L-idose, and L-idosan, for which suitable degradation techniques are available.

It is significant that during the esterification reaction with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide and the reduction with sodium borohydride, there was no loss of tritium from the polysaccharide, as shown by absence of label in the dialysate of the reaction mixture. Since, as shown in this paper, the glycuronosyl groups were labeled exclusively at C-5, these results indicate that the C-5 hydrogen atom is not involved in the sodium borohydride reduction step under the conditions employed. This is in contrast to the complete loss of the C-5 tritium atom which occurs during the reduction of methyl (methyl D-[5-^{3}H]glucopyranoside)uronate with sodium borohydride in anhydrous methanol (Prihar et al., 1978).

A final sodium borohydride concentration of 2 M and a reaction temperature of 50 °C for 2 h represent minimal requirements in the reduction step (Shively et al., 1976). We found that lower concentrations of the reducing agent or lower reaction temperatures for shorter times result in incomplete reduction of the carboxyl groups. Treatment of the hydrolysate with sodium nitrite at pH 3.9 (Shively & Conrad, 1976) effected complete conversion of the products to a mixture of monosaccharides. D-Glucuronic acid residues were converted to D-glucose, and L-iduronic acid residues were converted to L-idose. Based on previous studies (Perlin & Sanderson, 1970; Shively, 1975; Shively et al., 1976) it may be assumed that more than 90% of the L-idose was present as L-idosan (see Figure 2).

The precision of the degradation method used to locate the label in the D-glucose and L-idose was enhanced by use of a double-label technique. The results obtained with the experimental sample and the control indicate that at most 10% of the tritium could have been located at positions other than C-5. (This represents a maximum figure, since no tritium was found to be associated with the formate, which originated from C-3 of the parent uronosyl moieties.) The position of the label in the L-iduronosyl residue was also shown by the degradation of L-idosan. These findings were further confirmed by the demonstration that D-glucuronic acid, isolated from the parent tritiated O-desulfated heparin by the procedure of Höök et al. (1974) (deaminative cleavage in combination with acid hydrolysis), was labeled at C-5 only. A significant difference was noted between the ratios of D-[^{3}H]glucuronic acid to L-[^{3}H]iduronic acid determined by the procedures of Shively et al. (1976) and Höök et al. (1974), respectively. Whereas

the latter procedure gave values ranging between 1.4 and 1.6, the [^{3}H]hexose to [^{3}H]idosan ratio was 4:1; even after correction for the L-[^{3}H]idose present in the [^{3}H]hexose fraction, estimated at no more than 10% of the amount of L-[^{3}H]idosan, the minimum ratio was calculated to be 3.5. This discrepancy is likely due to the difference in stability toward acid hydrolysis between D-glucuronosyl and L-iduronosyl residues. As a consequence of the greater stability of the D-glucuronidic linkages, the yield of free D-glucuronic acid will be less than that of L-iduronic acid in the procedure of Höök et al. (1974), whereas results which reflect more accurately the proportion of the two uronic acids in the native polymer will be obtained by the method of Shively et al. (1976). The finding that a higher proportion of the tritium label was present in D-glucuronosyl than in L-iduronosyl residues assumes greater significance when we consider that the former account for 20% or less of the total uronic acid in the polysaccharide. Apparently, the specific activity of the D-glucuronosyl residues in the reaction product may be as much as 15-fold higher than that of the L-iduronosyl residues.

The evidence presented shows that incubation of O-desulfated heparin with ^3H_2O in the presence of heparosan-*N*-sulfate D-glucuronosyl 5-epimerase introduces a tritium atom at C-5 of the glycuronosyl moieties of the polymer, concomitant with the 5-epimerization of at least some of these moieties. Although 5-epimerization was not demonstrated in the tritium-incorporation experiments described in this paper, it can be assumed to have occurred on the basis of similar experiments with a biosynthetic substrate (Jacobsson et al., 1979). During preparation of tritiated O-desulfated heparin, reaction conditions were such that exchange and presumably also 5-epimerization reached equilibrium; however, the only carbon atom labeled was C-5. Therefore, no exchange of hydrogen atoms at C-2, C-3, or C-4 with protons of the medium, or with the C-5 hydrogen of the same glycuronosyl moiety, occurs during the epimerization, for had such an exchange taken place, label would have been found at carbon atoms other than C-5.

Labeling exclusively at C-5 is consistent with preliminary observations using a more complex biosynthetic system. In those experiments heparin precursor polysaccharides composed of alternating D-glucuronosyl and 2-deoxy-2-sulfamido-D-glucosyl moieties, in which the D-glucuronosyl moieties were labeled with tritium at C-2, C-3, C-4, or C-5, were used as substrates with a microsomal epimerase preparation from a mouse mastocytoma. Exchange of tritium with protons of the medium was observed only with the C-5-labeled substrate (Jacobsson et al., 1979).

The present results as well as earlier observations are in accord with an epimerization mechanism which involves only C-5 of the glucuronosyl moiety and rule out mechanisms by which label would be incorporated at other carbon atoms. Two such plausible mechanisms, now excluded, are (a) formation of a hexos-4-eenuronosyl intermediate by reversible dehydrogenation at C-4 and C-5 or (b) formation of a hexos-3-eenurosyl intermediate by reversible dehydration at C-3 and C-4. It is likely that the initial step in the epimerization reaction is abstraction of a proton from C-5, possibly with the aid of a nucleophilic group on the enzyme, resulting in formation of a carbanion at C-5. Readdition of a proton from the medium to this carbanion in either the D-glucuronosyl or L-iduronosyl configuration completes the reaction.

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