Biosynthesis of Chondroitin Sulfate

II. INCORPORATION OF SULFATE-35S INTO MICROSMAL CHONDROITIN SULFATE*

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SUMMARY

A microsomal preparation from chick embryo cartilage has been shown to catalyze the incorporation of sulfate-35S from 3'-phosphoadenosine 5'-phosphosulfate-35S into microsomal glycosaminoglycan. This enzyme preparation is the same preparation that previously has been shown to catalyze the formation of chondroitin (nonsulfated) from sugar nucleotide precursors. The microsomal sulfate acceptor appears to be of high sulfate content and appears to be similar to chondroitin sulfate found in a supernatant preparation from chick embryo cartilage. The presence in the microsomal fraction of both sulfotransferase activity and glycosaminoglycan-polymerizing activity, as well as chondroitin sulfate, suggests that sulfation in the cell may take place in close proximity to the location of glycosaminoglycan polymerization.

Soluble preparations from a variety of tissues have been utilized to show incorporation of sulfate-35S into chondroitin sulfate (3-6) and other glycosaminoglycans (mucopolysaccharides). These glycosaminoglycans were usually commercial preparations which had been separated from their major protein components, although endogenous soluble chondroitin sulfate has also been utilized as a sulfate acceptor with soluble enzymes from cartilage (7-9). Recently, evidence has been obtained by electron microscopy indicating that the sulfation of glycosaminoglycans might occur on the endoplasmic reticulum or in the Golgi zone, in close proximity to the polymerization of the glycosaminoglycan (10, 11). If this were the case, one might expect to find sulfotransferase activity in microsomal particles. In this regard, the synthesis of the sulfated glycosaminoglycan, heparin, has been shown with a microsomal fraction from mast cells (12-14). Sulfation was indeed found to be catalyzed by the same microsomal preparation that was involved in the glycosaminoglycan polymerization. Furthermore, newly synthesized as well as endogenous heparin was found to remain bound to these microsomal particles.

Previous work has already shown the formation of chondroitin with a microsomal preparation from chick embryo cartilage (2, 8). The present investigation shows the presence of microsomal sulfotransferase activity and the transfer of sulfate to endogenous glycosaminoglycan bound to these same microsomal particles.*

EXPERIMENTAL PROCEDURE

Unlabeled and 35S-labeled 3'-phosphoadenosine 5'-phosphosulfate was synthesized with yeast enzyme by the method of Robbins (16), chromatographed on Dowex 1-X8 according to the method of Wilson, Asahi, and Bandurski (17), and finally obtained as previously described (13).

Chondroitin sulfate from bovine nasal septa (mostly chondroitin-4-sulfate with some chondroitin-6-sulfate) and hyaluronic acid from umbilical cord (Grade I) were purchased from Sigma. H235SO4 was purchased from New England Nuclear. Pancreatin was purchased from Viobin (Monticello, Illinois), and testicular hyaluronidase from Sigma. Frozen 14-day-old chick embryos were purchased from Pel-Freez Biologicals (Rogers, Arkansas).

Twice washed microsomal preparations sedimenting between 10,000 and 105,000 × g were prepared from chick embryo epiphyses, as previously described (2, 18). An enzymic preparation was also obtained from the 105,000 × g supernatant fraction. The glycosaminoglycan of the supernatant fraction was obtained by precipitation with 0.1% cetyltrimethylammonium bromide and Celite followed by extraction with 0.4 M NaCl and 1.2 M NaCl (19, 20). All of the extracted material was pooled, and cetyltrimethylammonium was precipitated with potassium thiocyanate (19). The solution containing the glycosaminoglycan was then dialyzed overnight against several changes of water in order to remove the NaCl and excess potassium thiocyanate (19).

Suzuki et al. have also recently described the presence of sulfotransferase activity in particulate preparations from chick embryo cartilage (15).

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cyanoate. The solution within the dialysis tubing was evaporated under vacuum, and the glycosaminoglycans were dissolved in approximately 0.5 ml of water.

Microsomal and supernatant enzymic preparations were incubated at 37° together with 3'-phosphoadenosine 5'-phosphosulfate-35S for varying lengths of time. The resulting labeled glycosaminoglycan-35S was isolated from these reaction mixtures, as previously described (2, 19), by chromatography on Whatman No. 1 paper in ethanol-1 m ammonium acetate (7:3), pH 7.8. The origins of the chromatograms (containing all of the glycosaminoglycans) were cut out and incubated overnight at 37° with 2 ml of 1% pancreatin in 0.05 m Tris, pH 8.5. This solubilized all the radioactivity at the origins. The pancreatin suspensions containing the glycosaminoglycans were boiled and centrifuged at 20,000 X g for 10 min. Under these conditions it was found that varying amounts (usually 10 to 20%) of glycosaminoglycan-35S were precipitated with the boiled pancreatin. This radioactive material could be quantitatively eluted from the pellet by resuspension in 1 m NaCl and recentrifugation at 20,000 X g. Therefore, this procedure was carried out in all cases. The 1 m NaCl washing was added to the initial supernatant from the boiled pancreatin incubation and dialyzed overnight against 0.01 m K2SO4 and then against several changes of water.

After microsomal enzyme was incubated with 3'-phosphoadenosine 5'-phosphosulfate-35S, the following procedure was utilized for one experiment. (a) An aliquot of the reaction mixture was spotted on Whatman No. 1 paper. (b) The remainder of the reaction mixture was suspended in 12 ml of 0.25 m sucrose and centrifuged at 105,000 X g for 20 min. The pellet was resuspended in 0.1 ml of 1 m NaCl and an aliquot was spotted. (c) An additional 12 ml of 1 m NaCl were added and the suspension was centrifuged at 105,000 X g for 20 min. The pellet was resuspended in 0.1 ml of water and an aliquot was spotted. (d) The suspension was then heated to 100° in a boiling water bath for 3 min and centrifuged at 2,500 rpm and an aliquot of the supernatant was spotted. (e) The sediment was suspended in 0.1 ml of 1 m NaCl, the suspension was centrifuged at 2,500 rpm, and an aliquot of the supernatant was spotted. (f) The total remaining sediment was spotted. The chromatogram with these spots was then developed as above. The radioactive material at the origins was eluted with pancreatin, dialyzed, and assayed.

Samples of labeled and nonlabeled cartilage glycosaminoglycan were precipitated with 0.1% cetyltrimethylammonium bromide in the presence of Celite, and extracted with varying concentrations of NaCl.

Other samples of glycosaminoglycan-35S were placed, together with nonlabeled cartilage glycosaminoglycan or carrier hyaluronic acid and chondroitin sulfate, on a DEAE-cellulose column and eluted with a LiCl logarithmic gradient. Aliquots of each fraction were assayed for radioactivity and for the glycosaminoglycans.

A column of Sephadex G-200 was utilized for gel filtration of cartilage glycosaminoglycan and glycosaminoglycan-35S, together with standard hyaluronic acid and chondroitin sulfate. Another sample of microsomal glycosaminoglycan-35S was incubated at 25° with 0.5 × 106 m NaOH for 1 hour and filtered on Sephadex G-200 in identical fashion.

Samples of glycosaminoglycan-35S were incubated together with carrier chondroitin sulfate in the presence of testicular hyaluronidase. Gel filtration of the reaction mixtures was then performed on a column of Sephadex G-100 as previously described (2, 13). Aliquots of the fractions were assayed for radioactivity and the presence of uranic acid. Uronic acid-containing material was assayed by the Bitter and Muir (21) modification of the carbazole method of Diseche (22). Radioactivity was determined with a low background (0.5 cpm) gas flow counter or with a liquid scintillation counter. Bray's mixture (23) was used for the latter.

### RESULTS

Both microsomal and supernatant preparations were found to be capable of transferring sulfate-35S from 3'-phosphoadenosine 5'-phosphosulfate-35S to endogenous glycosaminoglycan in the microsomal or supernatant fractions, respectively. The sulfotransferase activity of the supernatant preparation appeared to be greater than that of the microsomal preparation. However, there was much less endogenous acceptor in the microsomal preparation, and this preparation also degraded 3'-phosphoadenosine 5'-phosphosulfate very rapidly. Either of these factors may have accounted for the apparently lower sulfotransferase activity of the microsomal preparation, so no meaningful conclusions could be reached regarding the relative activities of the two preparations.

Recovery of glycosaminoglycan-35S in the microsomal pellet after various washings is shown in Table I. The bulk of the glycosaminoglycan-35S remained with the particulate material on centrifugation and after washing with 0.25 m sucrose. The radioactivity was not significantly extracted from the particulate preparation with 1 m NaCl, either before or after denaturation of the preparation by boiling. However, digestion with pancreatin followed by extraction with NaCl quantitatively solubilized the radioactivity.

When sulfate-35S was substituted for 3'-phosphoadenosine 5'-phosphosulfate-35S, or when the microsomal enzyme was boiled

### Table I

<table>
<thead>
<tr>
<th>Washings</th>
<th>Sediment</th>
<th>Supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole reaction mixture</td>
<td>13,500</td>
<td></td>
</tr>
<tr>
<td>Centrifugation in 0.25 m sucrose</td>
<td>9,800</td>
<td>3,700</td>
</tr>
<tr>
<td>Centrifugation in 1 m NaCl</td>
<td>7,200</td>
<td>2,600</td>
</tr>
<tr>
<td>Centrifugation in water (after boiling)</td>
<td>6,600</td>
<td>100</td>
</tr>
<tr>
<td>Centrifugation in 1 m NaCl (after boiling)</td>
<td>5,800</td>
<td>50</td>
</tr>
<tr>
<td>Total remaining pellet</td>
<td>5,800</td>
<td></td>
</tr>
</tbody>
</table>

* Each total was corrected for the losses of radioactivity resulting from the taking of aliquots in the previous steps.

* Calculated by subtraction of counts per min in sediment from counts per min in sediment of preceding step.
prior to incubation with 3'-phosphoadenosine 5'-phosphosulfate-35S, there was negligible incorporation of sulfate-35S into the microsomal material (less than 3% of the amount with active enzyme and 3'-phosphoadenosine 5'-phosphosulfate-35S).

The incorporation of sulfate-35S into microsomal glycosaminoglycan (shown in Fig. 1) appeared to continue for at least 1 hour and then to reach a maximum. The microsomal preparation also degraded 3'-phosphoadenosine 5'-phosphosulfate-35S with the appearance of inorganic sulfate-35S. With the initial 3'-phosphoadenosine 5'-phosphosulfate-35S, there was negligible incorporation of sulfate-35S into the microsomal material (less than 3% of the amount with active enzyme and 3'-phosphoadenosine 5'-phosphosulfate-35S). The microsomal preparation also contained 3'-phosphoadenosine 5'-phosphosulfate-35S with cetyltrimethylammonium bromide and extraction with NaCl are shown in Table II. Most of the radioactive material was extracted with 1.2 M NaCl, the majority of NaCl necessary for elution of chondroitin sulfate. Some material, however, was extractable with the lower NaCl concentration (0.4 M) sufficient for extraction of glycosaminoglycans of low sulfate content. Some of the commercial chondroitin sulfate was also extractable at this lower NaCl concentration.

Chromatography on DEAE-cellulose of supernatant glycosaminoglycan, supernatant glycosaminoglycan-35S, and microsomal glycosaminoglycan-35S is shown in Fig. 2. Both of the glycosaminoglycan-35S samples were obtained after 5 min in incubations of 3'-phosphoadenosine 5'-phosphosulfate-35S with the enzymic preparations. Essentially all of the radioactivity (both microsomal and supernatant glycosaminoglycan-35S) was eluted from the column near carrier commercial chondroitin sulfate. However, the supernatant glycosaminoglycan-35S appeared to be somewhat more homogeneous than the microsomal glycosaminoglycan-35S. It is noteworthy that some of the glycosaminoglycan-35S was found in fractions appearing after the elution of the carrier chondroitin sulfate, indicating that this radioactive material either contains a higher amount of sulfate or is a larger molecule than the chondroitin sulfate standard. The nonlabeled glycosaminoglycan obtained from the supernatant cartilage preparation appeared in similar fractions to those in which the supernatant glycosaminoglycan-35S appeared. There is, however, a suggestion that the earlier fractions containing supernatant glycosaminoglycan accept more sulfate-35S than the later fractions. This might indicate that smaller molecules or molecules of lesser sulfate content act as slightly better sulfate acceptors. Amounts were too small to establish this point. Except for a very small but consistent peak of glycosaminoglycan and glycosaminoglycan-35S near the area of hyaluronic acid appearance, there seems to be no glycosaminoglycan appearing in areas indicating low sulfate content.

Microsomal glycosaminoglycan-35S (500 cpm) and supernatant glycosaminoglycan-35S (500 cpm) were each added to solutions containing commercial chondroitin sulfate (3.5 μmoles of uronic acid by carbazole test), 0.1% cetyltrimethylammonium bromide, 0.03 M NaCl, and 50 mg of Celite. A sample of nonradioactive supernatant glycosaminoglycan was added to cetyltrimethylammonium bromide, NaCl, and Celite in the same fashion. Each sample was centrifuged for 10 min at 2000 × g, and aliquots of the supernatants were assayed for radioactivity or uronic acid content or both. The sediments were then extracted repeatedly with NaCl as shown. Each supernatant was collected by centrifugation at 2000 × g for 5 min and assayed for radioactivity or uronic acid content or both.

<table>
<thead>
<tr>
<th>Sample analyzed</th>
<th>Microsomal glycosaminoglycan-35S</th>
<th>Supernatant glycosaminoglycan-35S</th>
<th>Supernatant glycosaminoglycan</th>
<th>Chondroitin sulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total radioactivity</td>
<td>Total uronic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CPM</td>
<td>μmoles</td>
<td>CPM</td>
<td>μmoles</td>
</tr>
<tr>
<td>Supernatant after precipitation with cetyltrimethylammonium and 0.03 M NaCl</td>
<td>28</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Extraction with 0.03 M NaCl</td>
<td>13</td>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Extraction with 0.4 M NaCl</td>
<td>105</td>
<td>70</td>
<td>0.39</td>
<td>0.83</td>
</tr>
<tr>
<td>Second</td>
<td>10</td>
<td>9</td>
<td>0.21</td>
<td>0.22</td>
</tr>
<tr>
<td>Third</td>
<td>4</td>
<td>5</td>
<td>0.22</td>
<td>0.08</td>
</tr>
<tr>
<td>Fourth</td>
<td>4</td>
<td>2</td>
<td>0.13</td>
<td>0.01</td>
</tr>
<tr>
<td>Extraction with 1.2 M NaCl</td>
<td>220</td>
<td>245</td>
<td>1.48</td>
<td>1.90</td>
</tr>
<tr>
<td>First</td>
<td>25</td>
<td>40</td>
<td>0.23</td>
<td>0.40</td>
</tr>
<tr>
<td>Second</td>
<td>7</td>
<td>12</td>
<td>0.07</td>
<td>0.09</td>
</tr>
<tr>
<td>Fourth</td>
<td>3</td>
<td>7</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>Extraction with 2.1 M NaCl</td>
<td>10</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>First</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>430</td>
<td>438</td>
<td>3.06</td>
<td>3.56</td>
</tr>
</tbody>
</table>

Fig. 1. Incorporation of sulfate-35S from 3'-phosphoadenosine 5'-phosphosulfate-35S into microsomal glycosaminoglycan with time. The reaction mixture contained, in a volume of 0.08 ml: 0.05 M Tris, pH 7.5; 0.01 M MgCl2; 3'-phosphoadenosine 5'-phosphosulfate-35S, 675 μmoles (80 × 106 cpm); and 0.03 M of the microsomal preparation. The reaction mixture was incubated at 37°. Aliquots of 0.002 ml were removed at the indicated intervals, and the radioactive glycosaminoglycan was isolated and assayed for radioactivity as described in the text.
There were 125 ml of water in the mixing flask and 1 M hyaluronidase, there was complete degradation to material resembling testicular hyaluronidase.

The degree of sulfation of the microsomal endogenous acceptor could not be measured directly. However, it is apparent that the addition of sulfate-\(^{35}\)S was not in great enough quantity to change measurably the anionic nature of the acceptor. This is shown by comparing the anionic characteristics of glycosaminoglycan-\(^{35}\)S formed after incubation of the microsomal preparation with 3'-phosphoadenosine 5'-phosphosulfate-\(^{35}\)S for 5 min and for 2 hours. Incorporation of sulfate-\(^{35}\)S was shown to increase for 1 to 2 hours, yet the elution from DEAE-cellulose of glycosaminoglycan-\(^{35}\)S formed after a 5-min incubation was similar to that of glycosaminoglycan-\(^{35}\)S formed after 2 hours of incubation. Therefore, the amount of sulfate-\(^{35}\)S incorporated would appear to be insignificant in proportion to the amount of sulfate already present in the acceptor. Furthermore, the glycosaminoglycan-\(^{35}\)S isolated from the microsomal fraction appears similar to that isolated from the supernatant fraction, and the nonsulfated glycosaminoglycan from the supernatant

**FIG. 2.** Gradient elution of glycosaminoglycan and glycosaminoglycan-\(^{35}\)S from a DEAE-cellulose column. A, microsomal glycosaminoglycan-\(^{35}\)S (7000 cpm), together with hyaluronic acid (0.8 mg) and chondroitin sulfate (1.6 mg), was eluted from a column of DEAE-cellulose (1 X 5 cm) with a LiCl logarithmic gradient. There were 125 ml of water in the mixing flask and 1 M LiCl in the reservoir. Fractions of 2.5 ml were collected. B, an identical elution was carried out with supernatant glycosaminoglycan-\(^{35}\)S (6500 cpm) and approximately 2 mg of nonradioactive supernatant glycosaminoglycan. Both \(^{35}\)S-labeled preparations were obtained after 5-min incubations of 3'-phosphoadenosine 5'-phosphosulfate-\(^{35}\)S with the enzymic preparations. Fractions from both columns were assayed for radioactivity and for glycosaminoglycan content (carbazole determination).

Gel filtration of cartilage glycosaminoglycan and glycosaminoglycan-\(^{35}\)S is shown in Fig. 3. Glycosaminoglycan-\(^{35}\)S from microsomal or supernatant sources was identically excluded from Sephadex G-200. Nonradioactive glycosaminoglycan from cartilage supernatant was likewise excluded, while commercial chondroitin sulfate was retarded by the gel. A sample of glycosaminoglycan-\(^{35}\)S that had been treated with NaOH (to remove any protein that might have remained attached after pancreatin digestion) was also excluded from the column identically with the other glycosaminoglycan-\(^{35}\)S samples.

After incubation of glycosaminoglycan-\(^{35}\)S with testicular hyaluronidase, there was complete degradation to material retarded by Sephadex G-100.

**DISCUSSION**

The present study describes a cartilage microsomal preparation which catalyzes incorporation of sulfate-\(^{35}\)S from 3'-phosphoadenosine 5'-phosphosulfate-\(^{35}\)S into glycosaminoglycan bound to the microsomal preparation. This glycosaminoglycan remains nondialyzable after it is solubilized from the microsomal preparation by pancreatin. Precipitation with quaternary ammonium salts followed by extraction with NaCl yields results comparable to those of the extraction of commercial chondroitin sulfate. Chromatography of this glycosaminoglycan-\(^{35}\)S on an anion exchange column also yields results which indicate that the microsomal bound acceptor is similar in degree of sulfation to chondroitin sulfate. The glycosaminoglycan-\(^{35}\)S appears to be larger than commercial chondroitin sulfate (by Sephadex column filtration), and by the same criterion is similar in size to the newly formed nonsulfated glycosaminoglycan previously shown to be formed by similar microsomal preparations (2). The glycosaminoglycan-\(^{35}\)S is totally susceptible to degradation by testicular hyaluronidase.

Similar experiments which utilize both enzyme and endogenous acceptor from the supernatant fraction show similar results.

**Fig. 3.** Gel filtration of cartilage glycosaminoglycan and glycosaminoglycan-\(^{35}\)S. Microsomal glycosaminoglycan-\(^{35}\)S (280 cpm) and supernatant glycosaminoglycan-\(^{35}\)S (280 cpm) were each added to samples of hyaluronic acid (1.5 mg) and filtered on a column of Sephadex G-200 (1 X 60 cm) with 0.05 M LiCl. Fractions of 1.2 ml were collected and analyzed for uronic acid (carbazole) and radioactivity. Samples of glycosaminoglycan-\(^{35}\)S were added to nonradioactive supernatant glycosaminoglycan (approximately 1.5 mg) and to commercial chondroitin sulfate (2 mg). Each mixture was filtered on Sephadex G-200 as above. Superimposed results of the filtrations are shown. Microsomal glycosaminoglycan-\(^{35}\)S and supernatant glycosaminoglycan-\(^{35}\)S were indistinguishable from each other in elution pattern.
fraction appears to be entirely of high anionic content. This implies that either the microsomal glycosaminoglycan is all highly sulfated or, if nonsulfated glycosaminoglycan is present in the microsomal preparation, it does not act as a sulfate acceptor. This latter supposition does not seem likely, particularly since a newly formed nonsulfated glycosaminoglycan has been shown to act as an effective sulfate acceptor in a similar microsomal system from mast cell tumor (14).

The relative activities of the microsomal sulfotransferase and the supernatant sulfotransferase could not be estimated, since factors such as amount of endogenous acceptors and hydrolysis of 3'-phosphoadenosine 5'-phosphosulfate could not be controlled. The supernatant sulfotransferase may be microsomal enzyme that has been solubilized, or it may be a separate enzyme. The present data could not distinguish between these possibilities.

The problem of the sequence of polymerization and sulfation still remains unsolved. Sulfation can certainly take place to some degree after polymerization, but this may not be the natural course of events. The presence of sulfated glycosaminoglycan and sulfotransferase activity in the same microsomal preparation that contains glycosaminoglycan-polymerizing activity suggests that these reactions might occur at the same location and perhaps at the same time. The apparent absence of nonsulfated glycosaminoglycan in the microsomal preparation reinforces this hypothesis.

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