Heparosan N-sulfate D-glucuronosyl 5-epimerase, which catalyzes the conversion of β-D-glucuronosyl to α-L-iduronosyl residues in the course of heparin biosynthesis, has been purified approximately 9000-fold from the high speed supernatant fraction of a homogenate of a mouse mastocytoma. Following ammonium sulfate fractionation, the material precipitating between 35 and 60% saturation was subjected to a series of affinity chromatography steps on matrices containing immobilized concanavalin A, heparan sulfate, O-desulfated heparin, and Cibacron blue, respectively. Epimerase purified by this procedure yielded two major components on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The purified enzyme had approximately the same $K_v$ as bovine serum albumin when chromatographed on Sepharose 6B.

The activity of the purified enzyme was increased 50-fold by addition of the fraction which was not adsorbed to concanavalin A-Sepharose. The stimulating factor is likely to be a protein since it was nondialyzable, heat labile, and lost activity on digestion with trypsin.

In a previous report (1), an assay was described for the glucuronosyl 5-epimerase which catalyzes conversion of polysaccharide-bound glucuronic acid to iduronic acid groups in the course of heparin biosynthesis. Some properties of the epimerase including its substrate specificity were also determined, with the microsome fraction from a homogenate of the Furth mastocytoma as a source of the enzyme. The present paper describes the purification of the epimerase from the soluble fraction of the tissue homogenate. Some additional properties of the enzyme are also reported, particularly the requirement for a protein factor which is necessary for full activity of the purified enzyme.

* This work was supported by grants from the Swedish Medical Research Council, B79-13X-00139-15C; Konung Gustaf Va 80-års fond, The Medical Faculty, University of Lund, and National Institutes of Health Grants AM 18190, DE 02670, and HL 11310. This is Paper VIII of a series in which the preceding reports are Refs 1 and 2. A preliminary report has appeared (3). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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EXPERIMENTAL PROCEDURES

Materials

Bovine serum albumin, ovalbumin, transferrin, myoglobin, catalase, cytochrome c, ribonuclease A, and 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Hepes) were purchased from Sigma. Trypsin, treated with L-1-lysylamido-2-phenyl)ethyl chloromethyl ketone, and soybean trypsin inhibitor were obtained from Worthington. Twice recrystallized papain was prepared according to Kimmed and Smith (4). Glucosamine and galactosamine were products of Pfannstiel. Fluorochrome was obtained from Hoffman-La Roche. Con A-Sepharose, Sepharose 4B and 6B, and Sephadex G-200 were purchased from Pharmacia Fine Chemicals. Cibacron blue F3G-A was obtained from Polysciences. All other chemicals were of reagent grade and obtained from commercial sources.

Heparin (U.S. 155 units/mg) was obtained from Inoklin, Pharmaceutical Division, Park Forest South, Ill. After desulfation, a portion of the product was N-acetylated, and another portion was deacetylated and re-N-sulfated. These procedures have been described previously (1).

Heparan sulfate from heparin by-products (Fraction 1.25 to 2 in Table VIII, Ref. 5) was a gift from Dr. J. A. Cifonelli, University of Chicago. This material was digested with papain in 0.01 M phosphate buffer, pH 7.4, containing 0.005 M EDTA, 0.005 M cycysteine hydrochloride, and 0.5 M NaCl. After digestion, the polysaccharide was recovered by precipitation with cetylpyridinium chloride and converted to the sodium salt (5).

Mice of the strain LAFI/J0305 were obtained from Jackson Laboratories, Bar Harbor, Maine.

Coupling of Heparan Sulfate, O-Desulfated Heparin, and Cibacron Blue F3G-A to Sepharose (Sephadex)

The purified heparan sulfate was coupled to Sepharose by the following procedure (6). Solid cyanogen bromide (1.8 g) was added to 50 ml of an aqueous solution of the polysaccharide (200 mg), the solution was cooled in ice, and 50 ml of Sepharose 4B, well rinsed in distilled water, was added all at once. The slurry was stirred in an ice bath, and the pH was raised to 11 with 5 M NaOH and maintained at this level for 10 min. The gel was then shaken gently at 4°C for 24 h, rinsed with 0.1 M ethanolamine, pH 8.5, and kept in this solution for 2 h to block remaining active sites. Finally, it was rinsed successively with 0.5 M NaHCO₃, water, 0.1 M acetate buffer, pH 5.0, 2 M NaCl, and water.

O-Desulfated heparin (200 mg), prepared from heparin by desulfation, deacetylation, and subsequent re-N-sulfation, was dissolved in 25 ml of water, the pH was adjusted to 4.5, and the solution was added to 50 ml of 1,6-diaminohexane-substituted Sepharose 4B (7), which had been extensively washed with water at pH 4.5. 0.5 g of 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride was dissolved

The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; SDS, sodium dodecyl sulfate.
solved in 50 ml of water, the pH was adjusted to 4.5, and the solution was combined with the slurry under stirring. The pH of the mixture was readjusted every 15 min during a 2-h period. After 16 h, the gel was rinsed with 2 M NaCl, followed by water at pH 4.5. The amount of polysaccharide to the gel was estimated by hexosamine analysis on a Bio-Rad 200 automatic analyser. Packed gel (0.2 ml) was hydrolyzed in 3 ml of suprapure HCl under argon at 100°C for 3 h. The samples were evaporated, dissolved in 0.8 ml of starting buffer (0.2 M citrate, pH 2.2), and, after filtration, 0.5 ml was taken for analysis as described. The amounts of bound glucosamine were 0.8 and 1.9 pmol/mg for the gels containing heparan sulfate and O-desulfated heparin, respectively.

Cibacron blue F3G-A was coupled to Sephadex G-200 according to Böhm et al. (9).

**Analytical Methods**

Protein was estimated by fluorometric analysis essentially as described by Böhlen et al. (10), with bovine serum albumin as standard. To 250 μl of sample was added 50 μl of 0.6 M sodium borate, pH 8.5. During mixing on a Vortex mixer, 100 μl of fluorescamine reagent (0.3 mg of fluorescamine/ml of acetone) was added. Finally, 1 ml of 0.2 M sodium borate was added, and the relative fluorescence was measured with an Aminco spectrofluorimeter, model SPF 125. The excitation and emission wavelengths were 390 and 475 nm, respectively. When the amount of protein was limited, the volumes of sample and reagents were proportionately reduced to yield a final volume of 30 μl.

Polyacrylamide gel electrophoresis in SDS was carried out in standard gels (11.1 × 0.9) as described by Neville (11). The dimensions of the gels were 0.5 × 7 cm, and the lower buffer was 0.030 M HCl. 0.4244 M Tris, pH 9.16. Before electrophoresis, enzyme solutions were dialyzed against 0.01% SDS in dialysis bags (6000 to 8000 molecular weight cut-off), which had been boiled in 0.5% EDTA, 1% NaHCO₃. The dialyzed samples were lyophilized and then dissolved in 50 μl of 0.05 M Hepes, 0.05 M KCl, pH 7.4. All incubation mixtures contained 0.05 M Hepes, 0.05 M KCl, pH 7.4. Additions of salts were made to give the final concentrations indicated in the table.

**Epimerase Assay**

Substrate—Radioactively labeled microsomal heparin precursor polysaccharides were prepared by incubating microsomal enzyme with UDP-[5-3H]glucuronic acid, UDP-N-acetylglucosamine, and 3'-phosphoadenylyl sulfate as described (1). The product was used as substrate without fractionation by ion exchange chromatography and contained the four polysaccharide species, PS-N-acetylglucosamine, PS-N-sulfate, PS-N/Gln-SO₄, and PS-N/H₂SO₄ (see Fig. 1 in Ref. 1).

**Reaction Conditions**—Reaction mixtures contained, in a final volume of 300 μl, 1100 to 1500 cpm of substrate in 25 μl of water and 275 μl of enzyme in 0.05 M Hepes, 0.05 M KCl, 0.015 M EDTA, pH 7.4 (Buffer A). After incubation for 1 h at 37°C, the reaction was terminated by heating at 100°C for 2 min, and the samples were distilled as described (1). Aliquots (200 μl) of the distillates were mixed with 10 ml of Scintiverse (Fisher Scientific), and radioactivity was measured in a Packard scintillation counter, model 2450. Samples were counted for 20 min each.

**Enzyme Purification**

The source of 5-epimerase was the Furth mast cell tumor, which was propagated in LAFl/J0305 mice by the following procedure. Mast cells were prepared by trypsinization of the tumor, and the cells were stored in Hanks' medium under liquid nitrogen until used (12). Before incubation, the cells were thawed in a 37°C bath and diluted with Krebs-Ringer buffer to a final cell density of 8 × 10⁶/ml. One-hundred microliters of the suspension was inoculated into each hind leg of the mice, and tumors were allowed to develop for 17 days, at which time the animals were sacrificed by cervical dislocation and the tumors were removed, cooled on ice, and dissected free of muscular tissue. Purification of the epimerase was carried out at 4°C in the following manner and is summarized in Table I.

**Step 1: Homogenization and Centrifugation**—The tumors were minced and homogenized in a Potter-Elvehjem homogenizer in an equal volume of 0.1 M Tris, 0.15 M potassium phosphate, 0.006 M MgCl₂, 0.0025 M cysteine hydrochloride, pH 7.3. The homogenate was centrifuged at 20,000 × g for 10 min, and the supernatant liquid, which contained at least 50% of the total enzymatic activity, was collected and recentrifuged for 70 min at 70,000 × g. The high speed supernatant was stored at −20°C until sufficient material had been produced. Additional soluble enzyme could be obtained by re-extraction of the 20,000 × g pellet.

**Step 2: Ammonium Sulfate Fractionation**—This step achieved only 1.5-fold purification. However, its omission interfered with subsequent purification and yielded less pure final product; therefore, it was routinely included. To 700 ml of the supernatant liquid, solid ammonium sulfate was added under slow stirring to 35% saturation. Stirring was continued for 15 min after the salt had dissolved, and after another 15 min, the precipitate was collected by centrifugation at 35,000 × g for 1 h. The ammonium sulfate concentration was then brought to 80% saturation, and the precipitate was collected as before and was dissolved in 140 ml of 0.05 M Hepes, 0.05 M KCl, pH 7.4 (Buffer B).

**Step 3: Chromatography on Concanavalin A-Sepharose**—In developing a procedure for the purification of heparosan N-sulfate glucuronyl 5-epimerase, the ability of several potential affinity ligands to inhibit tritium release in the standard assay was first examined (Fig. 1). On the basis of this study, Sepharose-coupled concanavalin A was selected for the purification of the epimerase.

**TABLE I**

<table>
<thead>
<tr>
<th>Salt added</th>
<th>Final concentration of added salt</th>
<th>Tritium liberated</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>EDTA</td>
<td>15 cpm</td>
<td>120</td>
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<tr>
<td>KCl</td>
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<tr>
<td>MnCl₂</td>
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<td>9</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>20</td>
<td>129</td>
</tr>
<tr>
<td>NaH₃PO₄</td>
<td>20</td>
<td>116</td>
</tr>
</tbody>
</table>

**Fig. 1. Effect of potential affinity ligands on epimerase activity**

The enzyme from Step 1 was incubated with tritium-labeled substrate in the presence of desulfated, acetylated heparin (△—△), heparosan sulfate (□—□), O-desulfated heparin (■—■), concanavalin A (Δ—Δ), and Cibacron blue F3G-A (X—X).
inhibitory compounds, all of which retained epimerase activity, although to different extents, were selected for use in the purification procedure.

The 35 to 60% ammonium sulfate fraction (140 ml) was diluted with 760 ml of Buffer B and applied to a column of Con A-Sepharose (2.1 x 15 cm), which had been equilibrated with the same buffer. Fractions of 22 ml were collected at a rate of 40 ml/h. The gel was subsequently washed with eluted volumes of 0.05 M Heps, 0.25 M KCl, pH 7.4, and the enzyme was eluted at a rate of 20 ml/h with 250 ml of 0.05 M Heps, 0.25 M KCl, 0.5 M methyl α-D-mannoside, pH 7.4 (Fig. 2).

Since the loss of activity in Step 3 was 79%, the possibility was considered that some factor required for full activity had been removed. When a reaction mixture containing Con A-Sepharose-purified enzyme was supplemented with nonadsorbed protein, the activity of the enzyme increased 3- to 4-fold. Calculated on the basis of this stimulated activity, the recovery in Step 3 was then 83%, with an overall recovery of 51%. Accordingly, enzyme activity was routinely assayed with and without "factor" for each step of the purification (Table I).

**Step 4: Chromatography on Heparan Sulfate-Sepharose—**The eluate from the preceding step was diluted with 1000 ml of 0.05 M Heps, 0.015 M EDTA, pH 7.4, and applied to a heparan sulfate-Sepharose column (2.1 x 16 cm), which had been equilibrated with Buffer A. After the matrix had been washed with Buffer A to remove nonadsorbed proteins, the epimerase was eluted with a linear salt gradient (mixing vessel, 250 ml of Buffer A; reservoir, 250 ml of 0.05 M Heps, 0.25 M KCl, 0.015 M EDTA, pH 7.4). Fractions of 20 ml were collected at a rate of 40 ml/h and assayed for epimerase, protein, and conductivity (Fig. 3).

**Step 5: Chromatography on Sepharose Coupled to O-Desulfated Heparin—**The pooled active fractions (182 ml) eluted from the heparan sulfate-Sepharose column were diluted with 437 ml of 0.05 M Heps, 0.015 M EDTA, pH 7.4 (Buffer C). This sample was applied to a column (1.5 x 15 cm) of Sepharose linked to O-desulfated heparin. After washing with Buffer A, the epimerase was eluted with a linear salt gradient as in Step 4, except that the volume in each vessel was 150 ml. Fractions of 19 ml were collected at a rate of 15 ml/h and assayed for epimerase activity.

**Step 6: Chromatography on Cibacron Blue-Sephadex—**Epimerase-containing fractions from Step 5 (132 ml) were diluted with Buffer C (225 ml) and applied to a column (2.5 x 6 cm) of Cibacron blue-Sephadex which had been equilibrated in Buffer A. After rinsing with 4 bed volumes of Buffer A, the enzyme was eluted with a linear salt gradient (mixing vessel, 200 ml of Buffer A; reservoir, 200 ml of 0.05 M Heps, 0.5 M KCl, 0.015 M EDTA, pH 7.4). Fractions of 18 ml were collected at a rate of 15 ml/h. The fractions which contained epimerase activity (9 to 17) were pooled.

**RESULTS AND DISCUSSION**

**Some Properties of Crude Epimerase—**Prior to purification, some basic properties of the epimerase were determined, as observed in the standard assay system with the 97,000 x g supernatant fraction as enzyme source. The release of tritium was linear with time for slightly more than 1 h (Fig. 4), and the reaction rate increased with increasing enzyme concentration to a plateau value at approximately 1 mg of protein/ml (Fig. 5). The enzyme was active over a narrow pH range, with an optimum at pH 7.4 (Fig. 6). Particularly important from a practical point of view was the marked dependence on ionic strength. As seen in Table I, the enzyme had negligible activity in 0.05 M Heps, 0.05 M KCl, pH 7.4, but addition of EDTA to a final concentration of 0.015 M (standard buffer) yielded maximal tritium release. Similar results were obtained when the KCl concentration was increased to 0.1 M or upon addition of other salts (Na_2SO_4, Na_2HPO_4, and MgCl_2) at appropriately chosen concentrations. Only moderate stimulation was observed with CaCl_2 and MnCl_2 was comparatively ineffective. In agreement with previous studies of the microsomal epimerase (1), it is concluded that a specific metal ion requirement does not exist. However, exact control of ionic strength is necessary for reproducible assay of epimerase activity since the enzyme is active only over a narrow ionic strength range. The failure of manganous ion to stimulate activity is not presently understood, but this observation should be contrasted with the finding that the uronosyl 5-epimerase involved in dermatan sulfate biosynthesis requires manganous ion for full activity (13).

A $K_a$ of 5.5 x 10$^{-9}$ M was determined for the substrate used in this study. This value is subject to upward revision since the amount of endogenous, unlabeled substrate in the prepa-
Biosynthesis of Heparin

FIG. 4 (left). Time course of tritium liberation. Seventy micrograms of protein from the 97,000 × g supernatant fraction was incubated with substrate (2 × 10⁶ cpm) for the indicated periods of time.

FIG. 5 (left center). Effect of concentration of enzyme (Step 1) on tritium release.

FIG. 6 (right center). Effect of pH on epimerase activity. Enzyme from Step 1 was diluted 200-fold with 0.05 M Hepes, 0.05 M KCl, 0.015 M EDTA of the appropriate pH.

Properties of Purified Epimerase—As seen from Table II, the most highly purified preparation of heparosan N-sulfate D-glucuronosyl 5-epimerase was obtained in an overall yield of 25% and purified approximately 8700-fold to a specific activity of 67 × 10⁶ cpm/mg of protein. Enzyme of similar specific activity has been obtained in three separate preparations.

On SDS-polyacrylamide gel electrophoresis, enzyme from Step 6 gave a broad band (possibly representing two overlapping components) with a migration rate corresponding to a molecular weight of 54,000. In the presence of 2-mercaptoethanol, two major components were observed which migrated at rates corresponding to molecular weights of 52,000 and 56,000, respectively (Fig. 7). Several extremely faint bands were also visible; one of these was observed in a blank run in which dialyzed buffer was analyzed by the same procedure. It has not been possible to establish whether one or both of the two major components are identical with the enzyme since no enzymatic activity could be detected in dialyzed extracts of the gel. No activity was detectable in the gel after electrophoresis in the absence of SDS.

On analysis of the purified enzyme (Step 5 or Step 6) by gel chromatography on Sepharose 6B (for details, see Ref. 14), the activity emerged as a symmetrical peak slightly behind bovine serum albumin. This elution position is consistent with the molecular weights of 52,000 and 56,000 calculated for the major components observed on SDS-polyacrylamide gel electrophoresis. It cannot be presently ruled out, however, that the fully active enzyme consists of smaller subunits which...
Biosynthesis of Heparin

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Enzyme (µg protein per assay)

Radioactivity (cpm)

100
50

0.025
0.050
0.075

Concentration of exogenous protein (µg per assay)

100
200

50

An interesting discrepancy should be pointed out which concerns the molecular weight of the epimerase at various stages of purification. Whereas the highly purified enzyme was eluted near bovine serum albumin (molecular weight, 68,000), crude enzyme (Step 2) gave a single peak of activity which emerged shortly after the void volume and well ahead of xylosyltransferase from embryonic chick cartilage (molecular weight, 95,000 to 100,000) when analyzed by chromatography on Sephadex G-200 (for details, see Ref. 14). This suggests that the epimerase may associate with other proteins present in the early stages of purification.

Properties of the Stimulating Factor—By recombining enzyme fractions with the protein fraction not adsorbed to Con A-Sepharose in Step 3, a 3- to 5-fold stimulation of epimerase activity was obtained. The degree of stimulation was related to the purity of the enzyme and was as high as 50- to 69-fold for the most active preparations (Table II). At low concentrations of highly purified enzyme, little or no activity was observed in the absence of factor (Figs. 8 and 9).

The nature of the stimulating effect has not yet been determined, nor is the identity of the factor known. However, the activator is likely to be a protein since it was nondialyzable and was destroyed by heating at 60°C for 30 min (Fig. 10) or by digestion with trypsin (Table III). In contrast to the

![Graph](image_url)

**Fig. 8.** Effect on epimerase activity of the addition of increasing amounts of extraneous proteins to a standard incubation mixture. Factor preparation (solid line), cytochrome c (dashed line), catalase (Δ-Δ), ribonuclease A (○-○), and bovine serum albumin (A-A) were added to a standard incubation mixture containing enzyme obtained in Step 6. The factor preparation consisted of protein not adsorbed to Con A-Sepharose in Step 3.

![Graph](image_url)

**Fig. 9.** Relationship between enzyme concentration and activity in the absence (○-○) of added protein and in the presence of 100 µg of factor (○-○) or 500 µg of bovine serum albumin (△-△). Enzyme was obtained from Step 5, and the factor preparation consisted of protein not adsorbed to Con A-Sepharose in Step 3. Similar results were obtained when ovalbumin and transferrin were substituted for bovine serum albumin.

would migrate to the positions of the trace components found on SDS-polyacrylamide gel electrophoresis.

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![Graph](image_url)

**Fig. 10.** Heat inactivation of crude factor. Protein not adsorbed to Con A-Sepharose in Step 3 was kept in a bath at 80°C until the temperature of the solution reached 60°C. It was then transferred to a water bath at 60°C, and at the indicated times, a sample was added to a standard incubation mixture containing enzyme obtained in Step 6. The zero time value was obtained with untreated factor, and the second value was obtained with factor that was removed when the temperature had reached 60°C.

**Table III**

Effect of trypsin digestion on factor activity

<table>
<thead>
<tr>
<th>Addition</th>
<th>Activity (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>19</td>
</tr>
<tr>
<td>Factor, 100 µg</td>
<td>93</td>
</tr>
<tr>
<td>Digested factor, 100 µg</td>
<td>39</td>
</tr>
<tr>
<td>Trypsin-soybean trypsin inhibitor complex, 100 µg</td>
<td>43</td>
</tr>
</tbody>
</table>

*Incubation of the factor preparation for 2 h at 37°C did not diminish stimulating activity. Trypsin-soybean trypsin inhibitor complex did not increase the stimulating activity of the factor preparation.*
epimerase, the factor did not lose activity on exposure to 4 M guanidinium chloride (followed by dialysis against Buffer A).

Since the stimulation might be nonspecific, a number of proteins were tested for their effect on purified epimerase from Step 6. As seen in Fig. 8, ribonuclease A, cytochrome c, and catalase were capable of stimulating epimerase activity to the same extent as the unknown factor, but only at much higher concentrations. Other proteins, such as bovine serum albumin (Figs. 8 and 9), ovalbumin, and transferrin (data not shown), had only slight stimulatory effect. From a qualitative point of view, these experiments would seem to support a nonspecific mechanism of stimulation caused by certain active structures present in some proteins but not in others. On the other hand, the high potency of the crude factor preparation, which consists of a multitude of proteins, rather suggests that a single compound with a specific function is responsible for the observed effect. However, attempts to isolate the active principle by gel chromatography and ion exchange chromatography have so far been unsuccessful.

Some kinetic aspects of the stimulation by factor deserve further comment. As shown in Fig. 9, a plot of epimerase activity versus enzyme concentration was not linear at low concentrations, but addition of factor resulted in a linear relationship. This was not due to protection of the enzyme by factor since linearity with time was observed even at the lowest enzyme concentrations. It should also be noted that the stimulatory effect was highest at low concentrations of enzyme. In the absence of knowledge of the mechanism of epimerase action, these observations cannot be properly interpreted at this time.

REFERENCES