THE METABOLISM OF S\textsuperscript{35}-SODIUM ESTRONE SULFATE IN THE ADULT FEMALE RAT

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In general it is considered that conjugated estrogens are less active \textit{in vivo} than the free form (1). This fact is attributed generally to the slow rate of hydrolysis of the conjugated form in the body or to an increased rate of excretion; however, it was shown recently (2) that pregnant and non-pregnant rats given S\textsuperscript{35}-sodium estrone sulfate by subcutaneous injection rapidly excrete S\textsuperscript{35} as inorganic sulfate in the urine and feces, indicating that this conjugated estrogen underwent rapid hydrolysis in the body.

More detailed data are presented in the present paper on the rate and possible pathway of excretion of the S\textsuperscript{35} from S\textsuperscript{35}-sodium estrone sulfate given to adult female rats and the possible sites of hydrolysis of this conjugated estrogen.

EXPERIMENTAL

Rate of Excretion—Single doses of 0.5 mg. of sodium estrone sulfate,\textsuperscript{1} labeled with S\textsuperscript{35}, in 1 ml. of 0.1 M phosphate solution, pH 7.45, were administered to each of eighteen adult female rats which were divided into groups of six. These rats were of the Sprague-Dawley strain and weighed from 200 to 250 gm. Administration of the radioactive estrogen was made via the subcutaneous, intravenous, or gastric route. The animals were placed in metabolism cages and fed \textit{ad libitum}. Urine and feces were collected at 3, 6, 9, 12, 24, and 36 hour intervals.\textsuperscript{2} These fractions were

\textsuperscript{1} We wish to thank Dr. Gordon A. Grant, Dr. Edward C. Reifenstein, and Dr. J. Murray Scott of Ayerst, McKenna and Harrison, Ltd., New York, for their interest in the investigation and for supplying us with generous quantities of S\textsuperscript{34}-sodium estrone sulfate. The S\textsuperscript{34} used in this investigation was supplied by the Clinton Laboratories on allocation from the Isotopes Division, United States Atomic Energy Commission. The S\textsuperscript{34}-sodium estrone sulfate solutions were kept at 4° in the dark when not in use. Although stable for 2 to 3 weeks, fresh solutions were prepared each week. The dry S\textsuperscript{34}-sodium estrone sulfate was stored over P\textsubscript{2}O\textsubscript{5} in an evacuated desiccator at 4°.

\textsuperscript{2} No detectable amount of free S\textsuperscript{34} could be found after S\textsuperscript{34}-sodium estrone sulfate was incubated with urine under toluene for 12 hours at room temperature. Isolation of the S\textsuperscript{34}-sodium estrone sulfate by isotope dilution showed no loss of radioactive estrogen. Under similar conditions, S\textsuperscript{34} sodium estrone sulfate incubated with fecal homogenates showed a production of only 5 to 10 per cent free S\textsuperscript{34}.
analyzed for their total $^{35}S$ and inorganic $^{35}S$ content by the method of Tarver and Schmidt (3). In the determination of the $^{35}S$-sodium estrone sulfate content of the urine, an aliquot was adjusted to pH 7.5, 25 mg. of non-labeled sodium estrone sulfate were added, and the isolation was carried out as described below. The $^{35}S$-sodium estrone sulfate content of the feces was determined as follows: A mixture of 1 part feces to 10 parts of water was homogenized in a Waring blender for 3 to 5 minutes, 25 mg. of non-labeled sodium estrone sulfate were added, and the isolation was carried out as described later. All measurements of radioactivity were made by conventional counting methods. A 1.3 mg. per sq. cm. mica end window counter was used in the assay of radioactive samples. The results were corrected for decay and self-absorption. The average results of these experiments are shown in Fig. 1.

The recovery of $^{35}S$ after 36 hours was 75, 86, and 86 per cent after administration by the intravenous, subcutaneous, and gastric routes, respectively. The principal pathway of excretion of $^{35}S$ varied with the method of administration of the radioactive estrogen. After the intravenous injection of the radioactive estrogen, 37 per cent of the $^{35}S$ excreted was found in the feces and 63 per cent was found in the urine; however, after subcutaneous or gastric administration of the estrogen, 64 per cent of the $^{35}S$ excreted was found in the feces and 36 per cent in the urine.

Of prime interest was the fact that all the $^{35}S$ found in the urine and feces in these experiments was present as inorganic sulfate. No $^{35}S$-sodium estrone sulfate could be detected at any time interval in the urine or feces. This indicated that the conjugated estrogen underwent rather rapid hydrolysis in the body and that apparently the free sulfate was not utilized by the animal to any appreciable extent. The latter finding is essentially in agreement with that of Dziewiatkowski (4), who found that approximately 96 per cent of the $^{35}S$ given by intraperitoneal injection in the form of sodium sulfate was excreted in the urine and feces in 120 hours. In his experiments, the major portion of the $^{35}S$ was found in the urine.

Pathway of Excretion—As was pointed out above, a rather large percentage of the $^{35}S$ released by hydrolytic cleavage of $^{35}S$-sodium estrone sulfate in the body was found in the feces. This observation prompted an investigation of the possible pathway for entry of the radioactive material into the intestine; hence, a study was made of the amount and the chemical form of any $^{35}S$ that might be excreted via the bile of animals receiving this radioactive estrogen.

Bile fistulas were made by introduction of a cannula into the common

3 Samples of blood taken 15 minutes after the intravenous injection of $^{34}S$-sodium estrogen sulfate contained very little radioactivity.
bile duct with a segment of a 23 gage needle. Small diameter plastic tubing carried the bile from the needle into a 4 ml. cylindrical plastic tube that was attached by skin sutures to the ventral surface of the rat. The distal end of the tube was equipped with a screw cap which permitted periodic withdrawal of bile. This method of bile sampling was advantageous in that it was unnecessary to immobilize the rats during the period of bile collection.

Bile fistula, female rats, 250 to 300 gm. in weight, were given single doses of 0.5 mg. of S\textsuperscript{35}-sodium estrone sulfate by intravenous or subcutaneous injection. The animals were placed in metabolism cages and were fed \textit{ad libitum}. Urine, bile, and feces were collected up to 24 hours after the injection of the estrogen and were analyzed for their total S\textsuperscript{35}, inorganic S\textsuperscript{35}, and S\textsuperscript{35}-sodium estrone sulfate.\textsuperscript{4} Again all the S\textsuperscript{35} present was found to be in the inorganic form. The distribution is shown in Table I. After subcutaneous or intravenous injection of S\textsuperscript{35}-sodium estrone sulfate, the amount of S\textsuperscript{35} found in the bile was approximately 10 per cent of the total S\textsuperscript{35} excreted in 24 hours. With subcutaneous injection this represents approximately 25 per cent of the total S\textsuperscript{35} found in the bile and feces, and with intravenous injection, approximately 15 per cent of the total S\textsuperscript{35} in the biliary and fecal excretions. It will be noted that in the bile fistula

\textsuperscript{4} Incubation of S\textsuperscript{35}-sodium estrone sulfate with bile for 6 hours at 37\textdegree{} caused no release of free S\textsuperscript{35}. In these animal experiments the interval of time between collections of bile samples for assay was no greater than 6 hours.
rats subcutaneous injection of the labeled estrogen resulted in a high urinary excretion of $S^{35}$, which could not be resolved with the distribution found in normal rats.

The results of these experiments indicate that the bile does not serve as the main excretory pathway for the $S^{35}$ found in the feces of animals given $S^{35}$-sodium estrone sulfate by intravenous or subcutaneous injection. The exact site of entry of the $S^{35}$ into the intestine is not known. The $S^{35}$ which does enter the bile has already been cleaved from the estrogen.

**Hydrolytic Action of Organ Homogenates**—It was of interest to investigate the possible sites of hydrolysis of sodium estrone sulfate in the body.

### Table I

24 Hour Excretion of $S^{35}$ in Bile Fistula, Female Rats Given Single Injection of 0.5 Mg. of $S^{35}$-Sodium Estrone Sulfate

<table>
<thead>
<tr>
<th>Experiments</th>
<th>Route of administration</th>
<th>Urine</th>
<th>Bile</th>
<th>Feces</th>
<th>Total recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Counts $S^{35}$ per min.</td>
<td>Per cent $S^{35}$ excreted</td>
<td>Counts $S^{35}$ per min.</td>
<td>Per cent $S^{35}$ excreted</td>
</tr>
<tr>
<td>I</td>
<td>Subcutaneous</td>
<td>16,000</td>
<td>53</td>
<td>2900</td>
<td>10</td>
</tr>
<tr>
<td>II</td>
<td>&quot;</td>
<td>20,800</td>
<td>57</td>
<td>5200</td>
<td>14</td>
</tr>
<tr>
<td>III</td>
<td>&quot;</td>
<td>14,700</td>
<td>53</td>
<td>2400</td>
<td>9</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
<td>14,800</td>
<td>57</td>
<td>1900</td>
<td>7</td>
</tr>
<tr>
<td>V</td>
<td>&quot;</td>
<td>14,600</td>
<td>43</td>
<td>1370</td>
<td>5</td>
</tr>
<tr>
<td>VI</td>
<td>&quot;</td>
<td>14,700</td>
<td>48</td>
<td>1730</td>
<td>6</td>
</tr>
<tr>
<td>VII</td>
<td>&quot;</td>
<td>19,760</td>
<td>60</td>
<td>2940</td>
<td>9</td>
</tr>
</tbody>
</table>

Consequently, a study of the hydrolytic activity of various organ homogenates on $S^{35}$-sodium estrone sulfate was carried out. The organs used in this study were liver, pancreas, small intestine, large intestine, ovary, uterus, adrenal, and kidney. The amount of $S^{35}$-sodium estrone sulfate remaining after incubation with the homogenates was determined by the "inverse" isotope dilution technique. The procedure used in these studies is described below.

Adult female white rats of the Sprague-Dawley strain, 200 to 250 gm. in weight, were anesthetized with ether and the blood was removed by severance of the jugular veins. The desired organ was removed immediately and was homogenized in distilled water at $4^\circ$ in a Waring blender or

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* No production of free $S^{35}$ was noted when $S^{35}$-sodium estrone sulfate was incubated with plasma for $2_{1}^{1}$ hours at $37^\circ$. 
a glass homogenizer tube; both methods gave the same results. The amounts of organ were chosen so that the final homogenate contained 20 mg. of wet tissue per ml. This homogenate was centrifuged at 10,000 r.p.m. in a Servall angle centrifuge for 30 minutes at 4°. The supernatant contained over 80 per cent of the enzymatic activity of those organ homogenates which effected a hydrolysis of the conjugated estrogen. Equal volumes of the supernatant and 0.1 M PO₄ buffer, pH 7.45, were mixed and were tested for activity as follows: 10 ml. of the buffered homogenate were transferred to a 50 ml. glass-stoppered Erlenmeyer flask, and 2.0 mg. of S²⁵-sodium estrone sulfate, specific activity 76,000 c.p.m. per mg., were added. The flask was stoppered tightly, and the contents were mixed by shaking. The flask was then placed in a water bath at 37° for 2½ hours.

The following controls were run on each homogenate: (a) 2.0 mg. of S²⁵-sodium estrone sulfate in 10 ml. of 0.1 M PO₄ buffer, pH 7.45, incubated at 37° for 2½ hours, (b) the same amount of substrate in 10 ml. of buffered boiled homogenate, pH 7.45, incubated at 37° for 2½ hours, and (c) 2.0 mg. of S²⁵-sodium estrone sulfate in 10 ml. of buffered homogenate which were not incubated but extracted immediately after mixing by the procedure described below.

At the end of the incubation period, 100 mg. of non-radioactive sodium estrone sulfate were added to the incubation mixture. The contents were mixed, and immediately thereafter 35 ml. of n-butanol were added. The flask was stoppered and the contents were mixed by vigorous shaking for 2 to 3 minutes. Inasmuch as an emulsion always formed, it was necessary to centrifuge the mixture at 3000 r.p.m. for 15 to 20 minutes at room temperature in order to break the emulsion. The butanol-soluble fraction was removed and, if necessary, the non-butanol-soluble residue was reextracted a second time with a fresh change of butanol.

The butanol extracts were transferred to a 125 ml. separatory funnel and washed successively three times with 1.0 ml. portions of N NaOH and three times with 1.0 ml. portions of water. After the final washing, the butanol fraction was evaporated to dryness in vacuo and then was taken up in a small volume of warm absolute methanol, and warm anhydrous ether was added slowly till a slight turbidity had developed. The mixture was allowed to stand overnight at 4°. The crystals were collected on a dry filter and then dried over P₂O₅. It was necessary to recrystallize this material three or four times from this same solvent. Usually this sufficed to bring the specific activity and melting point of the material to a constant value. The sodium estrone sulfate was recrystallized from n-butanol, but usually this did not change the specific activity or melting point of the compound. M.p. 226-228° (uncorrected). Admixture with pure sodium estrone sulfate did not depress the melting point.
The total $S^{35}$ content and the specific activity of the sodium estrone sulfate were determined. The amount of unchanged $S^{35}$-sodium estrone sulfate remaining in the incubation mixture was calculated by the "inverse" isotope dilution technique (5).

The results of these experiments showed that under the conditions used only the liver homogenate contained an enzyme system capable of hydrolyzing the sulfate ester of estrone. In 2½ hours at 37° the extract from liver was able to release completely the sulfate from sodium estrone sulfate. In the case of the other organ homogenates, less than 10 per cent free sulfate could be detected under similar conditions. None of the controls showed any significant loss of $S^{35}$-sodium estrone sulfate or the production of any free sulfate.

All the $S^{35}$ released from the $S^{35}$-sodium estrone sulfate by liver homogenate was inorganic in nature. This was ascertained as follows, after it was shown that there is no $S^{35}$-sodium estrone sulfate remaining in the liver homogenate: 20 mg. of non-labeled sodium sulfate were added to the incubation mixture and then 5 ml. of 10 per cent $\text{Cl}_2\text{C} \cdot \text{COOH}$; the mixture was filtered and all the $S^{35}$ was precipitated as benzidine sulfate and the total $S^{35}$ content was determined in the usual way. A control, containing $S^{35}$-sodium estrone sulfate and buffer and treated in a similar manner, showed that less than 10 per cent of the $S^{35}$ was released from the conjugated estrogen.

It is particularly interesting that liver homogenate is capable of effecting the hydrolysis of this conjugated estrogen. As was pointed out by Szego and Roberts (6), the ratio of free to conjugated estrogen is much lower in the blood than that in the bile. In addition, Pearlman et al. (7) found that the gall-bladder bile of pregnant cows contained free estrone as the major estrogen. On the basis of the results presented in the present paper, it is evident that the liver contains an enzyme system capable of hydrolyzing the sulfate ester of estrone. Thus, at least in part, the estrone in the bile could be derived from the estrone sulfate in the blood.

SUMMARY

The metabolism of a conjugated estrogen, $S^{35}$-sodium estrone sulfate, in the adult female rat was studied.

Over 75 per cent of the $S^{35}$ of the radioactive estrogen given to female rats by intravenous, subcutaneous, or gastric injection was found in the urine and feces in 36 hours. All the excreted $S^{35}$ was inorganic in nature.

Administration of $S^{35}$-sodium estrone sulfate by subcutaneous or intravenous injection to bile fistula, female rats resulted in the excretion of less
than 15 per cent of the $\text{S}^{35}$ in the bile; the remaining $\text{S}^{35}$ was found in the urine and feces. Again all the $\text{S}^{35}$ excreted was inorganic in nature.

Various organ homogenates were examined for enzyme systems capable of hydrolyzing this conjugated estrogen. Liver homogenate was the only one examined that was capable of effecting a hydrolysis of this ester.

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