Biosynthesis of Dermatan Sulfate

II. SUBSTRATE SPECIFICITY OF THE C-5 URONOSYL EPIMERASE*

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Epimerization of D-glucuronosyl residues to L-iduronic ones during biosynthesis of dermatan sulfate involves an abstraction of the C-5 hydrogen of the target sugar residue. After inversion, a hydrogen from the medium is reinserted into the uronosyl residue. In the present study, microsomal enzyme prepared from cultured embryonic skin fibroblasts was incubated with dermatan or chondroitin in the presence of \( ^3\text{H}_2\text{O} \) of high specific activity. Incubation resulted in incorporation of tritium on C-5 of uronosyl residues of the substrates. The rate of the reaction was highest for dermatan. Incubation of the products with chondroitinase ABC released essentially all the tritium. Dermatan sulfate and chondroitin sulfate were inactive as substrates, which indicates that epimerization takes place before sulfation.

Analyses of the product obtained after incubation of chondroitin in \( ^3\text{H}_2\text{O} \)-containing medium for different incubation times showed that tritium accumulated first in L-iduronosyl residues. Later, tritium was also found in D-glucuronosyl residues. The reverse situation was observed when dermatan was used as substrate. After extended incubation times, the ratio of D-[\( ^3\text{H} \)]glucuronic acid to L-\( [\text{H}] \)iduronic acid in both dermatan and chondroitin reached a value of 85/15, which may reflect the equilibrium value. Digestion of labeled chondroitin with chondroitinase AC and oxidation of labeled dermatan with periodate showed that after 96 h of incubation with the epimerase and \( ^3\text{H}_2\text{O} \), most of the uronic acid residues had been involved in the reaction. Both products were composed of long blocks of glucuronic acid-containing disaccharides interrupted by a few L-iduronic acid-containing disaccharides arranged singly in clusters of two to three.

Reincubation of the \( ^3\text{H} \)-labeled products originating from dermatan or chondroitin with the epimerase resulted in release of tritium, which was linear with time and with increasing protein concentration.

During the biosynthesis of proteodermatan sulfate, the side chains are formed by stepwise addition of N-acetylgalactosamine and glucuronic acid from their respective nucleotide sugars to the nonreducing terminus of the acceptor chain. After or in close conjunction with polymerization, C-5 inversion of D-glucuronosyl to L-iduronosyl residues occurs together with sulfation of the polysaccharide (1). The precise relationship between epimerization and sulfation has not yet been established.

Studies on the formation of L-iduronic acid in dermatan sulfates by fibroblasts and microsomes therefrom showed that the C-5 hydrogen of the glucuronic residues was released during epimerization (2). An assay for the epimerase was developed in which chondroitin with glucuronosyl residues labeled with tritium at C-5 was used as a substrate. Incubation with epimerase released tritium from the uronosyl residues, and after equilibration with water it could be quantitated after distillation (3). Using this assay, it was shown that the epimerase had a pH optimum around pH 6 and required Mn\(^{2+}\) for maximal activity. The heparan N-sulfate-D-glucurono-nosyluronate 5-epimerase of mouse mastocytoma has a higher pH optimum and is not dependent on divalent cations (4, 5). Furthermore, by incubating fibroblast microsomes with various epimerase substrates, it could be demonstrated that there is most likely one epimerase for heparan sulfate synthesis and one for dermatan sulfate.

In earlier studies (3), chondroitin containing 5-\( ^3\text{H} \)-labeled glucuronosyl residues was used as a substrate, which precludes the characterization of the product as the tritium disappears during the reaction. Furthermore, it is difficult to obtain other 5-\( ^3\text{H} \)-labeled substrate than chondroitin and chondroitin sulfate. This investigation was therefore carried out using the reverse reaction, that is incorporation of tritium from \( ^3\text{H}_2\text{O} \). This approach permitted studies on the substrate specificity of the epimerase. Furthermore, it was possible to study the structure of the resulting product, which is prerequisite for understanding how the final copolymeric structure of dermatan sulfate is achieved.

EXPERIMENTAL PROCEDURES

Materials—\( ^3\text{H}_2\text{O} \) (90 Ci/mol) was bought from the Radiochemical Centre, Amersham, United Kingdom. Heps, \( \text{Mes} \), and papain (type III) were obtained from Sigma. Chondroitinase AC and ABC were delivered by Miles Laboratories, Inc., Elkhart, IN. 3' Phosphoadenylylsulfate was prepared as described (5).

Preparation of Epimerase—Cell cultures of human embryonic skin fibroblasts were maintained in monolayer (1). The cells were detached by gentle treatment with 0.02% EDTA and then homogenized (1). The homogenate was fractionated into a 600 × g pellet fraction, a 10,000 × g pellet fraction, and 105,000 × g pellet fraction as described earlier (3). The 10,000 × g pellet fraction, which had the highest specific activity and contained the major part of the epimerase activity, was used for the incubations.

Preparation of Substrates—Chondroitin 4-sulfate and chondroitin 6-sulfate were prepared from nasal septum and intervertebral discs, respectively (6). N/O-Desulfated and re-N-sulfated heparan sulfate was prepared as described earlier (5). Dermatan sulfate were prepared from pig skin and fractionated into 0–25 and 25–50% ethanol fraction. The abbreviations used are: Heps, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; Mes, 2-(N-morpholino)ethanesulfonic acid.
tions in the presence of Ca acetate (7). The dermatan sulfate preparations were freed of contaminating heparan sulfate by treatment with HNO₂ at pH 1.5 (8) followed by gel chromatography. A part of the dermatan sulfate was subjected to chondroitinase AC digestion followed by gel chromatography on Sephadex G-100 to remove glucuronic acid-containing disaccharides. Chondroitin sulfate- and dermatan sulfate-containing disaccharides were desalted with methanol/HCl (9) to give a sulfate concentration less than 0.1% determined according to Terbo and Hartila (10). The desalted and demethylated products were fractionated on Sephadex G-100. The starting materials were excluded from the column. After desulfation, the polysaccharides chromatographed between a K₅₀ of 0.9 and 0.9. Material chromatographing between a K₅₀ of 0.15 and 0.35 comprising 27% of the total dermatan and 41% of the total chondroitin was selected as substrate if nothing else is stated. Dermatan chromatographing between a K₅₀ of 0.67 and 0.9 was a mixture of roughly equal proportions of hexa-, octa-, and decasaccharides as shown by co-chromatography on Sephadex G-50 with a standard octa- and decasaccharide preparation. These were prepared by chondroitin from bovine testes hyaluronic acid digestion followed by gel chromatography and were a gift from Drs. Firoz Rahemtulla and Lennart Roden, The University of Alabama in Birmingham, Birmingham, AL.

**RESULTS**

Characterization of the Epimerase Reaction—Incubation of dermatan with microsomal enzyme and tritiated water of high specific activity resulted in incorporation of tritium into dermatan (Fig. 1). Using large amounts of substrate (100 μg), the reaction was linear for more than 20 h. The pH optimum of the reaction was 6.5 (Fig. 2), and the K₅₀ of the reaction using dermatan as substrate was 3.7 × 10⁻⁴ M calculated as concentration of uronosyl residues. The radioactive product of the reaction was bound on DEAE-cellulose and eluted in the same position as dermatan. It chromatographed with the same K₅₀ as the dermatan substrate on Sephadex G-50. Digestion of the product with chondroitinase ABC, which is an eliminase and removes the hydrogen from C-5 of the target uronosyl residue, resulted in 98% liberation of the tritium (Table I). These data indicate that nearly all tritium was incorporated at C-5 of the uronosyl residues of dermatan. The optimal substrate for the epimerase was dermatan (Table I). In addition to dermatan, chondroitin was also a substrate, but only half as much tritium was incorporated in the latter during a 4-h incubation. In a more detailed study of the time course of the product formation, 10 μg of dermatan and chondroitin were incubated with microsomal enzyme for various times (Fig. 3). It is clear that tritium was incorporated much more rapidly (approximately five times faster) into dermatan than chondroitin (Fig. 3, inset). The difference gradually dimin-

**Fig. 1.** Incorporation of tritium into dermatan with time. 100 μg of polysaccharide were incubated with epimerase as described under “Experimental Procedures.”

**Fig. 2.** Effect of pH on incorporation of ³H₂O into dermatan by the epimerase. 100 μg of polysaccharide were incubated in the presence of Mes (●) and Hepes (○) for 4 h.
ished with incubation time, but even after 96 h, more radioactivity was found in dermatan than chondroitin. Dermatan sulfate of various copolymeric structure as well as chondroitin 4- and 6-sulfate were essentially inactive as substrate (Table I). Also, an N- and O-desulfated and re-N-sulfated heparin, a substrate for the heparosan N-sulfate-D-glucuramiduronate 5-epimerase, was inactive as substrate (Table I). Dermatan of various chain lengths from intact polysaccharide down to a preparation containing mainly hexa- to decasaccharides was incubated with enzyme, and no difference in the amount of tritium incorporated per µmol of uronic acid was noted.

**Structure of the Product—**[5-3H]Dermatan and [5-3H]chondroitin obtained after various times of incubation were subjected to chondroitinase AC and ABC digestion followed by distillation. In dermatan, there was a rapid increase in the amount of radioactive D-glucuronic acid residue (Fig. 4a). Only later did tritium-labeled L-iduronic acid residues start to accumulate. After 96 h of incubation, the ratio of D-[5-3H]glucuronosyl to L-[5-3H]iduronosyl residues was 85/15. In chondroitin, formation of radioactive L-iduronic acid started immediately (Fig. 4b) and reached a constant albeit low level after short incubation times. Formation of radioactive D-glucuronic acid residues took place at a constant but moderate rate. After 96 h of incubation, the ratio of D-[5-3H]glucuronosyl to L-[5-3H]iduronosyl residues was the same as for dermatan (85/15), and this value may correspond to the equilibrium constant for the reaction. The same proportion (85/15) of radioactive L-iduronic to D-glucuronic acid in [5-3H]chondroitin and [5-3H]dermatan was also shown using acid hydrolysis and N-acetylation followed by paper chromatography (data not shown).

In order to determine the distribution of L-iduronosyl- and D-glucuronosyl-containing disaccharides in [5-3H]chondroitin, [5-3H]chondroitin was subjected to chondroitinase AC digestion. Digestion of [5-3H]chondroitin obtained after 4 h of incubation released [3H]O, and yielded in addition L-iduronic acid-containing oligosaccharides (Fig. 5a). The oligosaccharides ranged from octa- to tetrasaccharides, the hexasaccharide being the most prominent. After longer times of incubation, the relative amount of L-iduronic acid-containing disaccharides decreased (Fig. 5b). The distribution of radioactivity in L-iduronic acid-containing disaccharides was, however, not changed.

[5-3H]Dermatan was characterized by selective periodate oxidation of L-iduronic acid residues followed by alkaline elimination of oxidized residues and gel chromatography on Sephadex G-50. [5-3H]Dermatan obtained after 4 h of incubation was mainly cleaved to hexasaccharides containing D-glucuronosyl residues (Fig. 6a). After 96 h of incubation, the D-glucuronosyl-acid-containing disaccharides of [5-3H]dermatan were extended to segments almost as large as the entire chain (Fig. 6b). This means that most of the nonradioactive L-iduronosyl residues had been epimerized to D-glucuronosyl residues during the reaction.

3'-Phosphoadenylylsulfate was included in some of the experiments. As shown earlier (3), a 50% inhibition of tritium incorporation was noted. Furthermore, in spite of the 10,000 x g pellet fraction being active in sulfating endogenous acceptor, no effective sulfation of dermatan was obtained as no radioactivity chromatographed as dermatan sulfate on ion exchange chromatography. A slight increase in the L-iduronic

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**Table I**

| Substrate                          | Radioactivity incorporated into polysaccharide (dpm) | Radioactivity released by chondroitinase ABC (%) |
|-----------------------------------|-----------------------------------------------------|-------------------------------------------------|
| Dermatan                          | 45,340                                              | 98                                              |
| Dermatan sulfate (0.25% fraction) | 980                                                 | 63                                              |
| Dermatan sulfate (25–50% fraction)| 410                                                 | 11                                              |
| Chondroitin                       | 20,340                                              | 96                                              |
| Chondroitin 4-sulfate             | 200                                                 | 34                                              |
| Chondroitin 6-sulfate             | 1,100                                               | 5                                               |
| N/O-Desulfated and re-N-sulfated heparin | 1,410                                              | 8                                               |

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**Fig. 4.** Amount of radioactive D-glucuronic acid (●) and L-iduronic acid (○) in [5-3H]dermatan (a) and [5-3H]chondroitin (b) after various times of incubation. 10 µg of polysaccharide were incubated with epimerase for different periods of time. The amount of tritium released from the product by chondroitinase AC is regarded as originating from D-glucuronic acid residues. Tritium in L-iduronosyl residues is obtained by subtracting tritium obtained by chondroitinase ABC digestion from that obtained by chondroitinase AC.

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**Fig. 3.** Incubation of dermatan (●) and chondroitin (○) with epimerase. 10 µg of polysaccharide were incubated with enzyme as described under "Experimental Procedures."
acid content (from 2 to 12%) was, however, obtained.

Liberation of Tritium from [5-3H]Dermatan/Chondroitin—
Reincubation of [5-3H]dermatan or [5-3H]chondroitin with a
microsomal epimerase preparation resulted in liberation of tritium which could be quantitated after distillation of 3H2O from the incubation mixture. Under appropriate conditions, the reaction is linear with time and with concentration of enzyme protein. Using this assay, it is possible to measure epimerase activity in 1.3 × 10⁶ cultured fibroblasts.

Prolonged incubation (48 h) of [5-3H]dermatan with the
enzyme resulted in a release of 60% of the tritium (Table II).

The radioactivity was preferentially lost from the L-iduronic acid residues.

DISCUSSION

C-5 inversion of D-mannuronic acid to L-glucuronic acid in
alginic acid and of D-glucuronic acid to L-iduronic acid in
heparin, heparan sulfate, and dermatan sulfate takes place on
the polymer level (1, 12, 13). The reactions are similar in the
sense that the epimerization starts with an abstraction of the C-5 hydrogen on the target uronosyl residue. During inversion, the configuration changes at C-5, and a hydrogen is exchanged with the surrounding aqueous medium (3, 4, 14, 15). The various epimerases, however, differ with regard to substrate specificity, cofactor requirement, and pH optimum. Even in fibroblasts which have the capacity to synthesize both heparan sulfate and dermatan sulfate (1, 16), the two epimerases seem to be different (3). This is also supported by the data in Table I which show that N1/O-desulfated heparin is a poor substrate when the conditions are optimal for epimerization of dermatan and chondroitin.

It is notable that dermatan incorporates tritium at a rate
approximately five times higher than that of chondroitin (Fig.
3). After extended incubation times, the products obtained
from chondroitin and dermatan are similar with a ratio of n-
glucuronosyl to L-iduronosyl residues around 85/15. This is
probably close to the equilibrium value. The low yield of L-
iduronic acid residues is in agreement with thermodynamic considerations which favor the D-gluco-configuration (C₃) over the L-ido-configuration (either C₄ or C₅) at equilibrium.

The accumulation of radioactive L-iduronosyl and D-
glucuronosyl residues in dermatan and chondroitin with time is
also in agreement with the equilibrium value. When dermatan is incubated, the rapid accumulation of labeled D-glucuronic acid residues reflects the increase of both labeled and unlabeled D-glucuronic acid residues. The much slower increase of labeled L-iduronic acid residues does not reflect the concentration of unlabeled L-iduronic acid residues as these decrease during the incubation (Fig. 6). When chondroitin is the substrate, the equilibrium concentration of L-iduronic acid

| Incubation time (h) | Released ³H | Radioactive IdoA in the product |
|---------------------|-------------|-------------------------------|
| 0                   | 0           | 18                            |
| 4                   | 9           | 11                            |
| 48                  | 57          | 6                             |

TABLE II

Uronosyl composition of [5-3H]dermatan after incubation with epimerase

[5-3H]Dermatan was purified after incubation with enzyme in
3H2O. It was then reincubated in the absence of 3H2O under otherwise
the same conditions. Release of tritium was measured after distilla-
tion. The [5-3H]dermatan remaining after incubation was character-
ized by chondroitinase AC and ABC digestion followed by distillation.

| Incubation time (h) | Released ³H | Radioactive IdoA in the product |
|---------------------|-------------|-------------------------------|
| 0                   | 0           | 18                            |
| 4                   | 9           | 11                            |
| 48                  | 57          | 6                             |

FIG. 5. Separation of split products obtained by chondroitinase AC digestion of [5-3H]chondroitin on a column of Sephadex G-50. [5-3H]Chondroitin obtained after 4 h (a) and after 96 h (b) of incubation was subjected to digestion and separation. Column size, 8 × 140 cm; fraction volume, 1.8 ml; flow rate, 5 ml/h.

FIG. 6. Separation of split products obtained by periodate oxidation-alkaline elimination of [5-3H]dermatan on a column of Sephadex G-50. [5-3H]Dermatan obtained after 4 h (a) and 96 h (b) of incubation was subjected to oxidation. For details about the separation, see the legend to Fig. 5. The small arrow indicates the elution position of the substrate dermatan.
is obtained within 4 h of incubation. The later increase of labeled D-glucuronic acid residues merely reflects the back-reaction and only results in increased specific activity of D-glucuronic acid residues of the product.

The initially faster increase of the amount of labeled L-iduronic than D-glucuronic acid residues in chondroitin (Fig. 4b) suggests that after the formation of the intermediate, possibly a carbanion, the uptake of a hydrogen induces the reaction to proceed to the L-ido-configuration rather than reverting to the original D-gluco-configuration. If the reaction after uptake of tritium had given D-glucuronyls residues directly, a much higher rate of accumulation of the latter residues would have been expected. The labeled D-glucuronic acid residues may instead be obtained secondarily by conversion of L-iduronic acid residues to D-glucuronic acid ones.

L-iduronic acid-containing disaccharides in chondroitin after both short and long incubation times and in dermatan after long incubation times are distributed singly or in clusters of two to three. This suggests that the epimerase does not leave the substrate chain after conversion of 1 residue but rather attacks the nearest neighbor to yield clusters of L-iduronic acid-containing disaccharides. In vivo, when polymerization, epimerization, and sulfation operate together, blocks of 40–70 L-iduronic acid-containing disaccharides may be obtained.

The epimerization is a crucial step in the biosynthesis of dermatan sulfate as regulation of the epimerase activity may determine the final structure of the polysaccharide chain. Polymerization of UDP-glucuronic acid and UDP-N-acetylgalactosamine yields chondroitin. After or during polymerization, epimerization and finally sulfation of the polysaccharide chain occur. As in the absence of sulfation mainly D-glucuronic acid residues are formed, it is likely that sulfation plays an important role in achieving the final copolymeric structure of dermatan sulfate. This can be envisaged if a close connection between epimerization and sulfation exists that results in a swift sulfation of newly formed L-iduronosyl-N-acetylgalactosamine units which prevents further epimerase attacks. The role of sulfation for L-iduronic acid formation is supported by studies on cell-free dermatan sulfate synthesis in the presence of 3'-phosphoadenylylsulfate (1) and by the slight increase of labeled L-iduronic acid residues obtained in this study in the presence of 3'-phosphoadenyl sulfate. The D-glucuronic acid-containing blocks of different lengths in various dermatan sulfates may be a result of sulfation preceding epimerization or a lack of epimerization followed by sulfation. A lack of sulfation of newly formed iduronosyl-containing regions opens possibilities for a re-attack by the epimerase resulting in D-glucuronic acid-containing regions. Further studies of the relation between sulfation and epimerization in dermatan sulfate biosynthesis may increase the understanding of the regulation of this complex process.

The tritium incorporated into uronosyl residues of dermatan and chondroitin can be released in subsequent incubations with epimerase. This opens possibilities to label dermatan in the presence of H2O. After isolation of the product, it can be used as substrate in a convenient release assay in which released tritium is quantitated after distillation (3, 4) or extraction (17, 18). The latter procedure involving extraction with 25% isoamyl alcohol or in toluene base scintillation is especially useful for enzyme purification work.

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