The Relative Transfer of Estrogens and Their Sulfates Across the Guinea Pig Placenta: Sulfurylation of Estrogens by the Placenta*

Mortimer Levitz, George P. Condon, William L. Money, and Joseph Dancy†

From the Departments of Obstetrics and Gynecology and Pediatrics, New York University-Bellevue Medical Center, and the Sloan-Kettering Institute for Cancer Research, New York, New York

(Received for publication, October 28, 1959)

In a recent report from these laboratories (2), an in situ perfusion method was described which could be used to measure the transfer of substances in either direction across the guinea pig placenta. When the procedure was applied to the estrogens, it was found that the unconjugated hormones, estrone, estradiol-17ß, and estriol were transferred quickly under conditions in which the placenta posed an effective barrier to their glucuronide conjugates. However, it was found that when estrogens were administered to the mother, conjugates other than glucosiduronates were detected in the umbilical circulation. In the present report, evidence is presented indicating that these conjugates are estrogen sulfates, that the placenta is impermeable to these conjugates, but that the placenta can sulfurate estrogens and release them into the umbilical circulation.

EXPERIMENTAL

Perfusion—Pregnant guinea pigs of no particular breed were purchased from the Dierolf Farms, Boyertown, Pennsylvania. Within the last two or at least six a gestation a fetus was delivered by hysterotomy into a 0.9% sodium chloride solution bath maintained at 38°C. The umbilical cord was clamped close to the fetus (Fig. 1a) and an artery and the vein were cannulated between the clamp and the placenta. The fetal side of the placenta was perfused with a mixture of guinea pig plasma-0.9% sodium chloride solution (1:2). To study maternal to fetal transfer the estrogen was injected into the mother intravenously and after a period of time comparison was made between the estrogens in the maternal plasma and the perfusate. For fetal to maternal transfer the estrogen was added to the perfusate and its disappearance in the course of the perfusion through the placenta was investigated. To study sulfurylation of estrogens in the placenta, the placenta was perfused with the estrogen in the usual way after the maternal circulation was excluded by a clamp through the maternal decidua close to the placenta (Fig. 1b).

Radioactive Estrogens—Estriol-16-C14 was synthesized as previously described (3). The by-product of the synthesis, 16-epiestriol-16-C14, was converted to estrone-16-C14 by treatment with pyridine hydrochloride at 200°C (4). The estrone-16-C14 was purified by chromatography on silica gel (5) and by crystallization from 95% ethanol. It was obtained in 40% yield. Chromatography on paper revealed less than 1% of radioactive impurity in each of the preparations. Estrone-C14 sulfate was prepared by treating 6 mg of estrone-C14 dissolved in 50 mg of dry pyridine with 22 mg of chlorosulfonic acid at room temperature for 2 days. The reaction flask was placed in an ice bath and 4 N potassium hydroxide was added dropwise to neutrality. After the addition of water the solution was extracted with ether and then n-butanol. The butanol was removed under vacuum and replaced with 5 ml of water. Chromatography of the product on paper revealed a single radioactive zone corresponding to authentic estrone sulfate. The solvent system was butanol-toluene (100:100)/ammonia-water (20:180) (6). The eluted zone was quantitatively hydrolyzed by phenolsulfatase giving estrone-C14 as the sole radioactive product. The yield of estrone-C14 sulfate was estimated from the radioactivity to be over 90%. In one year in which the solution was stored at 15°C except for occasions when samples were required, 3% hydrolysis occurred.

Analytical: Placental Transfer—The radioactivities in the perfusate, maternal plasma, and placenta were separated into ethereal solutions of the unconjugated estrogens and aqueous solutions of the conjugated estrogens as previously described (2). Usually aliquots of the aqueous solutions were analyzed separately for glucosiduronates, sulfates, and phosphates by enzymatic methods. In some instances, aliquots were treated with the enzyme sequentially. That is, after treatment with one enzyme and extraction of the other soluble estrogen the procedure was repeated with a second enzyme.

Glucosiduronates were hydrolyzed with β-glucuronidase 500 units (Fishman) per ml at 38°C, pH 5, for 16 hours. In each case a control containing the complete system except for the enzyme was run concurrently and the difference in the amount of released other soluble radioactivity was used as a measure of the glucosiduronate conjugates. In some instances the specific

* This investigation was supported in part by Research Grants PHS-CY-2071 from the National Institutes of Health; and the Association for Public Health Service; The American Cancer Society; PHS-G-4024 from the National Cancer Institute, United States Public Health Service; The American Cancer Society; PHS-G-4024 from the National Institutes of Health; and the Association for the Aid of Crippled Children. A preliminary account of this work was presented at the Fiftieth annual meeting of the American Society of Biological Chemists at Atlantic City, New Jersey, April 1959 (1).

† John and Mary Markle Scholar in Medical Science.


† Retodase, Warner-Chilcott Laboratories, New Jersey.
β-glucuronidase inhibitor, saccharolactone (7), was added at a concentration of 0.025 M to provide additional evidence for the presence of glucuronides.

Sulfates were hydrolyzed with phenolsulfatase (0.5 mg per ml) at 50°, pH 0, for 10 hours. Concurrent incubations were carried out with enzyme plus inorganic phosphate which inhibits phenolsulfatase (8). The percentage of radioactivity rendered ether-soluble by enzymatic treatment indicated the amount of phenolsulfate present.

An attempt was made to investigate the presence of estrogen phosphates with the use of acid and alkaline phosphatasés. Incubations were carried out at pH 5 and pH 10, respectively, at an enzyme concentration of 1 mg per ml at 37° for 16 hours. Sulfurylation by Placenta—After the perfusion with either estradiol-16-C14 or estrone-16-C14 aqueous solutions of the estrogen conjugates in the placenta and the perfusate were obtained. Sodium hydroxide was added to a final concentration of 0.1 N and the solution was extracted with n-butanol. The butanol was removed under reduced pressure and replaced with water. After extraction with ether to remove unconjugated estrogens which may have formed during the procedure, an aliquot was assayed for phenolsulfates as described above. When significant phenolsulfate radioactivity was indicated, the conjugate was chromatographed on paper with the butanol-toluene/ammonia-water system mentioned above. The paper was scanned for radioactivity in a windowless automatic paper scanner. The radioactive zone was eluted with methanol which was replaced with water. The aqueous solution was extracted with ether and incubated with phenolsulfatase. The hydrolyzed estrogens were extracted with ether and chromatographed on paper. The systems were chloroform-formamide for estriol and benzene-formamide for estrone. The development times were 2 days and 3 hours, respectively. In the estrone experiments two zones corresponding in Rf to estrone and estradiol-17β carriers, respectively. Each was crystallized from ethanolic constant specific activity. Estrone was converted to the monobenzoate and estradiol-17β to the dibenzoate. Each was crystallized from ethanolic constant specific activity.

Counting—Radioactivities were determined in either the D46A Nuclear windowless flow gas counter (50% efficiency) or the D47 Nuclear micromil window counter (25% efficiency). The counts were corrected to infinite thinness.

Enzymes + saccharolactone, further strengthening the identification of the glucuronides. In contrast phenolsulfatase hydrolyzed 24% of the maternal water-soluble radioactivity and 3% of the water-soluble radioactivity in the perfusate. Phenolsulfatase was inhibited about 48% by 0.12 M phosphate ion. In a control experiment a similar result was obtained for the inhibition of the hydrolysis of estrone-C14 sulfate synthesized in our laboratory.

**RESULTS**

Estradiol-16-C14 was injected into pregnant guinea pigs while the fetal placenta was perfused in situ. Water-soluble and ether-soluble radioactivity was detected in the maternal circulation and in the perfusate indicating rapid transfer and conjugation (Table I). By enzymatic hydrolysis it was shown that 43% of the maternal water-soluble radioactivity was in the glucosiduronate form whereas in the perfusate it was about 3% (Table II). The hydrolysis was inhibited 95% by saccharolactone, further strengthening the identification of the glucuronides.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Perfusion time</th>
<th>Maternal plasma</th>
<th>Fetal (perfusate) plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Injected</td>
<td>Water soluble</td>
<td>Ether soluble</td>
</tr>
<tr>
<td>1</td>
<td>5-14</td>
<td>3.9</td>
<td>5.825</td>
</tr>
<tr>
<td>2</td>
<td>2-11</td>
<td>3.7</td>
<td>13,300</td>
</tr>
<tr>
<td>3</td>
<td>2-11</td>
<td>3.3</td>
<td>22,000</td>
</tr>
</tbody>
</table>

**TABLE II**

Effect of β-glucuronidase and phenolsulfatase on water-soluble radioactivities in maternal plasma and perfusate of guinea pig

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Maternal Plasma</th>
<th>Fetal Perfusate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water soluble</td>
<td>Ether soluble</td>
</tr>
<tr>
<td>I</td>
<td>6.3</td>
<td>3.3</td>
</tr>
<tr>
<td>II</td>
<td>1.1</td>
<td>5.9</td>
</tr>
<tr>
<td>III</td>
<td>1.3</td>
<td>7.2</td>
</tr>
</tbody>
</table>

**TABLE III**

<table>
<thead>
<tr>
<th>Material</th>
<th>Experiment I</th>
<th>Experiment II</th>
<th>Experiment III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perfusate</td>
<td>% Hydrolysis</td>
<td>% Hydrolysis</td>
<td>% Hydrolysis</td>
</tr>
<tr>
<td>β-Glucuronidase</td>
<td>34</td>
<td>7.4</td>
<td>50</td>
</tr>
<tr>
<td>Control</td>
<td>1.3</td>
<td>1.9</td>
<td>5.1</td>
</tr>
<tr>
<td>Enzyme + saccharolactone</td>
<td>8.4</td>
<td>4.8</td>
<td>3.3</td>
</tr>
<tr>
<td>Phenolsulfatase</td>
<td>24</td>
<td>100</td>
<td>23</td>
</tr>
<tr>
<td>Control</td>
<td>1.9</td>
<td>8.7</td>
<td>2.0</td>
</tr>
<tr>
<td>Enzyme + phosphate</td>
<td>13</td>
<td>55</td>
<td>14</td>
</tr>
</tbody>
</table>

* Incubation was carried out with a minimum of 204 c.p.m. but most were between 1000 and 4000 c.p.m.
An attempt was made to identify the remaining one-third of the maternal water-soluble radioactivity. It was thought that it might be a phosphate since Oertel and Eik-Nes (10) found that injected dehydroepiandrosterone circulates in the peripheral blood of the dog largely as a phosphate. Alkaline phosphatase was ineffective but acid phosphatase hydrolyzed the water-soluble fraction to about the same extent as $\beta$-glucuronidase. However, the action of acid phosphatase was completely inhibited by saccharolactone indicating that the acid phosphatase was contaminated with $\beta$-glucuronidase. This fraction remains unidentified.

The possibility of significant cross-contamination between phenolsulfatase and $\beta$-glucuronidase was eliminated in the following manner. In Experiments 1 and 2 (Table II) aliquots of the maternal water-soluble radioactivities were treated with the enzymes sequentially. After treatment with $\beta$-glucuronidase and extraction with ether, the aqueous residue was treated with phenolsulfatase and again extracted with ether. The radioactivities in the ether fractions were determined. Other aliquots were similarly treated except that the order of enzyme incubations was reversed. These treatments had no effect on the results shown in Table II.

The origin of the estrogen sulfates in the perfusate was investigated. Estrone-16-C$^{14}$ sulfate was synthesized and used in placental transfer experiments. The placenta was virtually impermeable in both directions to estrone sulfate under the conditions of the experiment (Tables III and IV). In studying the transfer across the placenta from the fetal side it was demonstrated that under these same conditions the placenta was freely permeable to unconjugated estrone.

**Sulfurylation by Placenta**—The failure of estrone sulfate to traverse the placental barrier coupled with the finding of significant amounts of estrogen sulfates in the perfusate after giving unconjugated estrogens to the mother raised the possibility that the placenta was the site of sulfurylation. The placenta was isolated from the maternal circulation as described in “Experimental” and perfused with radioactive estrogens. Sulfurylation of estrone-16-C$^{14}$ and estrone-16-C$^{14}$ was demonstrated. The placenta was extracted in the Waring Blender with 80% ethanol. After removing the ethanol under reduced pressure the aqueous residue was extracted with ether. Between 95 and 98% of the radioactivity was extracted. The water-soluble radioactivity was chromatographed on Whatman No. 1 paper with n-butanol-toluene/ammonia-water as the solvent system. A parallel strip contained synthetic estrone-C$^{14}$ sulfate. The distributions of the radioactivities on each strip were similar. The $R_f$ was 0.25.

In the last two estrone experiments (Table V) further evidence was sought that the conjugates were sulfates. Some of the water-soluble radioactivity was chromatographed on Whatman No. 1 paper with n-butanol-toluene/ammonia-water as the solvent system. A parallel strip contained synthetic estrone-C$^{14}$ sulfate. The distributions of the radioactivities on each strip were similar. The $R_f$ was 0.25.

The radioactive zone corresponding to estrone sulfate was eluted with methanol and incubated with phenolsulfatase. About 95% of the eluted radioactivity became ether soluble after enzyme treatment. The ether-soluble radioactivity released by enzyme hydrolysis was identified as a mixture of estrone and...
TABLE VI

**Determination by reverse isotope dilution of estrone and estradiol-17β**

<table>
<thead>
<tr>
<th>Carrier</th>
<th>Before crystallization</th>
<th>After crystallization from ethanol</th>
<th>Derivative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrone</td>
<td>25</td>
<td>120</td>
<td>115</td>
</tr>
<tr>
<td>Estradiol-17β</td>
<td>20</td>
<td>28</td>
<td>30</td>
</tr>
</tbody>
</table>

* The c.p.m. were corrected for self-absorption. About 0.5 mg was plated. For estradiol-17β, the observed counts were only 10 to 15 c.p.m. above background (20 c.p.m.) hence a 25 to 30% error is expected. The observed counts were about 40 c.p.m. above background for estrone.

† Monobenzoate was corrected to c.p.m. per mg of estrone.

‡ Dibenzazete was corrected to c.p.m. per mg of estradiol-17β.

The currently available preparations of β-glucuronidase, phenol-sulfatase, and acid phosphatase are not pure. It was demonstrated that there was no significant cross-contamination between Ketodase and Mylase P, but that the acid phosphatase had β-glucuronidase activity. The acid phosphatase was contaminated also with phenol-sulfatase. The use of the specific β-glucuronidase inhibitor, saccharolactone, served further to identify glucuronides. The inhibition of phenol-sulfatase by inorganic phosphate is not specific. The identification of the estrogen sulfates was confirmed by paper chromatography.

Estradiol sulfate as well as estrone sulfate was obtained when the placenta was perfused with estrone. This is to be expected since the placenta is a rich source of 17α-dehydrogenase (15). The sulfates of estrone and estradiol were not separable by paper chromatography. Estradiol sulfate had a much lower Kf.

In the previous paper it was noted that the relative inability of the fetus to glucosiduronate was probably advantageous because the impermeability of the placenta would lead to accumulation. This would suggest that sulfurylation by the placenta and probably by the fetus should lead to accumulation of sulfates by the fetus. The answer to this is not yet clear. One possible explanation would be reversal of sulfurylation by fetal sulfatases. Activity of this nature has been demonstrated in vivo in a human adult.†

**SUMMARY**

With the use of an in situ perfusion technique the transfer of estrogens across the guinea pig placenta was studied. The placenta was freely permeable to the unconjugated estrogens, estrone-C14, and estriol-C14 but relatively impermeable to estrone-C14 sulfate. With a modification of the same technique it was possible to demonstrate the conversion of estrone-C14 to the sulfates of estrone and estradiol-17β in the placenta.

**REFERENCES**


† M. Levitz and G. H. Twombly unpublished observations.
The Relative Transfer of Estrogens and Their Sulfates Across the Guinea Pig Placenta: Sulfurylation of Estrogens by the Placenta
Mortimer Levitz, George P. Condon, William L. Money and Joseph Dancis