BIOSYNTHESIS OF HEPARIN. LOSS OF C-5 HYDROGEN DURING CONVERSION OF D-GLUCURONIC TO L-IDURONIC ACID RESIDUES

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SUMMARY: Microsomal fraction from mouse mastocytoma was incubated with UDP-N-acetylglucosamine and ^{3}H-, ^{14}C-labeled UDP-glucuronic acid. Enzymatic sulfation of the resulting heparin-precursor polysaccharide (by addition of 3'-phosphoadenylylsulfate) was accompanied by selective loss of ^{3}H from C5, but not from C2 or C4, of the uronic acid residues. Analysis of the sulfated product showed C5-^{3}H in the glucuronic acid but not in the iduronic acid component. The formation of L-iduronic acid residues by C5-epimerization of D-glucuronic acid units at the polymer level thus involves loss of hydrogen at C5.

The biosynthesis of heparin involves a number of reactions, initiated by the assembly of monosaccharide units into a nonsulfated polymer consisting of alternating GlcUA[1)] and GlcNAc residues (1,2). This polysaccharide is modified by a series of reactions to yield the final product, which contains N- and O-sulfate groups, and IdUA. The latter accounts for more than half of the total uronic acid (3). The modification reactions include N-deacetylation, N-sulfation and O-sulfation, and, in addition, uronic acid C5 epimerization, by which previously incorporated GlcUA residues are converted to IdUA units (4). The formation of IdUA is promoted by sulfation

1) Abbreviations; GlcUA, D-glucuronic acid; IdUA, L-iduronic acid; GlcNAc, 2-acetamido-2-deoxy-D-glucose; N-sulfate groups, sulfamino groups; O-sulfate groups, ester sulfate groups; PAPS, 3'-phosphoadenylylsulfate; HEPES, 2-(4-(2-hydroxyethyl)-1-piperazinyl)-ethane-sulfonic acid.

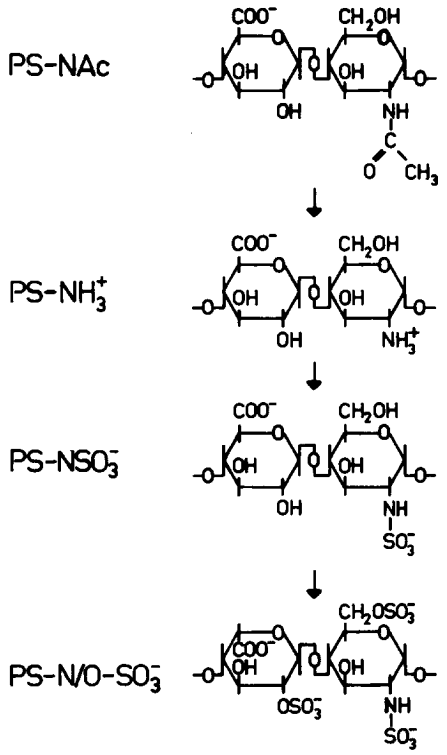


Fig. 1. Representative disaccharide units of microsomal polymeric intermediates formed during biosynthesis of heparin. The order of the various modification steps is indicated by arrows. The intermediates (which will be designated in the text as indicated in the figure) are separated into distinct peaks by ion-exchange chromatography (Fig. 3), and thus occur as discrete molecular species. With the exception of PS-NAc they have, however, structural features additional to those shown in the figure, such as the presence of GlcNAc residues in all fractions (3), and of small amounts of IdUA in PS-NSO_3^- (unpublished results). The structural representation of the markedly heterogeneous PS-N/O-SO_3^- is particularly oversimplified. The disaccharide shown is typical of the heparin end-product; however, in addition, PS-N/O-SO_3^- includes partially O-sulfated species, largely composed of nonsulfated or mono-O-sulfated IdUA- or GlcUA-containing disaccharide units (Ref. 3 and unpublished results).

of the polysaccharide (4,5). The polymer-modification process occurs in a stepwise manner, yielding intermediate components which are separable by ion-exchange chromatography. Characteristic structural features of such intermediates, isolated from a mouse mastocytoma microsomal fraction (3), are shown in Fig. 1.

To date little information has been available concerning the mechanisms

of the polymer-modification reactions. In this communication we show that formation by C5 epimerization of the IdUA units in heparin involves loss of hydrogen from C5 of the GlcUA residues.

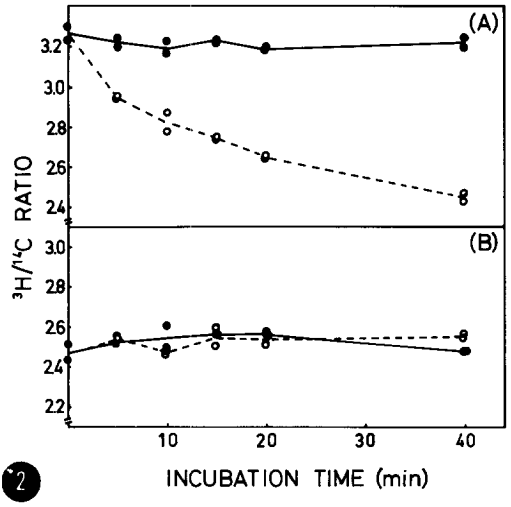
MATERIALS: The preparation of a microsomal fraction from transplantable mouse mastocytoma has been described previously, as were the glycosaminoglycans used as carrier or as reference standards (2). IdUA was prepared from crude dermatan sulfate by acid hydrolysis followed by preparative paper chromatography (5). PAPS was prepared using enzymes from high-speed supernatants of either rat liver (6) or mastocytoma tissue (7). ^{3}H-labeled D-glucose and UDP-[^{14}C]GlcUA (uniformly labeled; 278 μCi/μmole) were purchased from the Radiochemical Centre, Amersham, U.K. Unlabeled UDP-GlcUA and UDP-GlcNAc were obtained from Sigma Chemical Co., St. Louis, Mo. UDP-GlcUA, specifically ^{3}H-labeled in various positions, was prepared from 2-D-[^{3}H]glucose (500 μCi/μmole), 4-D-[^{3}H]glucose (6100 μCi/μmole), or 5-D-[^{3}H]glucose (1000 μCi/μmole) by incubating a mixture of D-[^{3}H]glucose, phosphoenol pyruvate, ATP, UTP, NAD, hexokinase, pyruvate kinase, UDPglucose pyrophosphorylase, inorganic pyrophosphatase, and UDPglucose dehydrogenase in 0.1 M Tris-HCl, pH 7.5. The product was purified by paper electrophoresis at pH 3.6 followed by paper chromatography in ethanol: 1M ammonium acetate, pH 7.5 (7:3, v/v).

METHODS: Analytical methods. Uronic acid was determined by the carbazole reaction (8), and protein by the procedure of Lowry et al. (9), with human serum albumin as standard.

Radioactivity was determined with a Packard Model 2450 liquid scintillation spectrometer, using Instagel (Packard Instrument Co.) as scintillation medium. Labeled components on filter paper were analyzed after cutting the paper strips into 1 cm pieces which were transferred to scintillation vials containing 1 ml of water. About 1 h later, 10 ml of Instagel was added; the vials were then shaken vigorously and counted. Whenever required, the counting efficiency for ^{3}H and ^{14}C was estimated by use of appropriate internal standards.

Incubation of mastocytoma microsomal fraction. The conditions for incubation of microsomal fraction with UDP-sugars and PAPS were essentially as established in a previous study (2). For details, see the legends to figures.

RESULTS AND DISCUSSION: Labeled heparin-precursor polysaccharide (2) was formed by incubating mastocytoma microsomal fraction with UDP-[^{14}C]GlcUA, unlabeled UDP-GlcNAc and either UDP-5-[^{3}H]GlcUA or UDP-2-[^{3}H]GlcUA (for further details, see legend to Fig. 2). After 30 min the incorporation of radioactivity was interrupted by adding unlabeled UDP-GlcUA in excess; the incubations were then continued in the presence or absence of PAPS. During the second stage of incubation (30-70 min from start) samples were withdrawn and polysaccharide was recovered by precipitation with trichloroacetic



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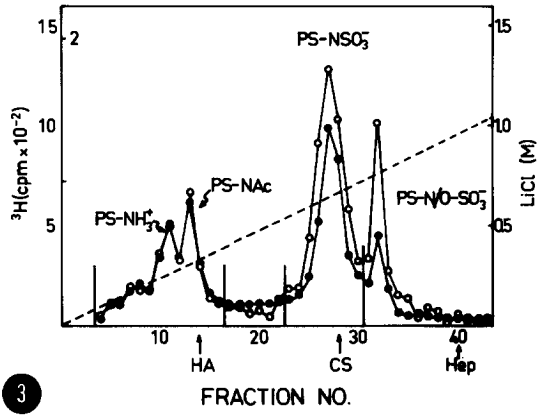

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Fig. 2. Effect of PAPS on the tritium content of microsomal polysaccharide, ^3H-labeled at (A) C5; (B) C2 of the uronic acid residues. Incubation mixtures contained, per ml of 0.05 M HEPES buffer, pH 7.4: a total of approximately 85 nmole UDP-GlcUA; 2.5 μCi UDP[^{14}C]GlcUA; 25 μCi UDP-5-[^3H] GlcUA or UDP-2-[^3H]GlcUA; 2.5 μmole UDP-GlcNAc; 10 μmole $MnCl_2$; 10 μmole $MgCl_2$; 5 μmole $CaCl_2$; and 10 mg of microsomal protein. After 30 min at 37°, 1.0 μmole UDP-GlcUA was added per ml of incubation mixture; half (0.3 ml) of the samples were then transferred to tubes containing 0.6 μmole of PAPS. Duplicate samples of 25 μl were withdrawn after varying periods of continued incubation (corresponding to 0 - 40 min in the diagram), and mixed with equal volumes of 1% serum albumin; polysaccharide was recovered by precipitation with trichloroacetic acid and was then analyzed by liquid scintillation counting, as described (2). The symbols denote incubations with (o) and without (•) PAPS, respectively.

Fig. 3. Chromatography on DEAE-cellulose of polysaccharide obtained by incubating mastocytoma microsomal fraction with UDP-5-[^3H]GlcUA and UDP-[^{14}C]GlcUA, in the presence of UDP-GlcNAc and PAPS. The incubation mixture (total volume, 3.0 ml) contained, per ml of 0.05 M HEPES buffer, pH 7.4: a total of approximately 60 nmole UDP-GlcUA; 2.5 μCi UDP-[^{14}C]GlcUA; 25 μCi UDP-5-[^3H]GlcUA; 2.5 μmole UDP-GlcNAc; 10 μmole $MnCl_2$; 10 μmole $MgCl_2$; 5 μmole $CaCl_2$; 1.0 μmole PAPS; and 10 mg of microsomal protein. After 1 h at 37°, 1.0 μmole of PAPS was added per ml, and the mixture was left at 37° for another hour. The incubation was terminated by heating the sample at 100° for 3 min: labeled polysaccharide was then isolated by gel chromatography (Sephadex G-50) after digestion with papain (2). The product was mixed with 0.5 mg of carrier heparin and fractionated by ion-exchange chromatography as described (3), at room temperature. Effluent fractions were analyzed for ^3H (•) and ^{14}C (o) and were then pooled as indicated by the vertical lines. The fractions obtained were desalted by passage through a column of Sephadex G-25 and eluted with 10% aqueous ethanol. The arrows indicate the peak elution positions of standard hyaluronic acid (HA), chondroitin 4-sulfate (CS) and heparin (Hep), respectively, -----, LiCl concentration. The four peaks correspond, in the order of elution, to the fractions I-IV described in a previous report (3).

acid. As seen from Fig. 2B the $^{3}H/^{14}C$ ratio of the polysaccharide prepared from C2 ^{3}H precursor, and hence tritiated at C2, remained unchanged throughout the incubation period, whether PAPS was present or not. Similar results were obtained with the 5-tritiated polymer in the absence of PAPS (Fig. 2A). In contrast, about 25% of the total tritium was lost from the 5-tritiated polysaccharide in the presence of PAPS. Sulfation of the microsomal polysaccharide, previously shown to promote the uronic acid C5 epimerization process, thus results in loss of hydrogen from C5 but not from C2 of uronic acid residues.

Experiments were designed to identify the source of labile C5 hydrogen with regard to the various species (see Fig. 1) of microsomal polymeric intermediates. Partially sulfated polysaccharide, labeled with [^{14}C]GlcUA and 5-[^{3}H]GlcUA, was prepared as described in the legend to Fig. 3 and was then fractionated by ion-exchange chromatography on DEAE-cellulose. The $^{3}H/^{14}C$ ratios of the resulting sulfated fractions (PS-NSO_3^-; PS-N/O-SO_3^-) were lower than those of the nonsulfated fractions (PS-NAc, PS-NH_3^+) Fig. 3); if the ratio for the combined fractions PS-NH_3^+ and PS-NAc is taken as 1.0, the ratios for PS-NSO_3^- and PS-N/O-SO_3^- are 0.7 and 0.5, respectively. When UDP-4-[^{3}H]GlcUA or UDP-2-[^{3}H]GlcUA was substituted for the 5-labeled compound, the $^{3}H/^{14}C$ ratio of the polymer products did not change. These results demonstrate a specific loss of C5 tritium from the sulfated polysaccharide fractions, which also contain IdUA residues.

Confirmation that the elimination of C5 hydrogen is obligately related to the uronic acid C5 epimerization reaction was provided by analysis of isolated uronic acid monosaccharides. Fractions of nonsulfated and sulfated microsomal polysaccharide were degraded by a combination of acid hydrolysis and deamination with nitrous acid (5) and the resulting monosaccharides were separated by paper chromatography. The chromatogram shown in Fig. 4A represents the uronic acids derived from PS-N/O-SO_3^- (see Fig. 3) of the 5-^{3}H-, ^{14}C-labeled polysaccharide. The IdUA component, which comprised 45%

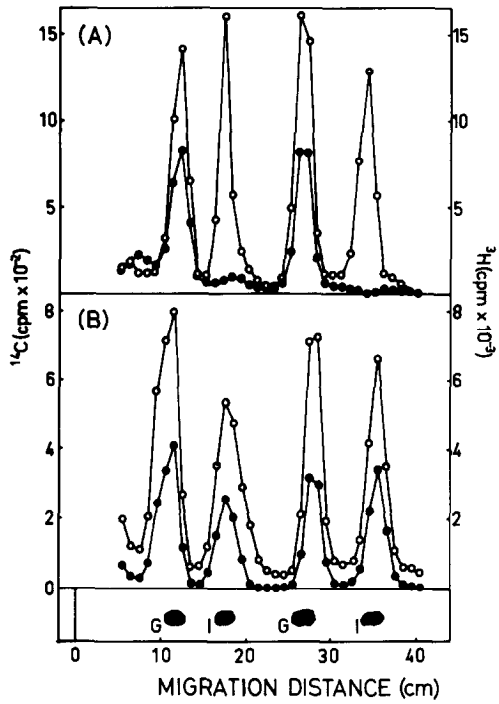

Fig. 4. Paper chromatography of uronic acids derived from fraction PS-N/O-SO_3^- of (A) ^{14}C-, 5-^{3}H-labeled polysaccharide (see Fig. 3); (B) ^{14}C, 2-^{3}H-labeled polysaccharide. The paper (Whatman No. 3 MM) was developed with ethyl acetate-acetic acid-water, 3:1:1; strips were analyzed for ^{3}H (•) and ^{14}C (o). The standards (uronic acids and corresponding lactones) shown below the chromatograms are: G, D-GlcUA; I, L-IdUA, the lactones migrating faster than the free acids.

of the total [^{14}C] uronic acid, was devoid of tritium, whereas the GlcUA had both ^{3}H- and ^{14}C-label. The $^{3}H/^{14}C$ ratio was the same in GlcUA and in IdUA in 4-^{3}H-labeled PS-N/O-SO_3^- (Fig. 4B) as well as in the 2-^{3}H-labeled polymer. These results indicate that conversion of GlcUA to IdUA residues involves loss of C5 hydrogen from GlcUA units. Results to be published elsewhere show that the released tritium equilibrates with water and may be quantitatively recovered by distillation; the procedure has been developed into an assay for epimerase activity (10). The 5-epimerization probably resembles the D-mannuronic acid-L-guluronic acid conversion which occurs during biosynthesis of alginic acid; when this reaction was conducted in tritiated water, the resulting L-guluronic acid residues were ^{3}H-labeled (11).

The mechanism of the C5-inversion remains unknown. The data available are compatible with the involvement in the reaction of a C5 carbonium ion, carbanion, or radical, or of a 4,5 unsaturated, intermediate.

Previous studies have shown that formation of IdUA in heparin (3,4) as well as in dermatan sulfate (13) and heparan sulfate (5) is associated with sulfation of the polysaccharide. The $^{3}H/^{14}C$ ratio of PS-N-SO_3^- as well as that of PS-N/O-SO_3^- (Fig. 3) is lower than that of the non-sulfated fractions PS-NAc and PS-NH_3^+. This finding and the absence of IdUA from the nonsulfated fractions suggest that in the biosynthesis of heparin N-sulfation may be a prerequisite to uronic acid C5-epimerization[2)].

Although O-sulfation enhances the extent of the epimerization (cf. the [^{14}C]uronic acid composition of PS-N/O-SO_3^-, it does not appear to be required for the reaction to occur. These postulates have been verified in recent experiments relating the introduction of various sulfate substituents to the loss of C5-tritium from GlcUA residues and to the formation of IdUA units (10).

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The present report is No. 5 in a series where the preceding paper is Reference 3.

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2) Due to inadequate analytical procedures the presence of IdUA in PS-NSO_3^- was overlooked in previous analysis of this material (3); the possible significance of N-sulfation was therefore not recognized.

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