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, estriadiol-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 two or at least a gestus a fetus was delivered by hysteroscopy 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 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.

Estriol-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 etheral 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

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Estrogen Sulfates—Synthesis and Transfer in the Placenta

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RESULTS

Estriol-16-C\textsuperscript{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 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. 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\textsuperscript{14} sulfate synthesized in our laboratory.

TABLE I

Maternal to fetal (perfusate) transfer of estriol-16-C\textsuperscript{14} and its metabolites in guinea pig

<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>1-5 hrs</td>
<td>3.9 x 10\textsuperscript{3}</td>
<td>5825</td>
</tr>
<tr>
<td>2</td>
<td>1-11 hrs</td>
<td>3.7 x 10\textsuperscript{3}</td>
<td>13300</td>
</tr>
<tr>
<td>3</td>
<td>2-11 hrs</td>
<td>3.3 x 10\textsuperscript{3}</td>
<td>22000</td>
</tr>
</tbody>
</table>

TABLE II

Effect of \(\beta\)-glucuronidase and phenolsulfatase on water-soluble radioactivities in maternal plasma and perfusate of guinea pig

<table>
<thead>
<tr>
<th></th>
<th>Experiment I</th>
<th>Experiment II</th>
<th>Experiment III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal</td>
<td>Fetus</td>
<td>Maternal</td>
<td>Fetus</td>
</tr>
<tr>
<td>Plasma</td>
<td>Perfusate</td>
<td>Perfusate</td>
<td>Perfusate</td>
</tr>
<tr>
<td>% Hydrolysis*</td>
<td>% Hydrolysis*</td>
<td>% Hydrolysis*</td>
<td>% Hydrolysis*</td>
</tr>
<tr>
<td>(\beta)-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>2.1</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-solu-
ble fraction to about the same extent as β-glucuronidase. How-
ever, the action of acid phosphatase was completely inhibited
by saccharolactone 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 fol-
lowing 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 radio-
activities in the ether fractions were determined. Other aliquots
were similarly treated except that the order of enzyme incuba-
tions was reversed. These treatments had no effect on the re-

sults shown in Table II.

The origin of the estrogen sulfates in the perfusate was in-
vestigated. Estrone-16-C14 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 demon-
strated 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 sig-
ificant 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 “Experi-
mental” and perfused with radioactive estrogens. Sulfurylia-
tion of estrol-16-C14 and estrone-16-C14 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 quantitatively 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>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 plasma</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 perfused</th>
<th>Estrogen-16-C14</th>
<th>Estrone-16-C14 sulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Start c.p.m./ml</td>
<td>End c.p.m./ml</td>
<td>Start c.p.m./ml</td>
</tr>
<tr>
<td>1. Sequential*</td>
<td>(a)</td>
<td>19,650</td>
<td>6,385</td>
</tr>
<tr>
<td></td>
<td>(b)</td>
<td>8,545</td>
<td>3,965</td>
</tr>
<tr>
<td></td>
<td>(c)</td>
<td>19,650</td>
<td>6,385</td>
</tr>
<tr>
<td></td>
<td>(d)</td>
<td>3,585</td>
<td>1,538</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.
† The placenta was perfused with 16 ml of plasma containing a mixture of estrone and estrone sulfate and the relative rates of disappearances noted.

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**TABLE V**

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

Placentas were perfused with radioactive estrogens for 15 min-
utes after maternal as well as the fetal circulations were excluded.
The conjugates were extracted from placental tissue. The pla-
cental 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 radioactivity was treated with
phenolsulfatase.

<table>
<thead>
<tr>
<th>Estrogen</th>
<th>Perfused c.p.m.</th>
<th>Butanol soluble c.p.m.</th>
<th>Hydrolyzed by phenolsulfatase %</th>
<th>Phosphate inhibition %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol</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 10^5</td>
<td>1.3 x 10^5</td>
<td>85</td>
<td>55</td>
</tr>
<tr>
<td>Estrone</td>
<td>7.3 x 10^4</td>
<td>9.3 x 10^4</td>
<td>67</td>
<td>60</td>
</tr>
<tr>
<td>Estrone‡</td>
<td>7.9 x 10^4</td>
<td>5.1 x 10^4</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 radioactivity 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 pla-
centa values. The other perfusates contained none.
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 placenta 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 estrone 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></td>
<td>mg</td>
<td>c.p.m./mg*</td>
<td>c.p.m./mg*</td>
</tr>
<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.

‡ 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 eluates 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 (12). 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 glucuronidates. These results suggested that the preferential transfer of sulfates, a puzzling explanation since the reasoning applied to the impermeability of the glucuronidates should also hold for the sulfates. An alternative explanation was that the free estrogens entered the placenta and a fraction of this was conjugated with sulfuric acid (but not with glucuronic acid) distal to the barrier and then released into the umbilical circulation.

These alternatives were explored in the following way. Estrone-C14 sulfate was synthesized and its transfer characteristics investigated. The placenta was found impermeable. When the isolated placenta was perfused with labeled estradiol and estrone, sulfuration but not glucosiduronidation was demonstrated.

Enzymatic sulfurylation requires a sulfate-activating system which generates adenosine 3'-phosphate 5'-phosphosulfate from adenosine triphosphate and inorganic sulfate, and a sulfokinase to transfer the sulfate from adenosine 3'-phosphate 5'-phosphosulfate to substrate (12). The sulfurylating enzyme system has been demonstrated in liver and to lesser extents in kidney, intestine, and chick embryo cartilage (13). It is expected that this is the first report of the presence of sulfurylating enzymes in the placenta.

Conjugates were identified primarily by enzymatic hydrolyses. The currently available preparations of β-glucuronidase, phenolsulfatase, 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 phenolsulfatase. The use of the specific β-glucuronidase inhibitor, saccharolactone, served further to identify glucuronides. The inhibition of phenolsulfatase 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 glucosiduronidate 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 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 estradiol-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.

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M. Levitiz, G. P. Condon, W. L. Money, and J. Dancis

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