Incorporation of $^{35}$SO$_4$ into Endogenous Heparin by a Microsomal Fraction from Mast Cell Tumors*

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**SUMMARY**

A mouse mast cell tumor microsomal preparation which catalyzes the incorporation of $^{35}$SO$_4$ from 3'-phosphoadenosine 5'-phosphosulfate-$^{35}$S into microsomal heparin has been described. This enzyme preparation is the same preparation that previously has been shown to catalyze the incorporation of sugars into a microsomal glycosaminoglycan related to heparin. It is suggested that polysaccharide polymerization and sulfation take place in close proximity in the cell.

**EXPERIMENTAL PROCEDURE**

$^{35}$S-Labeled and unlabeled 3'-phosphoadenosine 5'-phosphosulfate was synthesized with yeast enzyme according to the method of Robbins (16). The product was chromatographed on Dowex 1-X8 (200 to 400 mesh, chloride form) according to the method of Wilson, Asahi, and Bandurski (17). Fractions containing 3'-phosphoadenosine 5'-phosphosulfate were adsorbed on charcoal, eluted with 50% ethanol (made alkaline with NH$_4$OH), dried under vacuum, and chromatographed for 18 hours on Whatman No. 1 paper with ethanol-1 M ammonium acetate (7:3), pH 7.8 (18). The single ultraviolet absorbing radioactive spot ($R_{UMP} = 0.41$) was cut out, washed with absolute ethanol, and eluted with the paper with water.

Heparin was purchased from Calbiochem; chondroitin sulfate, hyaluronic acid, and ATP were purchased from Sigma, and $^{35}$SO$_4$ was obtained from New England Nuclear Corporation. Pancreatin was purchased from Viobin Corporation and testicular hyaluronidase from Sigma. Heparinase was prepared as previously described (12) from an acetone powder preparation of Flavobacterium heparinum (a generous gift of Dr. A. N. Payza). DBA mice bearing the mast cell tumors were initially obtained from Dr. T. Dunn (19).

The microsomal preparations were generally obtained as previously described (20). Homogenates were centrifuged at 20,000 $\times$ g removing the granules which contain the bulk of mast cell heparin. (These granules can be sedimented at 8,000 $\times$ g.) The supernatants were then centrifuged at 105,000 $\times$ g and the resulting microsomal pellets were then washed by centrifugation at 105,000 $\times$ g three times rather than twice as described previously.

Microsomal heparin was obtained as follows. The total microsomal preparation from 840 g of mast cell tumor was boiled and then incubated with 5 g of pancreatin for 18 hours at 37°C in 8 ml of 0.05 M Tris, pH 7.2, and centrifuged at 20,000 $\times$ g, the sediment was suspended in 5 ml of 1 M NaCl and the suspension was centrifuged again at 20,000 $\times$ g. The supernatant was added to the first supernatant and together they were dialyzed overnight against several changes of water. The solution within the dialysis tubing was concentrated under vacuum to 20 ml and then extracted with an equal amount of 88% phenol to remove residual protein. The aqueous layer was extracted twice with ether to remove phenol, and the total aqueous layer was then placed on a DEAE-cellulose column and eluted with a LiCl linear gradient.

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phosphonate-35S were incubated together at 37°, usually for 23 hours. The 35SO4-labeled product was isolated as follows. The total reaction mixtures were spotted on Whatman No. 1 paper, and the chromatograms with these spots were then developed in ethanol-l M ammonium acetate (7:3), pH 7.8. (In this chromatographic system, polysaccharides remain at the origin while 3'-phosphoadenosine 5'-phosphosulfate and SO4 are found with Rfu equal to 0.41 and 1.21, respectively.) The origins of the chromatograms were then cut out and eluted with pancreatin as previously described (12). The suspensions were centrifuged, the pellets extracted with 1 M NaCl as described above, and the combined supernatants were dialyzed against water. Aliquots of the solutions within the dialysis tubing were removed for assay of radioactivity. There was usually negligible loss of 35S during dialysis, but when amounts of microsomal preparation greater than 0.025 ml were used, there was occasionally some unidentified dialyzable radioactive material found at the origins of the chromatograms.

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 centrifuged at 2,500 rpm in an International centrifuge, and aliquots of the supernatant and sediment were spotted separately. (c) The sediment was resuspended 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. (d) An additional 12 ml of 1 M NaCl were added, and the suspension was centrifuged at 105,000 × g for 90 min. The pellet was resuspended in 0.1 ml of water, and an aliquot was spotted. (e) The suspension was then heated to 100° in a boiling water bath for 3 min, centrifuged at 2,500 rpm, and an aliquot of the supernatant was spotted. (f) 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. (g) 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.

After isolation of the 35SO4-labeled material a sample was precipitated with 0.5% cetyltrimethylammonium bromide in the presence of Celite, and eluted with varying concentrations of NaCl (21, 22).

Another sample of 35SO4-labeled material was placed together with carrier hyaluronic acid, chondroitin sulfate, and commercial heparin on a DEAE-cellulose column and eluted with a LiCl logarithmic gradient. Aliquots of each fraction were assayed for radioactivity and for the carrier glycosaminoglycans. A similar sample of 35SO4-labeled material was chromatographed identically without carrier glycosaminoglycans. The fractions containing radioactivity were pooled and dialyzed against several changes of water, and the solution within the dialysis tubing was evaporated under vacuum. Samples of this 35SO4-labeled material were incubated together with carrier hyaluronic acid, or carrier commercial heparin, in the presence of testicular hyaluronidase or bacterial heparinase. The reaction mixtures were then placed on a column of Sephadex G-100 and chromatography was performed. Aliquots of the fractions were assayed for radioactivity and the presence of uronic acid.

Uronic acid containing material was assayed by the carbazole method of Dische (23). Heparin was assayed by the carbazole method and by the Azure A method of Jacques, Monkhouse, and Stewart (24). Hexosamines were determined by the method of Gandolfi (25). Radioactivity was determined with a low background gas flow counter or with a scintillation counter.

RESULTS

Results of the chromatography of mast cell microsomal polysaccharide on DEAE-cellulose are shown in Fig. 1. The material obtained was eluted in an area similar to that of commercial heparin (see Fig. 3). No significant polysaccharide was found in the areas of elution of chondroitin sulfate or hyaluronic acid. The total yield of microsomal polysaccharide from the 845 g of DBA mouse mast cell tumor was calculated as 9.4 mg of heparin based on uronic acid determinations. Based on these determinations, 0.05 ml of the microsomal enzyme preparation would contain approximately 5 μg of heparin or 10 μmole of glucosamine. Azure A determinations and glucosamine determinations of this material yielded comparable results. Chromatography of the control pancreatin (no microsomes) showed no uronic acid in the fractions corresponding to the elution of heparin.

Recovery of 35SO4-labeled material in the microsomal pellet after various washings is shown in Table I. The bulk of the radioactivity 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 boiling. However digestion with pancreatin followed by extraction with NaCl solubilized the radioactivity quantitatively.

When 35SO4 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 35SO4 into the microsomal...
material (less than 2% of the amount with active enzyme and 3'-phosphoadenosine 5'-phosphosulfate-35S). When low concentrations (10^-7 M) of high specific activity 3'-phosphoadenosine 5'-phosphosulfate-35S were utilized, as much as 25% of the radioactivity of the 3'-phosphoadenosine 5'-phosphosulfate-35S was incorporated.

The incorporation of 35SO4 into glycosaminoglycan with time is shown in Fig. 2. Incorporation ceased after 2 hours, although the enzyme was still active at this time. (When microsomal preparations were incubated for 2 hours at 37°C before the addition of 3'-phosphoadenosine 5'-phosphosulfate-35S, there was only

- **Table I**

*Recovery of 35SO4-labeled material after incubation of 3'-phosphoadenosine 5'-phosphosulfate-35S with microsomal preparation*

<table>
<thead>
<tr>
<th>Washings</th>
<th>Sedimenta</th>
<th>Supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole reaction mixture</td>
<td>610</td>
<td></td>
</tr>
<tr>
<td>Low speed centrifugation</td>
<td>550</td>
<td>100</td>
</tr>
<tr>
<td>High speed centrifugation in 0.25 M sucrose</td>
<td>425</td>
<td>109</td>
</tr>
<tr>
<td>High speed centrifugation in 1 M NaCl</td>
<td>300</td>
<td>125</td>
</tr>
<tr>
<td>Low speed centrifugation after boiling</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Low speed centrifugation boiled 1 M NaCl</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total remaining pellet</td>
<td>965</td>
<td></td>
</tr>
</tbody>
</table>

a Each total was corrected for the losses of radioactivity due to the taking of aliquots in the previous steps.

b Calculated by subtraction of counts per min in sediment from counts per min in sediment of preceding step.

![Graph showing incorporation of 35SO4 from 3'-phosphoadenosine 5'-phosphosulfate-35S into microsomal glycosaminoglycan with time.](http://www.jbc.org/)

The reaction mixture contained, in a total volume of 0.1 ml: 0.05 M Tris, pH 7.5; 0.01 M MgCl2; 0.00015 M 3'-phosphoadenosine 5'-phosphosulfate-35S, 10^6 cpm total; 0.05 ml of microsomal preparation. Following incubation at 37°C for 13 hours, the radioactivity of the 3'-phosphoadenosine 5'-phosphosulfate-35S was determined.

The precipitation of the 35SO4-labeled material with cetyltrimethylammonium bromide is shown in Table II. Approximately half of the radioactive material was eluted from the column in the area of heparin, while the remainder of 35SO4-labeled material appeared in areas of elution of glycosaminoglycans of lesser sulfate content. These results are consistent with the findings of NaCl elution from the cetyltrimethylammonium bromide precipitate.

**Table II**

*Characteristics of precipitation of 35SO4-labeled material with cetyltrimethylammonium bromide*

<table>
<thead>
<tr>
<th>Sample analyzed</th>
<th>Total activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant after precipitation with cetyltrimethylammonium and 0.03 M NaCl</td>
<td>30</td>
</tr>
<tr>
<td>Extraction with 0.03 M NaCl</td>
<td>0</td>
</tr>
<tr>
<td>Extraction with 0.4 M NaCl</td>
<td>30</td>
</tr>
<tr>
<td>First</td>
<td>3</td>
</tr>
<tr>
<td>Second</td>
<td>0</td>
</tr>
<tr>
<td>Third</td>
<td>2</td>
</tr>
<tr>
<td>Fourth</td>
<td>0</td>
</tr>
<tr>
<td>Fifth</td>
<td>0</td>
</tr>
<tr>
<td>Extraction with 1.2 M NaCl</td>
<td>265</td>
</tr>
<tr>
<td>First</td>
<td>6</td>
</tr>
<tr>
<td>Second</td>
<td>10</td>
</tr>
<tr>
<td>Third</td>
<td>6</td>
</tr>
<tr>
<td>Fourth</td>
<td>0</td>
</tr>
<tr>
<td>Fifth</td>
<td>0</td>
</tr>
<tr>
<td>Extraction with 2.1 M NaCl</td>
<td>240</td>
</tr>
<tr>
<td>First</td>
<td>5</td>
</tr>
<tr>
<td>Second</td>
<td>3</td>
</tr>
<tr>
<td>Third</td>
<td>0</td>
</tr>
<tr>
<td>Fourth</td>
<td>0</td>
</tr>
<tr>
<td>Fifth</td>
<td>0</td>
</tr>
</tbody>
</table>

Radioactive material (700 cpm) was added to a solution containing 0.5% cetyltrimethylammonium bromide, 0.03 M NaCl, and 50 mg of Celite. After centrifugation for 10 min at 2000 X g, an aliquot of the supernatant was assayed for radioactivity. The sediment was then extracted repeatedly with NaCl as shown. Each supernatant was collected by centrifugation at 2000 X g for 5 min, and the radioactivity was determined.

A 30% reduction in 35SO4 incorporation.) The microsomal preparation also degraded 3'-phosphoadenosine 5'-phosphosulfate-35S with the appearance of inorganic 35SO4. With the initial 3'-phosphoadenosine 5'-phosphosulfate-35S concentration of 0.003 M, 61% was still intact after 2 hours.

The precipitation of the 35SO4-labeled material with cetyltrimethylammonium bromide and elution with NaCl is shown in Table II. Approximately half of the radioactive material was eluted with 1.2 M NaCl, the molarity of NaCl necessary for elution of chondroitin sulfate. The other half required 2.1 M NaCl, the molarity of NaCl necessary for elution of heparin.

Chromatography on DEAE-cellulose of 35SO4-labeled material together with carrier glycosaminoglycans is shown in Fig. 3. Approximately half of the radioactive activity was eluted from the column in the area of heparin, while the remainder of 35SO4-labeled material appeared in areas of elution of glycosaminoglycans of lesser sulfate content. These results are consistent with the findings of NaCl elution from the cetyltrimethylammonium bromide precipitate.

Results of the hyaluronidase and heparinase incubations with the radioactive material are shown in Fig. 4. Incubation with testicular hyaluronidase resulted in the degradation of added aluronic acid, whereas the 35SO4-labeled material was unaltered. In contrast, incubation with bacterial heparinase resulted in the degradation of both added commercial heparin and the 35SO4-labeled material.
Cpm), together with 2.5 mg of hyaluronic acid, 4 mg of chondroitin sulfate, and 4 mg of commercial heparin, was eluted from a column of DEAE-cellulose (1 X 5 cm) with a LiCl logarithmic gradient. The mixing flask contained 125 ml of water, and 2.5-ml fractions were collected. Initially the reservoir contained 1 M LiCl, which was changed to 2 M LiCl at Fraction 61. The carrier glycosaminoglycans were located by the carbazole method.

Fig. 3. Gradient elution of radioactive material from a DEAE-cellulose column. 35SO4-Labeled microsomal material (6000 cpm), together with 2.5 mg of hyaluronic acid, 4 mg of chondroitin sulfate, and 4 mg of commercial heparin, was eluted from a column of DEAE-cellulose (1 X 5 cm) with a LiCl logarithmic gradient. The mixing flask contained 125 ml of water, and 2.5-ml fractions were collected. Initially the reservoir contained 1 M LiCl, which was changed to 2 M LiCl at Fraction 61. The carrier glycosaminoglycans were located by the carbazole method.

Fig. 4. Elution of hyaluronidase and heparinase digests of 35SO4-labeled material from Sephadex G-100. Each reaction mixture contained 200 cpm of 35SO4-labeled material, and the following: A, 1 mg of hyaluronic acid; 0.1 M sodium acetate, pH 5; 0.2 M NaCl; and 2 mg of bacterial hyaluronidase in a total volume of 0.4 ml; B, 1 mg of commercial heparin; 0.1 M potassium phosphate, pH 7.0; and 2 mg of bacterial heparinase in a total volume of 0.5 ml. After incubation of A for 15 hours at 37° and B for 18 hours at 25°, the reaction mixtures were centrifuged. The supernatants were placed on a column of Sephadex G-100 (1 X 10 cm) and eluted with 0.05 M LiCl; 0.6-ml fractions were collected and assayed for radioactivity and uronic acid content. Undigested hyaluronic acid is eluted from this column at Fractions 5 to 9; undigested commercial heparin is eluted at Fractions 6 to 11.

**Discussion**

The present study describes a mouse mast cell tumor microsomal preparation which catalyzes the incorporation of 35SO4 from 3'-phosphoadenosine 5'-phosphosulfate-35S into a microsomal bound substance which can be solubilized by incubation with pancreatin. This substance is nondialyzable and behaves like a polysaccharide on paper chromatography. It can be precipitated with quaternary ammonium salts and can be eluted with the NaCl concentrations that are needed for elution of sulfated glycosaminoglycans. Chromatography of this material on an anion exchange column yields results similar to the elution of heparin and other sulfated glycosaminoglycans. The radioactive material is not degraded by testicular hyaluronidase, but is broken down by bacterial heparinase. In addition, heparin has been extracted in small quantity from similar microsomal preparations of this mast cell tumor. Therefore, it is concluded that the product into which 35SO4 is incorporated is microsomal heparin and heparin-like material of somewhat lesser sulfate content. Because these glycosaminoglycans are found in the active microsomal preparation, it is felt that they represent more nearly physiologically substrates than commercial deproteinized or otherwise chemically modified glycosaminoglycans.

Thus sulfation occurs with the same preparation which is involved in glycosaminoglycan polymerization (12). This suggests a close proximity of sulfating and polymerizing activities within the mast cell.

The maximum amount of sulfate incorporated into heparin by the microsomal preparation can be estimated as 1 sulfate for approximately 20 glucosamines on the basis of the measurements of microsomal heparin and 35SO4 incorporation. Since 3'-phosphoadenosine 5'-phosphosulfate-35S was still present and the enzyme was still active after incubation for 2 hours, it would appear that the cessation of incorporation of 35SO4 by 2 hours was due to saturation of the sites available for sulfation. The proportion of 35SO4 incorporated into glycosaminoglycan of lesser sulfate content may be somewhat higher than 1:20. This material was too small in amount to be positively identified.

There is evidence by electron microscopy that sulfation occurs in the Golgi apparatus (26), and glycosaminoglycan polymerization in chondrocytes is also thought to be associated with this organelle (27). In addition, the formation of some plant polysaccharides appears to be associated with the Golgi apparatus (26, 28). The present report of sulfating activity as well as the previous report of polysaccharide polymerizing activity in a microsomal fraction (which includes the Golgi) is consistent with this evidence. The presence of heparin and heparin-like glycosaminoglycans in the microsomal fraction suggests that heparin may be totally formed before being transported in some unknown manner to the mast cell granules which contain the bulk of mast cell heparin.

The stage of glycosaminoglycan formation at which sulfation takes place remains undetermined. As this and other reports (7-9) indicate, some sulfate can be incorporated into heparin subsequent to polymer formation, but the demonstrated amount is small. It is possible that most sulfation takes place at the same time as polymerization. Some additional unpublished data from this laboratory have indicated that this is probably the case.

**Acknowledgment**—I would like to express my appreciation to Silvana DeLuca for her expert technical assistance.
REFERENCES

CORRECTION
In the paper by John W. Little, J. R. Lehman, and A. D. Kaiser (Vol. 242, No. 4, Issue of February 23, 1967, page 672), on page 677, right-hand column, 3rd line from the bottom, the percentage given as "0.57%/e" should be "0.05%/e"; thus, the sentence should read as follows:
"This level of activity may not be significant, since the actual radioactivity measured in the assay was only 30% above background and represented hydrolysis of only 0.05% of the substrate."
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