SOME ASPECTS OF THE METABOLISM OF CHONDROITIN SULFATE-S\textsuperscript{35} IN THE RAT

BY DOMINIC D. DZIEWIATKOWSKI

(From the Hospital of The Rockefeller Institute for Medical Research, New York, New York)

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The use of sulfate of exogenous origin in the synthesis of chondroitin sulfate was suggested (1) and then demonstrated by the isolation of chondroitin sulfate-S\textsuperscript{35} from the cartilage of rats given sulfate-S\textsuperscript{34} (2, 3). Confirmatory reports have supplemented the earlier observations (4, 5). Autoradiography has been particularly effective in showing that administered sulfate-S\textsuperscript{35} is concentrated to a greater extent in regions which have a high acid mucopolysaccharide concentration (6-21) than in regions in which the concentration of such polysaccharides is low or in which they are absent. Results of experiments in which tissue cultures (22) and cartilage slices (23, 24) were used strongly suggest that the incorporation of sulfate into chondroitin sulfate is enzymatically controlled. It is likely that the reverse reaction, namely, the hydrolytic cleavage of the sulfate group from chondroitin sulfate, is similarly affected. However, the presence in mammalian tissues of an enzyme to catalyze such a reaction has not been demonstrated.

The following is a report of the fate of the sulfate group of chondroitin sulfate-S\textsuperscript{35} when the latter is administered to rats by stomach tube or by intraperitoneal injection.

EXPERIMENTAL

Adult male rats of the Sherman strain were used. Their average body weight was 310 gm., the range 290 to 330 gm. They were provided with Purina dog biscuits at all times except when urine was being collected.

In one set of experiments each of three rats received by stomach tube 3.3 mg. of sodium sulfate-S\textsuperscript{35} (2.90 X 10\textsuperscript{4} c.p.m.) in 1 ml. of water.\textsuperscript{1} The same amount of this material was injected intraperitoneally into each of three other rats. Each rat was then placed in a separate metabolism cage and allowed only water to drink. The urine excreted in the following 24 hours was analyzed for its content of inorganic sulfate sulfur, total sulfate sulfur, and total sulfur (25, 26). The S\textsuperscript{35} in each of these fractions was also determined by assay of the barium sulfate samples after isolation on

\textsuperscript{1} The S\textsuperscript{35} used in this investigation was supplied by the Oak Ridge National Laboratory on allocation from the United States Atomic Energy Commission.
filter paper disks (8). A week later the experiment was repeated with 20 mg. of potassium chondroitin sulfate-$S^{35}$ (0.75 mg. of sulfur, $2.70 \times 10^4$ c.p.m.) per rat and, after another week, with 25 mg. of fresh $S^{35}$-tagged cartilage ($4.72 \times 10^3$ c.p.m.) homogenized in 1 ml. of water. The knee-joints of 10 day-old rats that had received sodium sulfate-$S^{35}$ intraperitoneally 24 hours previously were the source of this cartilage.

In a second set of experiments each of three different rats was given by stomach tube 100 mg. of fresh $S^{35}$-tagged cartilage ($4.93 \times 10^4$ c.p.m.) homogenized in 1 ml. of water and into each of three other rats the same amount of the material was injected intraperitoneally. The urine excreted in the following 24 hours was collected and analyzed as in the first set of experiments. A week later, the experiment was repeated with 10 mg. of sodium chondroitin sulfate-$S^{35}$ (0.384 mg. of sulfur, $3.66 \times 10^4$ c.p.m.) in 1 ml. of water per rat.

An additional series of experiments was done, again with rats of approximately the same body weight, with sodium chondroitin sulfate-$S^{35}$ of much greater specific activity than that of the preparations mentioned above. Into each of three rats 1 mg. of the chondroitin sulfate ($1.82 \times 10^4$ c.p.m.) was injected intraperitoneally; into each of three rats in three other groups 2, 5, and 10 mg., respectively, were similarly injected. Each of three additional rats received by intraperitoneal injection 10 mg. of the chondroitin sulfate in 1 ml. of a homogenate of 51 mg. of fresh knee-joint cartilage, removed from 7 day-old rats. The urines were collected and analyzed as above. The unused portions of the urine samples from the rats that had received the same dose were pooled. Portions of the pools of urine from the rats given 1 and 2 mg. of the sodium chondroitin sulfate-$S^{35}$ were dialyzed against water in rocking dialyzers (27) at $0^\circ$ for 24 hours and the dialyzable and non-dialyzable $S^{35}$ was determined. The results obtained were checked by using urines immediately after collection from rats given 1 and 2 mg. of the same sodium chondroitin sulfate-$S^{35}$.

In the case of the pooled urine samples from rats given 5 and 10 mg. of the material, each of the pools in its entirety was dialyzed for a week against frequent changes of distilled water at $0^\circ$ in bags of Visking casing. The contents of the bags were then brought to dryness in small evaporating dishes kept in a vacuum desiccator over calcium chloride. The residues were taken up in 1 ml. of water and portions were used for analysis by paper chromatography according to Kerby (28) and by electrophoresis on paper and on starch (29, 30). To extend and verify the results, the urine excreted by a rat during a period of 17 hours following the intraperitoneal injection of 15 mg. of sodium chondroitin sulfate-$S^{35}$ and the urine from a rat given similarly about 100 $\mu$C. of $S^{35}$ as sodium sulfate were analyzed as follows. Each of these urine samples was diluted to 25 ml. and of this 20
ml. were then dialyzed for 1 week against frequent changes of distilled water at 0°, in bags of Visking casing. The non-dialyzable fraction of the urine was evaporated to dryness at room temperature over calcium chloride in a vacuum desiccator and the residue was taken up in 2 ml. of water. Portions of the concentrate and of the undialyzed urine were analyzed by electrophoresis in a starch block. The concentrate was also examined by paper electrophoresis and paper chromatography.

As an adjunct to the above experiments the possible coprecipitation of barium chondroitin sulfate and barium sulfate was determined under the conditions used for the determination of the S\textsuperscript{35} in the inorganic sulfate sulfur fraction of the urines: The urine collected for 24 hours from two adult male rats was diluted to 100 ml. with water. To 25 ml. portions 1, 2, and 3 mg. of sodium chondroitin sulfate-S\textsuperscript{35} in 1, 2, and 3 ml. of water, respectively, were added. A further dilution of each sample to 50 ml. was made and, after mixing, two 5 ml. portions were removed from each and further diluted to 100 ml. with water; the addition of 5 ml. of a 0.05 N solution of sodium sulfate, 1 ml. of a 2.5 N solution of hydrochloric acid, and 5 ml. of a 10 per cent solution of barium chloride followed. The barium sulfate was isolated 1 hour later by filtration onto paper disks for counting. After extensive acid hydrolysis of 5 ml. portions of the urine samples, the total sulfate sulfur was isolated similarly. 24 hours later, the analyses were repeated, the urine samples having been kept at 20° during this period of time.

Skeletons of suckling rats, Sherman strain, that had received sodium sulfate-S\textsuperscript{35} intraperitoneally at least 24 hours previously were the source of the chondroitin sulfate-S\textsuperscript{35} used. Potassium chondroitin sulfate-S\textsuperscript{36} was isolated according to a procedure reported by Einbinder and Schubert (31). On analysis it was found that it contained 2.86 per cent nitrogen, 3.75 per cent sulfate sulfur, 25.5 per cent hexuronic acid, and 20.4 per cent hexosamine. Sodium chondroitin sulfate-S\textsuperscript{36} was prepared according to Bostrijm (3). The preparation used in Experiments 9 and 10 (Table I) contained 3.96 per cent nitrogen, 3.84 per cent sulfate sulfur, 34.4 per cent hexuronic acid, and 25.9 per cent hexosamine; the preparation used in Experiments 11 through 15 contained 2.70 per cent nitrogen, 5.30 per cent sulfate sulfur, 32.4 per cent hexuronic acid, and 22.0 per cent hexosamine. The composition of the chondroitin sulfate preparations was ascertained as reported previously (5).

RESULTS AND DISCUSSION

The data on the excretion of S\textsuperscript{35} by the rats given sodium sulfate-S\textsuperscript{35}, chondroitin sulfate-S\textsuperscript{35}, and S\textsuperscript{36}-labeled cartilage are summarized in Table I. It can be seen that 52 to 86 per cent of the S\textsuperscript{36} administered as chondroitin
TABLE I

Excretion of $^{35}S$ in Urine of Rats Given Chondroitin Sulfate-$S^{35}$

The average of three values, each on a different rat, is listed in every instance. Corrections for radioactive decay and self-absorption were made.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Inorganic SO$_4$-S</th>
<th>Total SO$_4$-S</th>
<th>Total sulfur</th>
<th>Material and route of administration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \text{per cent of dose} )</td>
<td>( \text{c.p.m. per mg. S} )</td>
<td>( \text{per cent of dose} )</td>
<td>( \text{c.p.m. per mg. S} )</td>
</tr>
<tr>
<td>1*</td>
<td>62.5</td>
<td>2,110</td>
<td>73.9</td>
<td>2,160</td>
</tr>
<tr>
<td>2*</td>
<td>60.1</td>
<td>1,482</td>
<td>67.6</td>
<td>1,394</td>
</tr>
<tr>
<td>3†</td>
<td>45.3</td>
<td>1,074</td>
<td>52.0</td>
<td>1,055</td>
</tr>
<tr>
<td>4†</td>
<td>36.8</td>
<td>1,119</td>
<td>58.1</td>
<td>1,455</td>
</tr>
<tr>
<td>5†</td>
<td>61.2</td>
<td>371</td>
<td>71.8</td>
<td>369</td>
</tr>
<tr>
<td>6†</td>
<td>61.6</td>
<td>266</td>
<td>75.2</td>
<td>276</td>
</tr>
<tr>
<td>7§</td>
<td>61.6</td>
<td>3,108</td>
<td>70.7</td>
<td>3,041</td>
</tr>
<tr>
<td>8§</td>
<td>55.7</td>
<td>2,963</td>
<td>64.1</td>
<td>2,895</td>
</tr>
<tr>
<td>9‖</td>
<td>50.8</td>
<td>1,823</td>
<td>59.7</td>
<td>1,828</td>
</tr>
<tr>
<td>10‖</td>
<td>54.2</td>
<td>2,182</td>
<td>85.9</td>
<td>2,914</td>
</tr>
<tr>
<td>11¶</td>
<td>53.4</td>
<td>2,356</td>
<td>65.5</td>
<td>2,470</td>
</tr>
<tr>
<td>12¶</td>
<td>52.0</td>
<td>3,977</td>
<td>67.6</td>
<td>4,165</td>
</tr>
<tr>
<td>13¶</td>
<td>50.5</td>
<td>7,236</td>
<td>58.0</td>
<td>7,215</td>
</tr>
<tr>
<td>14¶</td>
<td>44.2</td>
<td>10,402</td>
<td>65.4</td>
<td>12,530</td>
</tr>
<tr>
<td>15¶</td>
<td>44.0</td>
<td>11,960</td>
<td>84.0</td>
<td>17,860</td>
</tr>
</tbody>
</table>

* Sodium sulfate was added to a solution of carrier-free sodium sulfate-$S^{35}$ so that each ml. contained 3.3 mg. of the salt and $2.90 \times 10^4$ c.p.m.

† The 20 mg. of potassium chondroitin sulfate-$S^{35}$ given to each animal were equivalent to $2.70 \times 10^4$ c.p.m. and 0.75 mg. of sulfur.

‡ 1 ml. of a homogenate containing 25 mg. of fresh knee-joint cartilage from 10 day-old rats was given to each of the adult rats. As a consequence of a previous administration of sodium sulfate-$S^{35}$ to the suckling rats, there were $4.72 \times 10^3$ c.p.m. of $S^{35}$ in 1 ml. of the cartilage homogenate and 97.0 per cent of this activity did not pass through a cellophane membrane.

§ Each of the rats was given 1 ml. of a homogenate containing 100 mg. of fresh knee-joint cartilage from 11 day-old rats that had previously received sodium sulfate-$S^{35}$. There were $4.93 \times 10^4$ c.p.m. per ml. of homogenate and of the activity 98.5 per cent did not pass through a cellophane membrane.
TABLE I—Concluded

To each rat 10 mg. of sodium chondroitin sulfate-S\textsuperscript{35} were given, equivalent to 0.384 mg. of sulfur and 3.66 × 10\textsuperscript{4} c.p.m. Each mg. of the sodium chondroitin sulfate-S\textsuperscript{35} was equivalent to 1.83 × 10\textsuperscript{4} c.p.m. The material was isolated from the skeletons of 8 day-old rats that had received sodium sulfate-S\textsuperscript{35} intraperitoneally 24 hours previously. For use in Experiment 15, the chondroitin sulfate-S\textsuperscript{35} was dissolved in a homogenate of knee-joint cartilage removed from 7 day-old rats. Each ml. of homogenate contained 10 mg. of the labeled chondroitin sulfate and 51 mg. of unlabeled cartilage.

Sulfate-S\textsuperscript{35} was excreted in 24 hours, the major portion, 37 to 62 per cent, was excreted as inorganic sulfate, and the remainder was found in the ester sulfate fraction. These values are not remarkably different from those found when sodium sulfate-S\textsuperscript{35} or S\textsuperscript{38}-labeled cartilage was given. In the experiments in which sodium sulfate or fresh cartilage was administered by stomach tube or injected intraperitoneally, the specific activity of the sulfur, counts per minute per mg. of sulfur, in the inorganic sulfate fraction of the urine was similar to that in the total sulfate fraction. This also appears to be the case when chondroitin sulfate was given by stomach tube, Experiments 3 and 9. On the other hand, when the chondroitin sulfate preparations were injected intraperitoneally, Experiments 4, 10, 14, and 15, the specific activity of the total sulfate sulfur in the urine was greater than that of the inorganic sulfate sulfur, except when the dose was 5 mg. or less per rat, Experiments 11, 12, and 13. The results of Experiments 4, 10, 14, and 15 indicate that the specific activity of the ester sulfate sulfur was markedly higher than that of the inorganic sulfate sulfur. For example, one of the rats in Experiment 4 excreted 8.12 mg. of inorganic sulfate sulfur and 10.15 mg. of total sulfate sulfur in 24 hours and the radioactivity associated with these sulfate sulfur fractions was 9560 c.p.m. and 15,560 c.p.m., respectively. The 2.04 mg. of ester sulfate sulfur are therefore associated with a radioactivity of 6000 c.p.m.; hence the specific activity of the ester sulfate sulfur is 6000 c.p.m. per 2.04 mg. or 2940 c.p.m. as compared to 1177 c.p.m. per mg. of sulfur in the inorganic sulfate fraction. This and similar results obtained following intraperitoneal injection of more than 5 mg. of chondroitin sulfate-S\textsuperscript{35} are interpreted tentatively as follows. The rat is able to break the bond between the sulfate group and the rest of the chondroitin sulfate moiety; this capacity, however, is a limited one. If an excessive amount of chondroitin sulfate is injected, a significant amount of the sulfate is excreted without prior release from the carbohydrate unit to which it is attached.

The urines were dialyzed to ascertain the nature of the S\textsuperscript{35}-labeled material excreted in the ester sulfate fraction by rats given chondroitin sulfate intraperitoneally. Some of these data are presented in Table II. It was
found that following a dose of 1 mg. practically all of the S$^{36}$ passed through the cellophane membrane. After 2 mg., however, 52.0 per cent was non-dialyzable. This latter finding appeared at first to be contradictory to the data in Table I, Experiments 12 and 13, in which no difference was

**Table II**

Fraction of S$^{36}$ in Rat Urines Which Is Non-Dialyzable after Intraperitoneal Injection of Small Amounts of Sodium Chondroitin Sulfate-S$^{36}$

Urine was collected for 24 hours following the administration of 1 mg. of the S$^{36}$-labeled chondroitin sulfate to one rat and 2 mg. to another. Duplicate portions of each urine were dialyzed against water in rocking dialyzers for 24 hours at 0°.

<table>
<thead>
<tr>
<th>Chondroitin sulfate-S$^{36}$ injected</th>
<th>Non-dialyzable S$^{36}$ in urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg.</td>
<td>per cent</td>
</tr>
<tr>
<td>1</td>
<td>1.07</td>
</tr>
<tr>
<td>2</td>
<td>52.13</td>
</tr>
</tbody>
</table>

**Table III**

Coprecipitation of Chondroitin Sulfate with Barium Sulfate

Sodium chondroitin sulfate-S$^{36}$ (1.83 X 10$^4$ c.p.m. per mg.) was added to diluted rat urine. Immediately thereafter and 24 hours later portions of the urine were taken for analysis: 1 hour after the addition of 5 ml. of a 0.05 N solution of sodium sulfate, 1 ml. of a 2.5 N solution of hydrochloric acid, and 5 ml. of a 10 per cent solution of barium chloride to each portion of urine, the barium sulfate was isolated by filtration onto paper disks for counting. The total S$^{36}$ added to the urine was determined similarly after extensive acid hydrolysis.

<table>
<thead>
<tr>
<th>Sodium chondroitin sulfate-S$^{36}$ added to urine</th>
<th>S$^{36}$ isolated in inorganic sulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Immediately</td>
</tr>
<tr>
<td>mg.</td>
<td>per cent</td>
</tr>
<tr>
<td>1</td>
<td>82.3</td>
</tr>
<tr>
<td>2</td>
<td>53.4</td>
</tr>
<tr>
<td>3</td>
<td>39.4</td>
</tr>
</tbody>
</table>

* The samples were held at 20° for 24 hours. During this time the pH changed from 7.3 to 8.7.

found between the specific activities of the sulfur in the inorganic sulfate and total sulfate fractions when as much as 5 mg. of the chondroitin sulfate-S$^{36}$ were injected. An explanation of the apparent discrepancy was found in experiments in which inorganic sulfate was precipitated as barium sulfate from urines to which chondroitin sulfate-S$^{36}$ had been added (Table III). Though the chondroitin sulfate-S$^{36}$ in the absence of inorganic sulfate did not precipitate as the barium salt, it is clear that, depending upon
Fig. 1. Electrophoretic analyses of $^{35}$-labeled components in the urine of a rat given sodium sulfate-$^{35}$ (100 µc.) by intraperitoneal injection. The urine was collected during the following 17 hours. For analysis of the undialyzed urine one-twentieth of the total volume was used, whereas in the case of the non-dialyzable fraction of the urine the amount used was equivalent to eighteen-twenty-fifths of the total. Barbital buffer, pH 8.6, µ 0.1, was used with a starch block that was 1 cm. thick, 5 cm. wide, and 62 cm. long. The separation of the $^{35}$-labeled components as shown was achieved in 5 hours at 12 ma. in a cold room at 0°. Segments 1 cm. wide were each eluted with 2 ml. of a 1 per cent sodium chloride solution; 1 ml. portions were subsequently dried for analysis.

Fig. 2. Electrophoretic analyses of $^{35}$-labeled components in the urine of a rat given 15 mg. of sodium chondroitin sulfate-$^{35}$ (2.75 $\times$ 10⁴ c.p.m.) by intraperitoneal injection. The urine was collected during the following 17 hours. For analysis one-tenth of the total volume was used in the case of the undialyzed urine; in the case of the non-dialyzable fraction of the urine the amount was equivalent to one-fifth of the total volume. Otherwise the analyses were the same as those in Fig. 1.
the amount present, a large or small fraction of it will either coprecipitate with or be occluded in barium sulfate during the precipitation of the latter. As a result it is likely that, in Experiments 12 and 13, if unchanged chondroitin sulfate was excreted in the urines, the specific activity of the sulfur in the inorganic sulfate fraction would appear to be as high as that of the sulfur in the total sulfate fraction.

So far the data only suggested the possible excretion of unchanged chondroitin sulfate or of some fragment (or fragments) of it with the sulfate still attached if the dose were 2 mg. or more. Convincing proof that some chondroitin sulfate was excreted unchanged was obtained by the use of paper chromatography and electrophoresis on paper and in a starch block.

For example, in the case of a urine from a rat that received sodium sulfate-$^{35}S$ and another from a rat given chondroitin sulfate-$^{35}S$ intraperitoneally, similar patterns were found on electrophoretic analysis by use of starch blocks (Figs. 1 and 2). In each of the urines there was $^{35}S$-labeled material that moved as did inorganic sulfate (cf. Fig. 3) and, in addition, materials with a slower mobility. Concentrates of the non-dialyzable fraction of the urines, however, gave dissimilar patterns. Practically all of the $^{35}S$ in the urine from the rat that received labeled sodium sulfate passed through the cellophane membrane (Fig. 1). In the concentrate of the non-dialyzable fraction of the urine from the rat which had been injected with chondroitin sulfate-$^{35}S$ there was material with a mobility like that of the administered chondroitin sulfate-$^{35}S$ (Figs. 2 and 3). Electrophoresis on paper and paper chromatography (Fig. 4), also indicate this. By using electrophoresis on paper, material akin to chondroitin sulfate was
detected in concentrates of dialyzed urine even when as little as 5 mg. of chondroitin sulfate-$\text{S}^{35}$ had been injected.

![Electrophoretic Analyses](image)

**Fig. 4.** A, electrophoretic analyses of materials in concentrates of the non-dialyzable fraction of the urines from rats given intraperitoneally 15 mg. of sodium chondroitin sulfate-$\text{S}^{35}$ and sodium sulfate-$\text{S}^{35}$ (100 µc.). In each instance an amount equivalent to one-two hundred and fiftieth of the original urine volume was used. Approximately 10$\gamma$ of sodium chondroitin sulfate-$\text{S}^{35}$ in 0.01 ml. of buffer were applied to the paper for reference. Acetate buffer, pH 4.3, $\mu$ 0.1, was used in conjunction with Whatman No. 3MM filter paper strips 3.7 X 14 inches; 1 ma. of current was allowed to flow for 17.5 hours at 0°. The papers were then dipped into a 0.1 per cent solution of toluidine blue in 30 per cent ethanol. Excess dye was removed by repeated washing in 30 per cent ethanol containing 0.5 per cent acetic acid. B, the possible separation of chondroitin sulfate from heparin and hyaluronic acid is apparent. Analysis as in (A) except that the running time was 16 hours. 5$\gamma$ of heparin and 10$\gamma$ of chondroitin sulfate and hyaluronic acid (umbilical cord) were applied to the paper, each in 0.01 ml. of water. C, paper chromatogram, prepared according to Kerby (28), of the materials used in (A). The amounts applied to the paper were also the same as those in (A).

A consideration of all the data leads to the conclusion that the intact adult rat can split the bond holding the sulfate in chondroitin sulfate. The rat's capacity to do so is limited when chondroitin sulfate isolated from the skeletons of rats is injected intraperitoneally. The injection of 5 mg. or more of such material results in the urinary excretion of some of
it unchanged. It is probable that the chondroitin sulfate had been de-polymerized in the process of isolation to give fragments of a size which could be excreted by the kidneys.

SUMMARY

Chondroitin sulfate-S\textsuperscript{35} isolated from the skeletons of suckling rats that had received sodium sulfate-S\textsuperscript{35} was injected intraperitoneally into or fed by stomach tube to adult rats. After tube feeding no difference was found between the specific activity of the urinary inorganic sulfate sulfur and the specific activity of the urinary total sulfate sulfur, even when the dose was 20 mg. of chondroitin sulfate-S\textsuperscript{35} per rat. On the other hand, after intraperitoneal injection an equivalence of the specific activities of the sulfur in these two fractions was found only when the dose was 5 mg. or less. After the intraperitoneal injection of 2 mg. or more of chondroitin sulfate-S\textsuperscript{35}, some of the S\textsuperscript{35} excreted in the urine was non-dialyzable. In concentrates of the non-dialyzable fractions of these urines material was found which on electrophoretic analysis and by paper chromatography resembled the chondroitin sulfate-S\textsuperscript{35} which had been injected.

It is concluded that the rat is capable of severing the ester linkage between sulfate and carbohydrate in chondroitin sulfate.

BIBLIOGRAPHY

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