Biosynthesis of Chondroitin Sulfate

II. INCORPORATION OF SULFATE-\(^{35}S\) INTO MICROSMAL CHONDROITIN SULFATE*

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SILVANA DE LUCA AND JEREMIAH E. SILBERT

From the Oral Disease Research Laboratory, Boston Veterans Administration Hospital, and the Department of Medicine, Tufts University School of Medicine, Boston, Massachusetts 02130

SUMMARY

A microsomal preparation from chick embryo cartilage has been shown to catalyze the incorporation of sulfate-\(^{35}S\) from 3'-phosphoadenosine 5'-phosphosulfate-\(^{35}S\) 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-\(^{35}S\) 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 \(^{35}S\)-labeled 3'-phosphoadenosine 5'-phosphosulfate was synthesized in 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. \(\mathrm{H}_{2}\mathrm{SO}_{4}\) 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 \(\times\) \(g\) were prepared from chick embryo epiphyses, as previously described (2, 18). An enzymic preparation was also obtained from the 105,000 \(\times\) \(g\) 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 \(\mathrm{m}\) \(\mathrm{NaCl}\) and 1.2 \(\mathrm{m}\) \(\mathrm{NaCl}\) (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 \(\mathrm{NaCl}\) and excess potassium thiocyanate.

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† To whom requests for reprints should be addressed (Veterans Administration Hospital, 150 South Huntington Ave., Boston, Massachusetts 02130).

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proximately 0.5 ml of water.

Microsomal and supernatant enzymic preparations were in-

culated at 37° together with 3'-phosphoadenosine 5'-phospho-
sulfate-35S for varying lengths of time. The resulting labeled
glycosaminoglycan-35S was isolated from these reaction mixtures,
as previously described (2, 13), by chromatography on What-
man No. 1 paper in ethanol-1 m ammonium acetate (7: 3), pH
7.2. 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 × g for 10 min. Under these conditions
it was found that varying amounts (usually 10 to 20%) of glycos-
aminoglycan-35 S 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 × g. Therefore, this procedure was carried out in all
cases. The 1 m NaCl washing was added to the initial super-
natant from the boiled pancreatin incubation and dialyzed over-
night against 0.01 m K2SO4 and then against several changes of
water.

After microsomal enzyme was incubated with 3'-phospho-
adename 5'-phosphosulfate-35S, the following procedure was
utilized for one experiment. (a) An aliquot of the reaction mix-

ture 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 × 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 × 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 ma-
terial 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 concen-
trations of NaCl.

Other samples of glycosaminoglycan-35S were placed, together
with nonlabeled cartilage glycosaminoglycan or carrier hyalu-
ronic 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 glycosamin-
oglycans.

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

Samples of glycosaminoglycan-35S were incubated together
with 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 de-
scribed (2, 13). Aliquots of the fractions were assayed for radio-
activity and the presence of uranic acid.

Uronic acid-containing material was assayed by the Bitter
and Muir (21) modification of the carbazole method of Dische
(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.

### Table I

**Recovery of glycosaminoglycan-35S after incubation of
3'-phosphoadenosine 5'-phosphosulfate-35S**

with microsomal preparation

<table>
<thead>
<tr>
<th>Washings</th>
<th>Sediment</th>
<th>Supernatant</th>
<th>Total cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole reaction mixture</td>
<td></td>
<td></td>
<td>13,500</td>
</tr>
<tr>
<td>Centrifugation in 0.25 m sucrose</td>
<td>9,800</td>
<td>3,700</td>
<td>13,500</td>
</tr>
<tr>
<td>Centrifugation in 1 m NaCl</td>
<td>7,200</td>
<td>2,600</td>
<td>10,800</td>
</tr>
<tr>
<td>Centrifugation in water (after boiling)</td>
<td>6,600</td>
<td>100</td>
<td>6,700</td>
</tr>
<tr>
<td>Centrifugation in 1 m NaCl (after boiling)</td>
<td>5,800</td>
<td>50</td>
<td>5,850</td>
</tr>
</tbody>
</table>

* Each total was corrected for the losses of radioactivity result-
ing 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.

### 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 sulfo-
transferase 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'-phospho-
adenosine 5'-phosphosulfate very rapidly. Either of these
factors may have accounted in part for the apparently lower
sulfotransferase activity of the microsomal preparation, so no
meaningful conclusions could be reached regarding the rela-
tive 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
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'-phosphate of 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 precipitation of cartilage glycosaminoglycan and glycosaminoglycan-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 mobility 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 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 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, 0.05 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.

![Figure 1: Incorporation of sulfate-35S from 3'-phosphoadenosine 5'-phosphosulfate-35S into microsomal glycosaminoglycan with time.](image-url)
The present study describes a cartilage microsomal preparation which catalyzes incorporation of sulfate-35S from 3'-phosphoadenosine 5'-phosphosulfate-35S 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-35S 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-35S 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-35S is totally susceptible to degradation by testicular hyaluronidase.

Similar experiments which utilize both enzyme and endogenous acceptor from the supernatant fraction show similar results. The degree of sulfation of the microsomal endogenous acceptor could not be measured directly. However, it is apparent that the addition of sulfate-35S 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-35S formed after incubation of the microsomal preparation with 3'-phosphoadenosine 5'-phosphosulfate-35S for 5 min and for 2 hours. Incorporation of sulfate-35S was shown to increase for 1 to 2 hours, yet the elution from DEAE-cellulose of glycosaminoglycan-35S formed after a 5-min incubation was similar to that of glycosaminoglycan-35S formed after 2 hours of incubation. Therefore, the amount of sulfate-35S incorporated would appear to be insignificant in proportion to the amount of sulfate already present in the acceptor. Furthermore, the glycosaminoglycan-35S isolated from the microsomal fraction appears similar to that isolated from the supernatant fraction, and the nonradioactive glycosaminoglycan from the supernatant...
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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