BIOSYNTHESIS OF ESTROGENS IN IN VITRO PERFUSION OF THE HUMAN PLACENTA

TETSUYA NAKAYAMA, KIYOSHI ARAI, TORU TABEI, TAKUMI YANAIHARA, KAZUO SATOH, KEIJI NAGATOMI AND YOSHIHISA FUJITA

Department of Obstetrics and Gynecology, Faculty of Medicine, University of Tokyo, Tokyo

SYNOPSIS

Perfusions of the human term placenta with radioactive androstenedione, testosterone, DHEA, progesterone, cholesterol and estradiol-17β as precursors were carried out and the formation of estrogens was investigated. After perfusion for three hours with human whole blood, added with human chorionic gonadotropin and cofactors, radioactive metabolites in the perfusate were studied. The radioactive metabolites were purified by chromatography at each step of purification. Estrogens were measured by the Kober reaction, and the radioactivity was counted by the gas flow counter. These experiments demonstrated that C-19 steroids were aromatized in a high yield by the perfused human placenta to estrone and estradiol-17β in the ratio of approximately 3 to 1, but that it was not capable of converting androgens to estriol. It was also noted that there was no conversion of estradiol-17β to estriol. In the perfusion study with DHEA, which is considered to be an important intermediate to estriol in the feto-placental unit during pregnancy, 10.7% of the initial radioactivity was recovered as a polar phenolic compound which behaved like 16-epiestriol on preliminary identification. Furthermore, neither progesterone nor cholesterol converted to estrogens in the human perfused placenta.

The human placenta is an abundant source of estrogen production in late pregnancy. It is well known that as human pregnancy advances urinary excretion of estriol* increases at a rate greater than that of estrone and estradiol-17β. Levitz et al. (1956) were unable to detect any evidence of conversion of estradiol-17β to estriol in the perfused human placenta. Troen (1961) demonstrated that the human placenta was capable of converting estradiol-17β to estriol, when HCG was added to the perfusing fluid. He also identified tentatively 16-epiestriol in the perfusate. Androstenedione has been shown to be converted to estrogens in a system consisting of human placental microsomes (Ryan, 1959a). Pathways of estrogen biosynthesis from C-19 steroids in the human placenta, have been elucidated to some extent (Bagget, 1959; Dorfman and Gual, 1959; Ryan, 1959b). In

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the opinion of Little and Shaw (1961), progesterone is probably an intermediate in the synthesis of estrone and estradiol-17β in this organ. They have also demonstrated the conversion of progesterone to 17α-hydroxyprogesterone in the placental homogenate.

Though the conversion of cholesterol to pregnenolone has been documented by Solomon (1954 and 1960), no attempt has been made to observe the synthesis of estrogen from cholesterol by perfusion of the human placenta in vitro.

Perfusion of the whole organ appears to be a more physiological approach to its metabolic and synthetic activities than does the study of homogenates, slices or perfused isolated cotyledons.

The present investigation was undertaken to examine the question of hydroxylation of estradiol-17β or estrone at C-16 and also of aromatization of the neutral steroids (androstenedione, testosterone and DHEA) by means of in vitro perfusion of the human placenta. In addition, the metabolism of progesterone and cholesterol was also studied.

MATERIALS AND METHODS

Materials

1) Radioactive steroids as tracers

<table>
<thead>
<tr>
<th>Perfusion</th>
<th>Steroid</th>
<th>Activity/Amount</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Androstenedione-4-14C</td>
<td>5μCi/200μg</td>
<td>New England, NEC 136, Lot No. 134-64-22, 1μCi/13μg</td>
</tr>
<tr>
<td>II</td>
<td>Testosterone-4-14C</td>
<td>5μCi/200μg</td>
<td>Amersham, CFA 129, Batch 7, 1μCi/13μg</td>
</tr>
<tr>
<td>III</td>
<td>Dehydroepiandrosterone-4-14C</td>
<td>3μCi/200μg</td>
<td>New England, NEC 206, Lot No. 134-98a-46b, 1μCi/7.09μg</td>
</tr>
<tr>
<td>IV</td>
<td>Progesterone-4-14C</td>
<td>5μCi/200μg</td>
<td>Amersham, CFA 148, Batch 16, 1μCi/12μg</td>
</tr>
<tr>
<td>V</td>
<td>Cholesterol-4-14C</td>
<td>10μCi/150μg</td>
<td>Amersham, CFA 128, Batch 43, 1μCi/15μg</td>
</tr>
<tr>
<td>VI</td>
<td>Estradiol-17β-16-14C</td>
<td>1μCi/200μg</td>
<td>Amersham, CFA 237, Batch 2, 1μCi/28μg</td>
</tr>
</tbody>
</table>

Radiochemical purity of these steroids was checked by paper chromatography in the Bush system A.

2) Reference standards

Estradiol-17β, estrone, estriol and 16-epiestriol were obtained from commercial sources. Purity of these standards was checked by paper chromatography and thin layer chromatography.

3) Gonatropin (Teikoku Hormone Mfg. Co., Ltd.) was used as HCG. NADH was purchased from the Sigma Chemical Co. All reagents were analytical grade and all solvents were redistilled before use.

Technique of perfusion

The human placentas were obtained at normal term delivery. The two umbilical arteries and the umbilical vein were cannulated by the Nelaton catheters and flushed out via arteries with 300 ml of heparinized saline. The placenta was attached to the perfusion apparatus, which is illustrated diagrammatically in Figure 1. The perfusion fluid was circulated at a rate of 80 to 100 ml/min., while being continuously oxygenated by rotating filmoxygenator. A pump of DeBakey type gave a pulsatile movement with adjustable pressure and speed. The pressure varied from 100 to 110 mmHg. The whole circuit was kept in an airconditioned cabinet maintained at 37°C. As perfusate, 150 ml of human whole blood was used. The above-mentioned steroid precursors dissolved in 0.2 ml of propylene glycol, 6,000 I.U. of human chorionic gonadotropin, 15 mg of NADH, and 250 mg of oxytetracycline were added to the perfusate. The perfusion time was three hours. Preliminary perfusions were performed to demonstrate that under the conditions used there was no histological evidence of rupture of blood vessels or destruction of cells and nuclei. Histochemical examinations of the placenta were made after the perfusion experiment, and the activities of acid phosphatase, alkaline phosphatase, succinic dehydrogenase, nonspecific esterase and 3β-hydroxysteroid dehydrogenase were assessed.
Measurement of radioactivity and determination of estrogens

These procedures were carried out as described elsewhere (Nakayama et al., 1966a).

Formation of derivatives

Estrogens were methylated with dimethyl sulfate according to Brown (1955).

Chromatography

Paper chromatography was developed on Whatman No. 1 filter paper in the Bush’s solvent systems (A, B 1 and C) (Bush, 1961). Localization of the standard estrogens was made by spraying with potassium ferric cyanide-ferric chloride reagent (Eberlein and Bongiovanni, 1958). Δ-3 Ketosteroids were visualized by ultraviolet light.

Gradient elution partition chromatography on Celite column according to Engel et al. (1961) was employed.

Alumina column chromatography of the methylated phenolic fractions was carried out according to the Brown’s method (1955).

Thin layer chromatography was developed in cyclohexane and ethyl acetate (1 : 1), (1 : 2). The standard estrogens and their derivatives were visualized by spraying with sulfuric acid and heating at 100°C for 10 mins.

Recrystallization to constant specific activity

The samples were recrystallized as the parent compound, using alternating or different solvent systems. An allowable limit for the establishment of radiochemical purity was 5~8% difference in the specific activity of the last two sets of crystals. These criteria are in agreement with those reported by Axelrod et al. (1965).

Extraction and purification

Immediately after perfusion, the perfusate was collected and centrifuged at 3,000 rpm for 15 mins. Plasma was separated and the red blood cells were then suspended in saline. The suspension was centrifuged and the supernatant was added to the plasma. It was felt that hydrolysis was not necessary in this case since other studies have shown that the vast bulk of the recovered steroids would be non-conjugated. Accordingly, the extraction of plasma without hydrolysis was carried out three times with the same volume of an ethyl acetate-ether mixture (1 : 1). The residue was dissolved in 70% methanol, stored at −15°C overnight, and lipid was removed by refrigerated centrifuge. In perfusion V, the residue of 70% methanol was partitioned between pentane and 90% methanol. The 90% methanol fraction was subjected to the separation of the phenolic fraction (Ryan and Smith, 1965). The pentane-soluble fraction contained unchanged labelled cholesterol.

Before separation of the phenolic fraction, known amount of estrone, estradiol-17β, estriol and 16-epiestriol were added to the extract as carriers. The residue was dissolved in benzene-petroleum ether (1 : 1), from which the “estril” fraction was extracted with distilled water and then the “estrone-estradiol” fraction was extracted with 0.4N NaOH (Brown, 1955; Roy and Brown, 1960). The remainder of the extraction was considered to contain the “neutral” fraction.

The phenolic fraction from perfusions II, IV, V and VI were separated by paper chromatography in the Bush systems B 1 and C, and by thin layer chromatography in cyclohexane-ethyl acetate (1 : 1). The methylated derivatives of estrogens thus separated, were further purified on alumina column chromatography (Brown, 1955). The phenolic fractions from other perfusions (I and III) were separated by gradient elution partition chromatography on a Celite column according to Engel et al. (1961); as the stationary phase 90% methanol was used, and 2, 2, 4-trimethylpentane and 1, 2-dichlorethane were used as the mobile phase. Subsequent to these separations and the formation of derivatives, paper chromatography in the Bush systems B 1 and C, and thin layer chromatography in cyclohexane-ethyl acetate (1 : 1), (1 : 2) were employed for further purification. After the purification by chromatographies, eluted samples were added with standard carriers and recrystallized as the parent compounds from different solvents.

“Neutral” fractions from each perfusion experiment were tentatively separated by paper chromatography in the Bush system A.
Table 1. Conversion of C-19 steroids in perfusion of human placenta

<table>
<thead>
<tr>
<th>Perfusion</th>
<th>Precursors</th>
<th>Initial cpm</th>
<th>Phenolic fraction</th>
<th>estrone</th>
<th>estradiol</th>
<th>estriol</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>androstenedione-14C</td>
<td>1,281,800 (100)</td>
<td>1,087,120 (84.8)</td>
<td>562,041 (43.8)</td>
<td>256,560 (20.0)</td>
<td>—</td>
</tr>
<tr>
<td>II</td>
<td>testosterone-14C</td>
<td>589,000 (100)</td>
<td>481,020 (81.5)</td>
<td>278,008 (47.2)</td>
<td>93,650 (15.9)</td>
<td>—</td>
</tr>
<tr>
<td>III</td>
<td>DHEA-14C</td>
<td>475,750 (100)</td>
<td>378,490 (79.6)</td>
<td>201,800 (42.4)</td>
<td>36,500 (7.7)</td>
<td>—</td>
</tr>
</tbody>
</table>

RESULTS

In perfusions I, II and III, the evidence for the conversion of C-19 steroids (androstenedione-4-14C in perfusion I, testosterone-4-14C in perfusion II and DHEA-4-14C in perfusion III) was presented (Table 1). It can be seen that these androgens are easily aromatized to estrogens in almost the same amount in each perfusion.

In perfusion I, the initial extract of the perfusate contained the radioactivity of 1,281,800 cpm, and 84.8% of which were found in the phenolic fraction. Following gradient elution partition chromatography of this fraction, 43.8% was found in the estrone fraction and 20.0% in the estradiol-17β fraction. There could be found only 1.5% and 0.8% of the radioactivity in the 16-epiestriol and estriol fractions (Fig. 2). Each radioactive peak was then purified by paper chromatography. The radiochemical purity of the estrone and estradiol-17β fractions was checked by the specific activity obtained as cpm per μg Kober chromogen, and then standard carriers of 8.26 mg of estrone and 6.35 mg of estradiol-17β were added and recrystallized to a constant specific activity (Table 2). How-
ever, the specific activity in the estriol and 16-epiestriol fractions fell so rapidly in the steps of purification that no conversion of the perfused androgen to those substances could be demonstrated.

The crude extract of the perfusion fluid from perfusion II contained 589,000 cpm, and 76.9% of the radioactivity was recovered from the "estrone-estradiol" fraction while 4.6% from the "estriol" fraction. Each fraction was further separated by paper chromatography; 47.2% was found in the estrone fraction and 15.9% in the estradiol-17β fraction. In this experiment, methylated derivatives were made and then purified on alumina column chromatography. No marked change in the specific activity was observed in these procedures (12,300 cpm/μg to 10,300 cpm/μg in estrone and 13,000 cpm/μg to 10,100 cpm/μg in estradiol-17β). The identities of radioactive materials in the "estriol" fraction was not established.

The conversion of DHEA-4-14C, a C-19 steroid which is considered to be an important intermediate to estriol during pregnancy, was studies in perfusion III. The initial extract of the perfusate contained 475,750 cpm. The conversion of DHEA into phenolic steroids occurred in a high yield similar to perfusions using other C-19 steroids. The three distinct peaks appeared on the Celite column chromatogram of the "estrone-estradiol" fraction (Fig. 3). The specific activities of estrone, estradiol-17β and estriol expressed as cpm per μg Kober chromogen are shown in Table 3. The radioactive metabolites recovered in the estrone and estradiol-17β fractions behaved exactly as the standard estrone and estradiol-17β. The radiochemical purity of the estriol fraction was not confirmed.

It was noted that more than 10% of the radioactivity was found in the 16-epiestriol fraction (P_{III} in Fig. 4), and this radioactive metabolite behaved exactly as the added carrier standard. The constant specific activity of this 16-epiestriol is presented in Table 4. But, it was difficult to separate 16-epiestriol from estradiol-17β with hydroxyl group at the 6 or 15 position by our method, so that contaminations in the 16-epiestriol fraction were not completely excluded.

In perfusions IV and V, in which progesterone-4-14C (perfusion IV) and cholesterol-4-14C (perfusion V) were added as precursors, no conversion to the phenolic steroids could be demonstrated.

In perfusion IV, the initial extract contained 963,900 cpm, but there could be found only 1% of the radioactivity in the phenolic fraction. And no estrogen could be identified. In perfusion V, more than 80% of the radioactivity was found in the pentane-soluble
Fig. 4. Celite column chromatogram of the "estradiol" fraction. Solid lines indicate 14C measurements. Dotted lines indicate weight of the standard carriers. P1; estrone, PII; estradiol-17β, PIII; 16-epiestriol, PIV; estriol. The gradient with dichloroethane was applied at the tube No. 26.

Table 4. 16-epiestriol like radioactive product (peak III in Fig. 4) in each step of purification

<table>
<thead>
<tr>
<th>Procedure</th>
<th>cpm</th>
<th>µg</th>
<th>specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water soluble fraction</td>
<td>81,940</td>
<td>50.0</td>
<td>1,639</td>
</tr>
<tr>
<td>Celite column chromatography</td>
<td>50,983</td>
<td>37.5</td>
<td>1,359</td>
</tr>
<tr>
<td>Paper chromatography</td>
<td>41,200</td>
<td>29.4</td>
<td>1,401</td>
</tr>
</tbody>
</table>

fraction, and only 14.2% was in the 90% methanol fraction. In the phenolic fraction, there could be found only 0.1% of the initial count. Following the separation procedure by thin layer chromatography, identifications of three estrogens in this fraction were not established.

Eighty one percent of the initial radioactivity was included in the "estrone-estradiol" fraction when estradiol-17β-16-14C was used as a precursor (perfusion VI). Following the separation by paper chromatography, 60.3% of the radioactivity was found in the estrone fraction. Sequential chromatography of the estrone fraction before and after methylation caused no significant change in the specific activity (1,105 cpm/µg after the first paper chromatography, and 1,083 cpm/µg after the alumina column chromatography). However, bulk of the radioactive metabolite in the "estradiol" fraction was so different from the standard estriol with a decrease in its specific activity during the steps of purification that no identification of radioactive estriol could be made.

Table 5 summarizes the results obtained from perfusions of the human placenta under the same conditions. This reveals a similar extent of aromatization for the three C-19 steroid precursors (androstenedione, testosterone and DHEA) with values ranging between 50.1% and 63.8% of the initial radioactivity recovered. No conversion to estriol was demonstrated from those C-19 steroids and from estradiol-17β.

It is interesting to note that the ratio of estrone to estradiol-17β was approximately 2.5 to 1 when C-19 steroid was used as a precursor, whereas 11.6 to 1 when estradiol-17β was used.

It is of great interest that the high yield of 16β-hydroxylated metabolite was formed from DHEA, while no epiestriol was formed from other C-19 precursors.

No conversion of progesterone and cholesterol to estrogens was demonstrated.

**DISCUSSION**

The aromatization of C-19 steroids, the formation of estrogens, were investigated in the human placenta. With respect to these results it is evident that a full term placenta
possesses an extremely high capacity of aromatization. This suggests to us that the principal route of placental estrogen synthesis may consist of aromatization of steroid precursors reaching the placenta via the fetal and/or the maternal circulation. The conversion of neutral steroids such as testosterone, androstenedione and DHEA to estrone and estradiol-17β by placental preparation in vitro is remarkably efficient, as first demonstrated by Ryan (1958). Indeed, recent in vitro (Longchampt et al., 1960; Ryan, 1958 and 1959) and in vivo (Bolté et al., 1964a) studies have shown that several C-19 steroids can be converted to estrogens in mid and late pregnancy. The results of our study further indicated that the neutral J4C-19 steroids such as androstenedione, testosterone and J5C-19 steroid such as DHEA, were easily aromatized by the perfused placenta to yield a similar ratio of estrone and estradiol-17β; there was always two or three times more estrone than estradiol-17β in the perfusate. It is of interest that ten times more estrone than estradiol-17β was found following perfusion with estradiol-17β.

It is also noteworthy from this study that there was no conversion of these neutral steroids to estriol. This lack of placental 16α-hydroxylation was also apparent when estradiol-17β was perfused. This fact suggests that the fetus with placenta must be responsible for the formation of estriol during pregnancy. It has been known, from the studies of Diczfalusy (1964), Bolté et al. (1964b and c) and Nakayama et al. (1965 and 1966b), that the feto-placental compartments have the capacity to convert DHEA or estradiol-17β to estriol. Recently, Diczfalusy (1964), Solomon (1966) and Siiteri and MacDonald (1966) proposed that DHEA sulfate formed in the fetal adrenal from placental pregnenolone was hydroxylated at C-16 in the fetal tissue. Bolté et al. (1966) suggested from their in vivo experiment with previable perfused fetus that the fetal liver at mid term had the capacity of 16α-hydroxylation of DHEA sulfate. Slaunwhite et al. (1965), Heinrichs et al. (1966) and Nakayama et al. (1967) showed by the in vitro experiment that the fetal liver had 16α-hydroxylase of DHEA. Nakayama et al. (1967) also reported that the fetal adrenal could convert DHEA to 16α-hydroxylated DHEA in an incubation experiment. An assumption may be made that the 16α-hydroxylated DHEA thus formed reaches the placenta, where it is aromatized to estriol. Our results from in vivo experiment, in which double isotopes were used in the feto-placental compartments, revealed that estriol was formed from DHEA more efficiently than androstenedione, and that 16α-hydroxylation might precede to aromatization in the feto-placental unit (unpublished results.).

It should also be emphasized from our data of perfusions I through III, that the ratio of the radioactivity recovered in the phenolic fraction was always the same regardless of the precursors used. Exclusively in perfusion III, where DHEA was added, 10.7% of the radioactivity was found in the 16-epiestriol fraction, and it was suggested that 16β-hydroxylation occurred in the placenta. Whereas, 16α-hydroxylation, which might be essential for the formation of estriol from DHEA, could not be demonstrated in the present investigation. Now the problem remains of the origin of the neutral steroids which can be aromatized to estrogens, particularly estriol, in the placenta.

Cholesterol is found in a high concentration in the umbilical blood, placenta and maternal peripheral blood. The most abundant neutral steroid elaborated during pregnancy is progesterone, which is formed by the placenta (Venning, 1938). And the conversion of cholesterol into progesterone by placental tissue has been demonstrated in vitro (Solomon et al., 1954; Morrison et al., 1956; Solomon, 1960) and also in vivo (Bloch, 1945). The present study indicated that neither cholesterol-14C nor progesterone-14C, which had been circulated in the isolated human placenta, converted into estrogen. This fact suggests that the placenta possesses little, if any,
ability to split the side chains of those steroids. These findings corroborate the recent view of the feto-placento-maternal compartments in the biosynthesis and metabolism of estrogens, particularly in the formation of estriol, in pregnancy proposed by Diczfalusy (1964) and Solomon (1966).

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REFERENCES