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

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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-17β, 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 sulfurylate 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 week or two of gestation a fetus was delivered by hystero tomy 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).

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† John and Mary Markle Scholar in Medical Science.


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 ether 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 ether soluble radioactivity was used as a measure of the glucosiduronate conjugates. In some instances the specific

* The authors are indebted to the Schering Corporation and Ayerst Laboratories for generous gifts of estrone and estrone sulfate.

† P. Mylase, Wallerstein Laboratories, New York, New York.

‡ Ketodase, 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°C, 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 conjugates in the placenta and in the perfusate. In the studies on sulfurylation of estrogens by the placenta and fetus (F) before perfusion of fetal side of the placenta. In the experiments on sulfurylation of estrogens by the placenta clamps (a) and (b) are applied.

**Results**

Estriol-16-C^14 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 glucuronidate 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. In contrast phenolsulfatase hydrolyzed 24% of the maternal water-soluble radioactivity and 93% 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-C^14 sulfate synthesized in our laboratory.

**Table I**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Perfusion time</th>
<th>Maternal plasma</th>
<th>Fetal (perfusion) 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

Aliquots of the water-soluble radioactivities in Table I were incubated with enzymes and the amounts of hydrolysis were determined. Incubations with β-glucuronidase were for 18 hours at 37°C at pH 5; with phenolsulfatase (Mylase P), for 18 hours at 37°C at pH 5; with phenolsulfatase (Mylase P), for 18 hours at 37°C at pH 5.

**Table III**

<table>
<thead>
<tr>
<th>Experiment I</th>
<th>Experiment II</th>
<th>Experiment III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal</td>
<td>Perfusate</td>
<td>Maternal</td>
</tr>
<tr>
<td>β-Glucuronidase</td>
<td>34</td>
<td>7.4</td>
</tr>
<tr>
<td>Control</td>
<td>1.3</td>
<td>1.9</td>
</tr>
<tr>
<td>Enzyme + saccharolactone</td>
<td>8.4</td>
<td>4.8</td>
</tr>
<tr>
<td>Phenolsulfatase</td>
<td>24</td>
<td>100</td>
</tr>
<tr>
<td>Control</td>
<td>1.9</td>
<td>8.7</td>
</tr>
<tr>
<td>Enzyme + phosphate</td>
<td>13</td>
<td>55</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 β-glucuronidase. However, the action of acid phosphatase was completely inhibited by saecharalactone indicating that the acid phosphatase was contaminated with β-glucuronidase. This fraction remains unidentified.

The possibility of significant cross-contamination between phenolsulfatase and β-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 β-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\textsubscript{14} sulfate was synthesized and used in placental transfer experiments. The placenta was virtually impermeable to unconjugated estrone.

### Table III

**Maternal to fetal (perfusate) transfer of estrone-16-C\textsubscript{14} sulfate in guinea pig**

The estrone-16-C\textsubscript{14} sulfate was injected into the maternal jugular vein at "0" time and the umbilical vessels were perfused for the times indicated. Maternal samples were taken by cardiac puncture at the conclusion of the perfusion.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Time</th>
<th>Injected</th>
<th>Maternal</th>
<th>Perfusate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min</td>
<td>c.p.m.</td>
<td>c.p.m./ml</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>2.4 x 10^4</td>
<td>8,700</td>
<td>27</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>2.0 x 10^4</td>
<td>3,550</td>
<td>28</td>
</tr>
</tbody>
</table>

### Table IV

**Fetal (perfusate) to maternal transfer of estrogens in guinea pig**

Radioactive estrogens were added to the perfusate and the rates of transfer inferred from the drop in radioactivity during perfusion through the placenta.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Estrogen (c.p.m.)</th>
<th>Start</th>
<th>End</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estrone-16-C\textsubscript{14}</td>
<td>c.p.m./ml</td>
<td>c.p.m./ml</td>
</tr>
<tr>
<td></td>
<td>Estrone-16-C\textsubscript{14} sulfate</td>
<td>c.p.m./ml</td>
<td>c.p.m./ml</td>
</tr>
<tr>
<td>1. Sequential*</td>
<td>(a)</td>
<td>19,660</td>
<td>6,385</td>
</tr>
<tr>
<td></td>
<td>(b)</td>
<td>13,075</td>
<td>14,900</td>
</tr>
<tr>
<td></td>
<td>(c)</td>
<td>3,965</td>
<td>12,140</td>
</tr>
<tr>
<td></td>
<td>(d)</td>
<td>6,675</td>
<td>12,140</td>
</tr>
<tr>
<td>2. Combination†</td>
<td></td>
<td>3,582</td>
<td>1,538</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13,210</td>
<td>12,150</td>
</tr>
</tbody>
</table>

* The placenta was perfused in 4 stages containing (a) estrone, (b) estrone sulfate, (c) a mixture of the two, and (d) estrone. Between stages the placenta was flushed with 4 ml of plasma.
† The placenta was perfused with 16 ml of plasma containing a mixture of estrone and estrone sulfate and the relative rates of disappearances noted.

### Table V

**Sulfurylation of estrogens in perfused guinea pig placenta**

Placentas were perfused with radioactive estrogens for 15 minutes after maternal as well as the fetal circulations were excluded. The conjugates were extracted from placental tissue. The placental radioactivity was fractionated into ether soluble (90 to 98%) and water soluble (conjugated) forms. The conjugates were extracted from 0.1 m NaOH with butanol. The butanol was replaced with water and the resulting solution was treated with phenolsulfatase.

<table>
<thead>
<tr>
<th>Estrogen</th>
<th>Perfused</th>
<th>Butanol soluble</th>
<th>Hydrolyzed by phenolsulfatase</th>
<th>Phosphate inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c.p.m.</td>
<td>c.p.m.</td>
<td>%*</td>
<td>%*</td>
</tr>
<tr>
<td>Estriol</td>
<td>2.0 x 10^4</td>
<td>2.9 x 10^4</td>
<td>80</td>
<td>65</td>
</tr>
<tr>
<td>Estradiol</td>
<td>1.8 x 10^4</td>
<td>1.3 x 10^4</td>
<td>95</td>
<td>53</td>
</tr>
<tr>
<td>Estrone</td>
<td>7.3 x 10^3</td>
<td>9.3 x 10^3</td>
<td>67</td>
<td>60</td>
</tr>
<tr>
<td>Estrone†</td>
<td>7.9 x 10^3</td>
<td>5.1 x 10^3</td>
<td>60</td>
<td>53</td>
</tr>
<tr>
<td>Estrone†</td>
<td>8.3 x 10^4</td>
<td>8.15 x 10^4</td>
<td>84</td>
<td>66</td>
</tr>
<tr>
<td>Estrone†</td>
<td>7.6 x 10^4</td>
<td>2.1 x 10^4</td>
<td>85</td>
<td>60</td>
</tr>
</tbody>
</table>

* Percentage of butanol soluble radioactive hydrolyzed by phenolsulfatase. See Table II for details of hydrolysis and inhibition.
† In these experiments radioactive sulfates also were found in the perfusate. The amounts were about one-third of the placenta values.

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 estradiol.
Table VI
Determination by reverse isotope dilution of estrone and estradiol-17β

In the last two experiments of Table V the estrogen sulfates made in the placentas perfused with estrone-C¹⁴ were hydrolyzed with phenolsulfatase. The ether extract was chromatographed on paper. Two radioactive zones corresponding in mobilities to estrone and estradiol-17β were obtained in each experiment. The estrogen zones from the two experiments were combined as were the estradiol zones. Each was analyzed by reverse isotope dilution.

<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 mg</td>
<td>120 c.p.m./mg*</td>
<td>115 c.p.m./mg*</td>
</tr>
<tr>
<td>Estradiol-17β</td>
<td>20 mg</td>
<td>28 c.p.m./mg*</td>
<td>30 c.p.m./mg*</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.
‡ Dibenzoate was corrected to c.p.m. per mg of estradiol-17β.

estradiol-17β in the following way. First, it was chromatographed on paper for 3 hours with benzene-formamide. Two radioactive zones corresponding in mobilities to estrone and estradiol were obtained. These were eluted. The 2 estrone and the 2 estradiol-17β esters were combined and the identifications were confirmed by the method of reverse isotope dilution (Table VI).

Discussion

The present study is an extension of the previous investigation on the relative transfer of estrogens and their glucosiduronates (2). In that study it was noted that the unconjugated estrogens rapidly passed across the placenta in both directions but the glucosiduronates did not. This difference in behavior was attributed to water solubility. Without specific transport mechanisms water-soluble substances of high molecular weight traverse membranes with difficulty (11).

In those studies and in these a significant water-soluble fraction was noted in the perfusate after injection of the unconjugated estrogens into the mother. Further analysis showed that the perfusate conjugates were virtually all sulfates despite the fact that the greater percentage of the conjugates in the mother were glucosiduronates. These results suggested the preferential transfer of sulfates, a puzzling explanation since 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 the 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.7

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


7 M. Levitz and G. H. Twombly unpublished observations.
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