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Biosynthesis of Heparin

I. Transfer of *N*-Acetylglucosamine and Glucuronic Acid to Low-Molecular Weight Heparin Fragments

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The mechanism of alternation of monosaccharide units in the heparin molecule has been investigated. A microsomal fraction from heparin-producing mouse mastocytoma catalyzed the transfer of *N*-acetylglucosamine from UDP-*N*-acetylglucosamine to an exogenously added carbohydrate-serine compound, isolated from heparin. Preliminary treatment of the acceptor with β-glucuronidase resulted in an almost quantitative loss of acceptor activity. No evidence was obtained for the transfer of *N*-acetylglucosamine to residues of iduronic acid.

Treatment of the microsome particles with Tween and alkali resulted in little solubilization of the *N*-acetylglucosaminyltransferase under conditions where more than half of the glucuronosyltransferase previously described, was brought into solution. The results indicate that two separate enzyme proteins may be involved in the stepwise transfer of the two sugar units to the non-reducing terminal position of the growing polymer.

Studies on the biosynthesis of heparin in cell-free systems have largely utilized homogenates from mast-cell tumors since heparin is the predominant polysaccharide in this tissue. Silbert [1] observed the formation of heparin-like material by incubating a particulate enzyme from DBA mouse mast-cell tumors with UDP-GlcNAc * and UDP-GlcUA. Subsequently, the transfer of sulfate to such a polysaccharide product was demonstrated.[2]

The technique of employing endogenous precursors as substrates for polysaccharide synthesis may, however, be less meaningful in studies directly aimed at the solubilization and purification of the glycosyltransferases involved. Indeed, a quantitative evaluation of the enzyme may not be performed with

* The abbreviations used, are: UA, uronic acid; GlcUA, D-glucuronic acid; IdUA, L-iduronic acid; GlcNAc, 2-acetamido-2-deoxy-D-glucose; UDP, uridine diphosphate. In oligosaccharides composed of β-D-pyranosides the carbon atoms involved in glycosidic linkages are indicated by a prefix, *e.g.* GlcUA-3-Gal, 3-*O*-β-D-glucuronosyl-D-galactose.

an endogenous substrate, as the concentration and availability of such an acceptor, rather than the activity of the enzyme may be the rate-limiting factor. Consequently, the use of exogenous substrates for the enzyme permits the development of a more convenient assay. In the present work, several fragments from the heparin molecule have been tested as acceptors in the transfer of *N*-acetylglucosamine-^{14}C and glucuronic acid-^{14}C from their respective uridine diphospho derivatives, using a cell-free system from FMS mouse mast-cell tumor. A procedure for the partial solubilization of the glycosyl transferases is described.

EXPERIMENTAL PROCEDURE

Materials. UDP-*N*-Acetylglucosamine-^{14}C (42 μCi/μmol) was purchased from New England Nuclear Corporation, Frankfurt, Germany. UDP-Glucuronic acid-^{14}C (33 μCi/μmol) was obtained from The Radiochemical Centre, Amersham, England. Tween 20 was supplied by Koch-Light Industries, Colnbrook, Bucks., England. Liver β-glucuronidase (Type B-1; 220 Fishman units/mg) was purchased from Sigma Chemical Co., St. Louis, Mo., USA.

A heparin-producing FMS mast-cell tumor [3,4] was maintained in the solid state in (A/Sn × Leaden)F_1 mice by subcutaneous and intramuscular transplantation in the hind legs every 10 – 14 days.

Carbohydrate-serine compounds were isolated from heparin after deaminative cleavage with nitrous acid, as described.[5] The structures of Fractions A_2, B_2, B_1, and B_1-β (obtained by treatment of Fraction B_1 with β-glucuronidase [6]) are shown in Fig. 1. The

Hyaluronic acid hexasaccharide:	GlcUA-GlcNAc-GlcUA-GlcNAc-GlcUA-GlcNAc
Compound A_2:	IdUA-GlcNAc-GlcUA-Gal-Gal-Xyl-Serine
Compound B_2:	GlcUA-GlcNAc-GlcUA-Gal-Gal-Xyl-Serine
Fraction B_1:	IdUA-GlcNAc-UA-GlcNAc-GlcUA-Gal-Gal-Xyl-Serine GlcUA-GlcNAc-UA-GlcNAc-GlcUA-Gal-Gal-Xyl-Serine
Fraction B_1-β:	IdUA-GlcNAc-UA-GlcNAc-GlcUA-Gal-Gal-Xyl-Serine GlcNAc-UA-GlcNAc-GlcUA-Gal-Gal-Xyl-Serine

Fig. 1. Structures of a hexasaccharide from hyaluronic acid and of carbohydrate-serine compounds derived from the polysaccharide-protein linkage region of heparin. Fractions B_1 and B_1-β each consist of two types of fragments, as indicated, present in approximately equimolar amounts.[6]

isolation from heparin of the oligosaccharides GlcUA-3-Gal, GlcUA-3-Gal-3-Gal, and GlcUA-3-Gal-3-Gal-4-Xyl has been reported previously.[7] A hexasaccharide from hyaluronic acid was isolated as described,[6] after degradation of the polysaccharide with testicular hyaluronidase.

Analytical methods. Protein was determined according to the method of Lowry *et al.*[8]

Paper chromatography was carried out in solvent (A), ethyl acetate-acetic acid-water (3:1:1), or (B), ethyl acetate-pyridine-water (2:1:2, upper phase). The papers were stained with aniline hydrogen phthalate.[9] Paper electrophoresis was performed in buffers (C), 0.08 M pyridine-0.046 M acetic acid, pH 5.3 (90 min; 80 V/cm), (D), 0.5 M formic acid-1.4 M acetic acid, pH 2.0 (2 h; 40 V/cm) or (E) 6 % formic acid, pH 1.7 (4 h; 40 V/cm). Paper electrophoretograms were stained with a silver dip reagent [10] or with ninhydrin.[11] Strips of paper electrophoretograms containing labeled compounds were analyzed for radioactivity with a Packard model 7201 strip scanner and the products were quantitated, after elution, by a Beckman liquid scintillation spectrometer.

Preparation of enzyme. Tumors from 20 mice were disrupted in a Virtis 45 homogenizer with two volumes of 0.05 M tris-acetate buffer, pH 7.4, containing 0.07 M KCl and 0.001 M EDTA, and were then subjected to differential centrifugation as described.[12]

Partial solubilization of glycosyltransferases was achieved by suspending the microsomal fraction, sedimenting at 1×10^5 *g* in 10 ml of buffer of the above composition, followed by the addition of Tween 20 (final concentration, 2 %). The pH of the solution was raised to 10.4 – 10.6 by the addition of NH_4OH and was then quickly readjusted to 7.4 by treatment with glacial acetic acid.[12] After centrifugation at 1.59×10^5 *g* for 2 h, the top 10 ml of each tube (total volume, 13 ml) were withdrawn and used as solubilized enzyme.

Enzyme assays. In testing for glycosyltransferase activity, the exogenous substrate (0.01 – 0.25 μmol) was incubated with solubilized or particulate enzyme (0.05 ml; 1 – 3 mg protein/ml) and ^{14}C-labeled sugar nucleotide (0.05 – 0.1 μCi) in 0.06 ml of the Tris-acetate buffer described above. For exact experimental conditions, see the legends to figures and tables. After incubation at 37° for varying periods of time, the reaction mixtures were spotted on strips of Whatman No. 3 MM paper for electrophoresis. The glucuronosyltransferase activity was determined after electrophoresis in buffer C as described.[6] Although a similar procedure was applicable for determination of *N*-acetylglucosaminyltransferase activity, interference from radioactive breakdown products on the electrophoretogram prompted the development of a more reliable assay for this enzyme. After removal of negatively charged compounds by electrophoresis at pH 2.0 (buffer D), the papers were dried and ^{14}C-*N*-acetylglucosamine-labeled carbohydrate-serine compounds were separated from free N-acetylglucosamine by paper chromatography in solvent A. After chromatography in this solvent for 10 h the labeled free monosaccharide had migrated off the paper, whereas the reaction product had not moved appreciably. In each instance, the product was eluted with water and the radioactivity was determined as described above.

RESULTS

Transfer of N-*acetylglucosamine to low-molecular weight heparin fragments.* Incubation of the particulate enzyme with Fraction B_1 (*cf.* the schematic structure, Fig. 1) and UDP-GlcNAc-^{14}C resulted in the formation of a compound which migrated as a cation in buffer E but as an anion in buffer C (*cf.* Fig. 4 in Ref. 5). The product was not stable in the incubation medium, as treatment of the labeled material with the particulate enzyme overnight resulted in the liberation of all radioactivity as *N*-acetylglucosamine (paper chromatography, solvent B). Similarly, *N*-acetylglucosamine was the only radioactive component detected after partial acid hydrolysis (dilute hydrochloric acid, pH 1.5, at 100° for 3 h) followed by passage of the neutralized hydrolysate through columns of Dowex 1-X2 and Dowex 50-X4. Further characterization of the product was achieved by gel chromatography, as described in the legend to Fig. 3. The radioactive product was eluted slightly ahead of Fraction B_1, as expected, assuming that transfer of *N*-acetylglucosamine to this fraction had occurred. Preliminary treatment of Fraction B_1 with β-glucuronidase [6] resulted in almost complete loss of acceptor activity (Fraction B_1-β; Table 1). Taken together, these data indicate the presence in the tumor homogenate of an enzyme capable of transferring a residue of *N*-acetylglucosamine from UDP-*N*-acetylglucosamine to a glucuronic acid unit at the nonreducing terminal position of Fraction B_1.

Table 1 shows the acceptor properties of some low-molecular weight heparin fragments in the *N*-acetylglucosaminyltransferase reaction. It is seen that significant product formation was observed only with Fraction B_1. Smaller

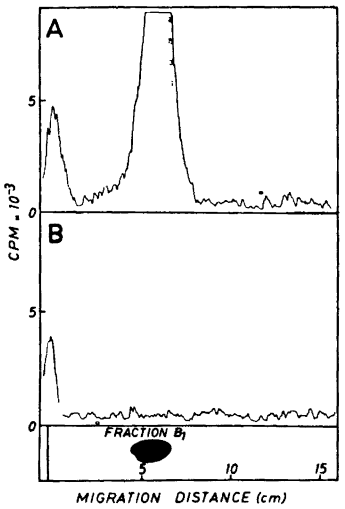

Fig. 2. Transfer of *N*-acetylglucosamine-^{14}C from UDP-*N*-acetylglucosamine-^{14}C to Fraction B_1. Assay conditions are described in Experimental procedure. (A), Tracing obtained after paper electrophoresis and paper chromatography following incubation of 1.59×10^5 *g* pellet fraction (0.15 mg of protein), Fraction B_1 (0.1 μmol) and UDP-*N*-acetylglucosamine-^{14}C (0.05 μCi; 42 μCi/μmol); (B), Control incubation without added Fraction B_1. Fraction B_1 (shown below the tracings) did not appreciably separate from the labelled product under the conditions of the assay.

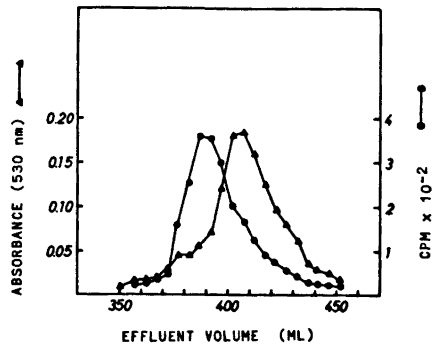

Fig. 3. Chromatography on Sephadex G-50 (superfine) of a mixture of Fraction B_1 (0.6 μmol, based on uronic acid content) and ^{14}C-labelled reaction product (3000 cpm) from Fraction B_1 and UDP-*N*-acetylglucosamine-^{14}C. The gel column (2 × 180 cm) was eluted with 0.2 M NaCl at a rate of 15 ml/h. Effluent fractions of 5 ml were collected and analyzed for uronic acid (▲) and for radioactivity (●).

Table 1. Substrate specificity of *N*-acetylglucosaminyltransferase system.

Acceptor	^{14}C-Product cpm
Fraction B_1	3020 [a]
Fraction B_1-β	252 [a]
Fragment A_2	< 10 [a]
Fragment B_2	< 10 [a]
GlcUA-3-Gal	< 10 [b]
GlcUA-3-Gal-Gal	< 10 [b]
GlcUA-3-Gal-Gal-Xyl	< 10 [b]
Hyaluronic acid hexasaccharide	593 [b]

[a] Each tube contained acceptor (0.05 μmol), particulate enzyme (0.12 mg of protein), UDP-GlcNAc-^{14}C (0.05 μCi) and $MnCl_2$ (20 mM). After 60 min at 37°, the reaction was stopped and the mixtures were analyzed by the standard assay procedure (electrophoresis in buffer D followed by paper chromatography in solvent A), described in Experimental procedure.

[b] Data from a separate but similar experiment with 0.2 μmol of acceptor and UDP-GlcNAc-^{14}C added as indicated above. Acceptor activity was determined after paper electrophoresis in buffer C except for the hexasaccharide, which was determined by the standard procedure.

Table 2. Effect of detergent and alkali on UDP-GlcNAc: Fraction B_1 *N*-acetylglucosaminyltransferase.

Aliquots (13 ml each) of the microsomal fraction (protein concentration, 1.1 mg/ml) were treated with NH_4OH and/or Tween 20 (final concentration, 2 %) as described and centrifuged at 1.59×10^5 *g* for 2 h. Samples of the pellet fractions and from the top 10 ml of each of the supernatant fractions were subsequently assayed for enzyme activity in the presence of Fraction B_1 (0.25 μmol) and UDP-GlcNAc-^{14}C (0.05 μCi).

Preliminary treatment of microsomal fraction	Total enzymatic activity	Amount of protein solubilized	Amount of enzyme solubilized	Amount of free *N*-acetylglucosamine liberated during the incubation period	
				pellet	supernatant
	cpm [a]	% of total	% of total	cpm	
Treatment at pH 10.4: No detergent	1 380	35	3	10 111	273
Treatment with Tween 20	3 900	53	5	1 770	14 927
Treatment with Tween 20 at pH 9.2	2 400	61	7	1 439	12 442
Treatment with Tween 20 at pH 9.2 (12 h)	2 000	64	13	1 004	13 659
Treatment with Tween 20 at pH 10.4	3 750	75	5	987	7 315
Treatment with Tween 20 at pH 10.9	2 290	75	7	197	4 737

[a] Sum of two incubations with corresponding 0.05 ml supernatant and 0.05 ml pellet fractions.

Table 3. Partial separation of glycosyltransferases involved in heparin biosynthesis, by selective solubilization of UDP-GlcUA: Fraction B_1-β glucuronosyltransferase.

The microsomal fraction was treated with NH_4OH and Tween 20 as described in Experimental procedure. Following centrifugation at 1.59×10^5 *g* for 2 h, 50 μl aliquots of the pellet and the supernatant fractions were incubated with (A), Fraction B_1-β (0.015 μmol), UDP-GlcUA-^{14}C (0.1 μCi; 33 μCi/μmol) and $MnCl_2$ (20 mM); or (B), Fraction B_1 (0.1 μmol), UDP-GlcNAc-^{14}C (0.05 μCi; 42 μCi/μmol) and $MnCl_2$ (20 mM). Product formation was determined by the standard assay procedures described in Experimental procedure.

Enzyme fraction	Enzymatic activity		Protein	Specific enzymatic activity		Solubilized enzyme	
	A	B		A	B	A	B
	cpm/incubation		μg/incubation	cpm/mg protein		% of total	
1.59×10^5 *g* pellet	817	4 122	74	11 020	56 000	–	–
1.59×10^5 *g* supernatant	2 001	218	130	15 400	1 680	71	5

molecules, such as compounds B_2 or A_2, or GlcUA-3-Gal-3-Gal-4-Xyl, were completely devoid of acceptor activity. In addition, hexasaccharide isolated after digestion of hyaluronic acid with testicular hyaluronidase was assayed as substrate. This molecule, which contains a $\beta 1 \rightarrow 3$ linked residue of glucuronic acid at its nonreducing terminal position, could only partially replace Fraction B_1 as acceptor of *N*-acetylglucosamine.

Effect of detergent and alkali on the glycosyltransferases. Treatment of the microsomal fraction with Tween and alkali (see Experimental procedure) resulted in the solubilization of 71 % of the glucuronosyltransferase, as determined with Fraction B_1-β as substrate (Table 3). In contrast, most (85–95 %) of the UDP-GlcNAc: Fraction B_1 *N*-acetylglucosaminyltransferase remained attached to the particles sedimenting at 1.59×10^5 *g* (Tables 2 and 3). As the latter fraction possessed the highest specific *N*-acetylglucosaminyltransferase activity (Table 3), it was utilized in subsequent experiments to determine the kinetic parameters.

In addition to the glycosyltransferases, the microsomal preparation also contained substantial amounts of hydrolytic enzyme(s) responsible for the conversion of UDP-*N*-acetylglucosamine to free *N*-acetylglucosamine. Thus, the nucleotide precursor was completely degraded after 30 min of incubation. UDP-Glucuronic acid was more stable in the enzyme preparation and the liberation of free glucuronic acid was comparatively small. A major portion of the enzymatic activity responsible for the liberation of *N*-acetylglucosamine was brought into solution by the standard detergent-alkali treatment (Table 2).

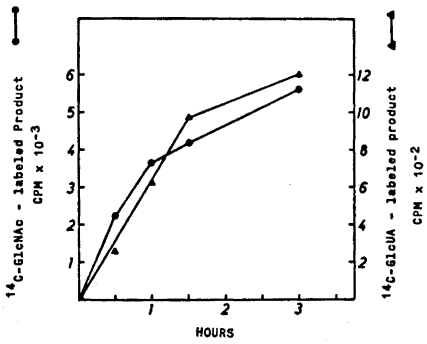

Fig. 4. Transfer of *N*-acetylglucosamine-^{14}C to Fraction B_1 or of glucuronic acid-^{14}C to Fraction B_1-β as a function of time. Each tube contained particulate enzyme (0.15 mg of protein) and $MnCl_2$ (20 mM) and either (a) Fraction B_1 (0.2 μmol) and UDP-*N*-acetylglucosamine-^{14}C (0.05 μCi; 42 μCi/μmol) or (b) Fraction B_1-β (0.015 μmol) and UDP-glucuronic acid-^{14}C (0.1 μCi; 33 μCi/μmol). After incubation during the periods of time indicated, the reaction mixtures were spotted on paper strips for analysis as described in Experimental procedure.

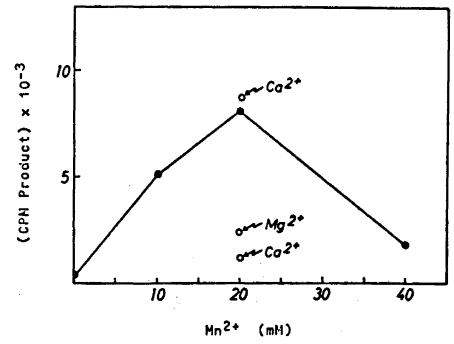

Fig. 5. Effect of divalent cations on the transfer of *N*-acetylglucosamine to Fraction B_1. The conditions of incubation were similar to those described in the legend to Fig. 4 except that metal ions were added as indicated.

However, the activity of the enzyme(s) responsible for the destruction of UDP-GlcNac was apparently impeded by the solubilization procedure since the 1.59×10^5 *g* pellet and supernatant fractions obtained after treatment of the microsomes with Tween and alkali both contained significant quantities of the nucleotide sugar after incubation for 60 min under standard conditions.

Kinetic studies. Using Fraction B_1 or B_1-β as substrates and the 1.59×10^5 *g* pellet fraction, obtained as described above, as enzyme source, the following kinetic parameters were determined for the *N*-acetylglucosaminyltransferase or glucuronosyltransferase, respectively. In both cases, product formation increased essentially linearly with time for 60 min (Fig. 4). Within the range tested (0 – 2.5 mg of protein/ml), the rate of incorporation was proportional to the concentration of protein. The *N*-acetylglucosaminyltransferase was stimulated by the addition of Mn^{2+} ions (Fig. 5). However, the Mn^{2+} could be replaced by Co^{2+} and partially by Mg^{2+} or Ca^{2+} (Fig. 5). Although the metal requirement for the transfer of glucuronic acid to fraction B_1-β was not investigated in detail, the addition of Mn^{2+} was necessary for product forma-

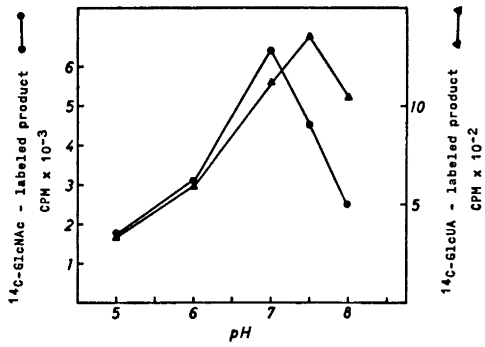

Fig. 6. Effect of pH on the transfer of *N*-acetylglucosamine-^{14}C and glucuronic acid-^{14}C to fractions B_1 and B_1-β, respectively. Aliquots of particulate enzyme were washed with Tris-acetate buffers of pH values ranging between 5.0 and 8.0. After centrifugation and suspension of the particles in the appropriate buffer, the two enzymes were assayed under the conditions described in the legend to Fig. 4.

tion. Both reactions occurred over a wide pH range with optimum at pH 7.0 for the transfer of glucuronic acid, and 7.5 for the transfer of *N*-acetylglucosamine (Fig. 6).

DISCUSSION

The mechanism for polysaccharide formation has attracted considerable interest in recent years and it now appears fairly well established that a large variety of carbohydrate compounds from mammalian sources are synthesized by stepwise addition of monosaccharide units at the non-reducing terminus of the growing chain. The product of each reaction serves as the substrate in the following step.[13] In a previous study on the biosynthesis of heparin the presence of an *N*-acetylglucosamine unit in nonreducing terminal position was found to be mandatory for the transfer of glucuronic acid to an exogenous acceptor.[6] Similarly, the transfer of *N*-acetylglucosamine would seem to require an acceptor compound with a terminal glucuronic acid residue (Table 1). These findings conform to the mechanism of alternation of monosaccharide

units proposed for the biosynthesis of chondroitin sulfate.[14] Furthermore, they support the postulate of Silbert,[15] that the biosynthesis of heparin occurs *via* the formation of an *N*-acetylated intermediary polymer.

It is unclear whether the incorporation of the alternating units of uronic acid and hexosamine in polysaccharides such as heparin is mediated by distinctly separate enzymes, or by a single enzyme protein having more than one catalytic site. Thus, an enzyme with three catalytic sites, responsible for the polymerization of hyaluronic acid chains from the requisite nucleotide precursors, was proposed by Markovitz *et al.*[16] The attachment of the polymerizing enzymes to subcellular membranes has largely prevented an experimental approach for the evaluation of such a hypothesis. The present study has indicated, however, that the incorporation of the alternating monosaccharide units into the heparin molecule is mediated by two separate enzyme proteins, since treatment of the microsome particles resulted in the preferential solubilization of the glucuronosyltransferase with only a small amount of the UDP-GlcNAc: Fraction B_1 *N*-acetylglucosaminyltransferase present in the supernatant fraction (Table 3). It is realized that some caution must be exercised in reaching such a conclusion, since the presence in the supernatant of the latter enzyme may be masked by the action of hydrolytic enzymes responsible for the destruction of UDP-*N*-acetylglucosamine or of the product. However, this possibility appears unlikely, as significant quantities of UDP-*N* acetylglucosamine remained in the supernatant fraction throughout the incubation period (Table 2).

The acceptor requirements of the *N*-acetylglucosaminyltransferase were apparently more exacting than those observed for the glucuronosyltransferase involved in the biosynthesis of heparin. Whereas the latter enzyme was essentially equally active with substrates ranging in size from trisaccharide to heptasaccharide,[17] the transfer of *N*-acetylglucosamine was demonstrated only with large substrates, such as Fraction B_1 or hexasaccharide from hyaluronic acid.

It is notable that no evidence was obtained for the transfer of *N*-acetylglucosamine to residues of iduronic acid. Recently, Malmström and Fransson [18] observed that a decasaccharide with nonreducing terminal iduronic acid acts as acceptor for *N*-acetylgalactosamine in a dermatan sulfate-synthesizing system. Treatment of Fraction B_1 with β-glucuronidase would seem to provide a similar kind of fragment (see Fraction B_1-β in Fig. 1), yet this procedure resulted in almost complete loss of acceptor activity (Table 1). Conceivably, the transfer of *N*-acetylglucosamine to residues of iduronic acid might require conditions other than those of the *in vitro* assay system employed; the absence of such transfer would then become trivial. On the other hand, the restricted acceptor properties of Fraction B_1-β may be highly significant in relation to the mode of heparin chain elongation. Recent studies with the mastocytoma microsomal system have demonstrated that the iduronic acid unit of heparin * is formed by epimerization on the polymer level of glucuronic acid residues previously incorporated into the polysaccharide chain.[22] Assuming that

* Iduronic acid constitutes more than half of the total uronic acid content of heparin,[19,20] and is a major radioactive uronic acid component of mastocytoma polysaccharide labelled *in vivo* with ^{14}C-glucose.[21]

epimerization may occur at internal positions of the polymer, this mechanism should allow for the presence of iduronic acid in heparin, yet not require the transfer of *N*-acetylglucosamine to terminal iduronic acid residues. Obviously, further studies are necessary to fully elucidate the biosynthesis of the iduronic acid-containing glycosaminoglycans.

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