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The Development of Placental Androstenedione and Testosterone Production and Their Utilization by the Ovary for Aromatization to Estrogen during Rat Pregnancy[1]

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ABSTRACT

During rat pregnancy the placenta may provide androgens as a source of precursor for estradiol (E_2) formation by the ovary. However, the relative importance of testosterone (T) and Δ^4-androstenedione (Δ^4A) for ovarian E_2 production is unknown. The present study therefore determined the ability of the rat placenta to convert [^{3}H] pregnenolone (P_5) substrate to [^{3}H] Δ^4A and [^{3}H] T, and to [^{3}H] progesterone (P_4) in vitro on Days 12, 14, 16, and 18 of gestation. The placental formation of Δ^4A and T was correlated with the uterine vein and peripheral sera concentrations of both androgens, and with their ability to be aromatized to E_2 in vitro by the ovary.

Placental androgen formation from P_5 increased and formation of P_4 decreased with advancing gestation, with the formation of Δ^4A being approximately 2- to 4-fold greater ($P<0.01$) than the formation of T on Days 12 to 16 of gestation. The conversion of P_5 to Δ^4A increased ($P<0.001$) from 18 ± 0.9 (mean percent conversion ± SEM) on Day 12 to 53 ± 3 and 57 ± 4 on Days 14 and 16, respectively, then decreased ($P<0.05$) to 42 ± 2 on Day 18. The uterine vein and peripheral sera concentrations of Δ^4A were 2- and 3-fold greater ($P<0.05$–0.001) than T, respectively, on Days 12 to 16. The ovarian percentage conversion of [^{3}H] Δ^4A to [^{3}H] E_2 was 2- to 4-fold greater ($P<0.001$) than the conversion from [^{3}H] T during the second half of pregnancy. Moreover, there was a progressive increase ($P<0.001$) in the ability of the ovary to aromatize both Δ^4A and T from Day 12 to Day 16 of gestation. The data indicate that there is a shift in placental steroidogenesis from P_4 at midgestation to androgens, primarily Δ^4A, during the second half of rat pregnancy. Furthermore, Δ^4A appears to be produced within the placenta and secreted into the uterine venous circulation in greater quantity than T and is used preferentially by the ovary for E_2 formation. We suggest, therefore, that placental Δ^4A may be the predominant androgen for ovarian E_2 production during the second half of rat pregnancy.

INTRODUCTION

Estrogen is essential for ovarian progesterone (P_4) production throughout rat pregnancy (Gibori et al., 1977; Rothchild, 1981). Although luteal and nonluteal tissues are capable of aromatization in vitro (Elbaum and Keyes, 1976; Gibori and Keyes, 1978), the formation of estradiol (E_2) is relatively low in these tissues without the addition of androgen substrate during the second half of rat pregnancy (Taya and Greenwald, 1981). Moreover, although peripheral serum testosterone (T) levels increase after Day 12 of rat gestation, the ovarian androgen content remains the same (Gibori et al., 1979). This suggests an extraovarian source of androgen for ovarian E_2 production. Since the placenta has the capacity to form androgens during rat (Rembiesa et al., 1972; Chan and Leathem, 1977) and mouse (Rembiesa et al., 1971; Soares and Talamantes, 1983) pregnancy, it was suggested by Rembiesa et al. (1972) that the placenta may be the source of androgen substrate for ovarian estrogen formation during the second half of rat pregnancy.

Although T has generally been considered the androgen of primary importance for ovarian

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E_2 production (Gibori and Sridaran, 1981), we have recently observed that Δ^4-androstenedione (Δ^4A) was formed by placentas from pregnenolone (P_5) substrate in far greater quantity during rat pregnancy. In the present study, therefore, we determined the ability of the placenta to convert P_5 substrate to Δ^4A and T, and to P_4 during the second half of rat pregnancy. The placental formation of Δ^4A and T was correlated with the concentrations of these steroids in the uterine vein and peripheral sera and with their utilization for E_2 formation in vitro by the ovary.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats were obtained from Zivic Miller Laboratories, Inc. (Allison Park, PA) and maintained in a temperature-controlled room (74°F) with a lighting schedule of 14L:10D. Purina Laboratory Rodent Chow (Ralston Purina Co., St. Louis, MO) and water were provided ad libitum. Virgin females (2.5 mo old) were paired with males on the day of proestrus. Females were examined for the presence of vaginal spermatozoa, which represented Day 1 of pregnancy.

Between 0900 and 1100 h on Days 12, 14, 16, or 18 of gestation animals were anesthetized with ether and a ventral midline incision was made in order to collect approximately 1 ml of uterine vein blood via a 27-gauge needle. Animals were decapitated, the peripheral trunk blood was collected, and the ovaries and uterus were immediately removed. The placentas and ovaries were dissected free of adhering tissue, weighed on a Mettler balance, and then placed in ice-cold Krebs-Ringer phosphate buffer for determination of steroid biosynthesis in vitro (described below).

Steroids

Nonradiolabeled steroids (P_4, Δ^4A, T, E_2) were purchased from Sigma Chemical Co. (St. Louis, MO) and recrystallized before use. Radiolabeled steroids were purchased from Amersham (Arlington, Heights, IL) and purified on LH-20 Sephadex columns with hexane/benzene/methanol (85:5:5). Steroids used were [7-^{3}H]P_5 (sp. act. 21 Ci/mmol); [1,2,6,7-^{3}H]T (sp. act. 93 Ci/mmol); [1,2,6,7-^{3}H]Δ^4A (sp. act. 97 Ci/mmol); [4-^{14}C]P_4 (sp. act. 57 mCi/mmol); [4-^{14}C]Δ^4A (sp. act. 59 mCi/mmol); [4-^{14}C]T (sp. act. 52 mCi/mmol); and [4-^{14}C]E_2 (sp. act. 56 mCi/mmol).

Placenta [^{3}H]P_5 Metabolism

Placental conversion of [^{3}H]P_5 to [^{3}H]P_4, [^{3}H]Δ^4A, and [^{3}H]T was determined in vitro. Placentas were homogenized in glass Ten Broek homogenizers in ice-cold Krebs-Ringer phosphate buffer (pH 7.4) at a concentration of 100 mg/ml. Two milliliters of homogenate were placed in a vial that contained approximately 2.5 μCi of [^{3}H]P_5 dissolved in 0.2 ml propylene glycol and 2.5 mM NAD^+. Homogenates were incubated with gentle shaking in a Dubnoff metabolic shaker for 2 h at 37°C in an atmosphere of 95% O_2/5% CO_2. Incubations were terminated by placing flasks in an acetone-solid CO_2 bath, and samples were stored at −20°C until assay.

Incubates were thawed to room temperature and 0.06 μCi each of [^{14}C]P_4, [^{14}C]Δ^4A, and [^{14}C]T were added for estimation of recovery losses. The incubates were extracted twice with 15 ml of diethyl ether. Unlabeled P_4, Δ^4A, and T (100 μg each) were added to the organic phase as carriers, and the extracts were evaporated under nitrogen gas. Sequential paper and affinity column chromatography were used in steroid purification. The placental steroids formed from P_5 substrate were initially separated on the paper chromatography system heptane/propylene glycol (50:1). This procedure gave R_f values of 0.05, 0.31, and 0.68 for T, Δ^4A, and P_4, respectively. Steroids were eluted from the first paper with methanol and placed on a second paper chromatography system of benzene/hexane/formamide (25:25:1) for T (R_f value = 0.48) and hexane/formamide (50:1) for Δ^4A and P_4 (R_f values = 0.08 and 0.58, respectively). The third paper chromatography system was heptane/methanol/water (4:3:1) for P_4 (R_f = 0.82) and benzene/hexane/formamide (25:25:1) for Δ^4A (R_f = 0.87). A Sephadex LH-20 18-cm column was used for the final purification of T with hexane/benzene/methanol (85:5:5) as solvent. Ten milliliters of scintillation reagent (Ready-Solv; Beckman Instruments, Inc., Fullerton, CA) were added to the purified metabolites in glass scintillation vials and ^{3}H and ^{14}C activity was determined in a liquid scintillation spectrometer (Nuclear-Chicago, Des Plaines, IL). Results are expressed as percent conversion of precursor to product (i.e., dpm of metabolite·dpm of P_5 added^{-1}·200 mg placental tissue^{-1}).

Ovarian [^{3}H]Δ^4A and [^{3}H]T Metabolism

Ovarian conversion of [^{3}H]Δ^4A and [^{3}H]T to [^{3}H]E_2 was determined in vitro. Both ovaries were homogenized in Krebs-Ringer buffer at a concentration of 10 mg/ml. Two milliliters of homogenate were placed in a vial containing 2.5 μCi [^{3}H]Δ^4A or [^{3}H]T dissolved in 0.2 ml propylene glycol and 2.5 mM NADH. Incubation and extraction were as described above with 0.06 μCi [^{14}C]E_2 and 100 μg of E_2 added for recovery estimation and carrier, respectively. The paper chromatographic systems used in the purification of E_2 were: 1) benzene/formamide (50:1) (R_f=0.14); 2) benzene/hexane/formamide (25:25:1) (R_f=0.48); and 3) benzene/methanol/water (2:1:1) (R_f=0.87). The results are expressed as percent conversion of precursor to product (i.e., disintegrations per min of E_2·disintegrations per min precursor added^{-1}·20 mg ovarian tissue^{-1}).

Determinations of radiochemical purity of [^{3}H]P_4, [^{3}H]Δ^4A, [^{3}H]T, and [^{3}H]E_2 for placental and ovarian incubations were made by identical mobility with reference standards and three recrystallizations to constant specific activity and ^{3}H/^{14}C ratio. All steroid products were found to be pure by these methods.

Radioimmunoassay of Serum Δ^4A and T

The uterine vein and peripheral serum concentrations of Δ^4A and T were determined by radioimmunoassay (RIA). The T and Δ^4A antisera were obtained from Radioassay System Laboratories (Carson, CA) and Dr. Pemmaraju N. Rao, respectively. The results obtained for Δ^4A and T analysis with prior column chromatographic purification were approximately 50% lower than without purification. Therefore, all samples were purified on Sephadex LH-20 columns prior to RIA.

Duplicate 0.4-ml peripheral and 0.1-ml uterine vein serum samples, to which approximately 0.002 μCi [^{3}H]T or [^{3}H]Δ^4A had been added for estimation of procedural losses, were extracted once with 5 ml of hexane (J. T. Baker Co., Phillipsburgh, NJ; analyzed reagent grade). The samples were evaporated under nitrogen gas, dissolved in 1 ml of 2,2,4-trimethylpentane (iso-octane)/toluene/methanol (90:5:5), and purified on 18-cm Sephadex LH-20 columns. The Δ^4A and T fractions were evaporated under nitrogen gas and dissolved in 1 ml diluent consisting of 0.01% bovine γ-globulin (Sigma) and 0.1% sodium azide in 0.9% saline. Recoveries of Δ^4A and T were determined in 0.1 ml of the extracts by scintillation spectrometry and were 73.9 ± 0.7% and 52.0 ± 1.0%, respectively.

Duplicate samples of the extracts (0.8 ml) and Δ^4A or T standards (Sigma Chemical Co.; 0–2000 pg/0.2 ml) were incubated overnight at 4°C with approximately 0.015 μCi [^{3}H]Δ^4A or [^{3}H]T and Δ^4A or T antisera (final dilutions 1:45,000 and 1:80,000, respectively) in a reaction volume of 1.2 ml. Bound [^{3}H]Δ^4A or [^{3}H]T was determined after removal of unbound ligand with charcoal (0.2 ml, 0.25%). Allowance was made for blank contributions by the addition of reagent residues to the Δ^4A and T standard curves.

The sensitivity of the Δ^4A and T assays were 10 pg/ml and 15 pg/ml of serum, respectively. When 500 pg Δ^4A or T were added to diluent and carried through the procedure, the observed values were 90.0 ± 3.5% (mean ± SEM) and 86.6 ± 2.0%, respectively, of those expected. The intra- and interassay coefficients of variation determined on a serum pool were 4.7% and 4.0% for Δ^4A and 10.9% and 6.8% for T, respectively.

Radioimmunoassay of Ovarian Δ^4A, T, and Placental P_5

The percent conversion of radiolabeled precursor to product in vitro is influenced by the tissue content of endogenous substrate (i.e., tissue pools of precursor). Thus, the concentrations of ovarian Δ^4A and T and the concentrations of placental P_5 were determined by RIA. Duplicate 0.4-ml aliquots of the ovarian homogenate (10 mg/ml) or 0.5-ml aliquots of placental homogenates (100 mg/ml), to which approximately 0.002 μCi [^{3}H]Δ^4A, [^{3}H]T, or [^{3}H] P_5 had been added for procedural losses, were extracted with 5 ml of hexane or petroleum ether, respectively. The samples were evaporated and dissolved in diluent, and aliquots (0.8 ml) were then analyzed by RIA essentially as described above for Δ^4A and T. Highly specific P_5 antiserum was obtained from Radioassay Systems Laboratories, Inc. Since similar results were obtained with and without prior chromatographic purification, all samples were analyzed without prior purification.

Analysis of Data

Data were analyzed by analysis of variance. Comparison of the means of formation or concentration of each steroid on various days of gestation was performed using the least significant difference method. Comparison of the formation or concentration of different steroids on the same day of gestation was analyzed by the Student's *t*-test.

RESULTS

The placental conversions of [^{3}H]P_5 substrate to [^{3}H]P_4, [^{3}H]Δ^4A, and [^{3}H]T are shown in Fig. 1. The conversion of P_5 to P_4 decreased ($P<0.001$) from 15 ± 2.7 (mean percent conversion ± SEM) on Day 12 of gestation to 3.8 ± 0.4, 3.2 ± 0.4, and 2.8 ± 0.4 on Days 14, 16, and 18, respectively. In contrast, the placental formation of androgens increased with advancing gestation, with the formation of Δ^4A approximately 2- to 4-fold greater ($P<0.01$) than the formation of T on Days 12 to 16 of gestation. Thus, the conversion of P_5 to Δ^4A increased ($P<0.001$) from 18 ± 0.9 (mean percent conversion ± SEM) on Day 12 to 53 ± 3 and 57 ± 4 on Days 14 and 16, respectively. There was a decrease ($P<0.05$) in the conversion of P_5 to Δ^4A on Day 18 (42 ± 2) compared to Days 14 and 16 of gestation. The percent conversion of P_5 to T increased ($P<0.05$–0.01) progressively from 6.2 ± 0.6 on Day 12 to 13.7 ± 3, 29.0 ± 2, and 39.0 ± 7 on

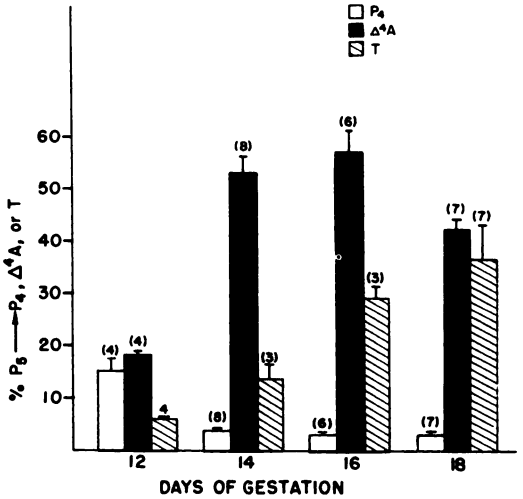

FIG. 1. Placental conversions of [^{3}H]P_5 to [^{3}H]P_4, [^{3}H]Δ^4A, and [^{3}H]T (mean ± SEM percent of disintegrations/min of P_5 converted to disintegrations of metabolite·min^{-1}·200 mg tissue^{-1}) on Days 12, 14, 16, and 18 of rat gestation. The number of rats in each group is shown in parentheses.

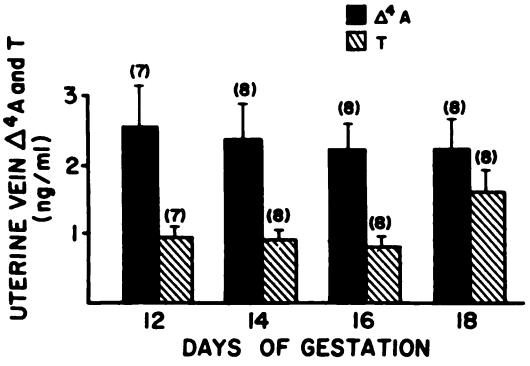

FIG. 2. Uterine venous serum Δ^4A and T concentrations (ng/ml) on Days 12, 14, 16, and 18 of rat gestation. Each bar represents the mean ± SEM and the number of animals in each group is shown in parentheses.

Days 14, 16, and 18 of rat pregnancy, respectively. Therefore, the data show a shift in placental steroidogenesis from P_4 at midgestation to an ability of the placenta to convert over 66%, 86%, and 81% of the P_5 to androgens, particularly Δ^4A, on Days 14, 16, and 18, respectively.

Uterine vein concentrations of Δ^4A were approximately 2-fold greater ($P<0.05-0.01$) than T on Days 12 to 16 of gestation (Fig. 2). There was no significant change in the Δ^4A and T uterine vein levels on Days 12 to 16. However, on Day 18 the uterine vein T concentration increased ($P<0.05$) to a value that was not significantly different from that of Δ^4A. The peripheral serum concentrations of Δ^4A were approximately 3-fold greater ($P<0.001$) than T during the second half of gestation (Fig. 3). The peripheral concentrations of Δ^4A and T increased ($P<0.01$) progressively from Days 12 to 18 of gestation.

The ability of the ovary to use either [^{3}H]T or [^{3}H]Δ^4A for the formation of [^{3}H]E_2 in vitro is depicted in Fig. 4. On Days 12, 14, 16, and 18 of gestation the ovarian formation of E_2 from Δ^4A was 2- to 4-fold greater ($P<0.001$) than the formation from T. Moreover, there was an increased ability of ovarian tissue to aromatize both Δ^4A and T from Day 12 to Day 14 ($P<0.05$) and from Day 14 to Day 16 ($P<0.001$), with no further increase in aromatization on Day 18 of gestation.

The endogenous concentrations of ovarian Δ^4A and T (ng/10 mg tissue) are shown in Table 1. The ovarian concentrations of Δ^4A were 6- to 8-fold greater ($P<0.05-0.001$) than those of T on Days 12 to 18 of gestation. Therefore, the greater formation of E_2 from Δ^4A compared to the formation from T reflects preferential use of Δ^4A by the aromatase enzyme complex. Moreover, there was no significant change in the ovarian concentrations of Δ^4A or T with advancing days of gestation. This indicates that the increased formation of E_2 from Δ^4A and T between Days 12 and 16 reflects a development of the ovarian aromatase enzyme system, rather than simply an enhanced conversion of radiolabeled precursor due to a decrease in the endogenous pool of androgen substrate.

The endogenous concentrations of placental P_5 (mean ng/100 mg tissue ± SEM) were 4.6 ± 0.3, 2.2 ± 1, 8.1 ± 0.4, and 10.3 ± 0.9 on Days 12, 14, 16, and 18, respectively. Therefore, with the exception of Day 14, placental P_5 concentrations increased ($P<0.01$) between Days 12 and 18 of gestation, at the same time that there was an increased ability of the placenta to form [^{3}H]Δ^4A and [^{3}H]T from [^{3}H]P_5 substrate. The 50% decline in endogenous P_5 concentrations between Days 12 and 14 does not account for the 3-fold rise in conversion of [^{3}H]P_5 to [^{3}H]Δ^4A at this interval. This indicates development of placental enzymes for the production of androgens with advancing gestation, rather than simply enhanced utilization of [^{3}H]P_5 because of decreased placental precursor pools.

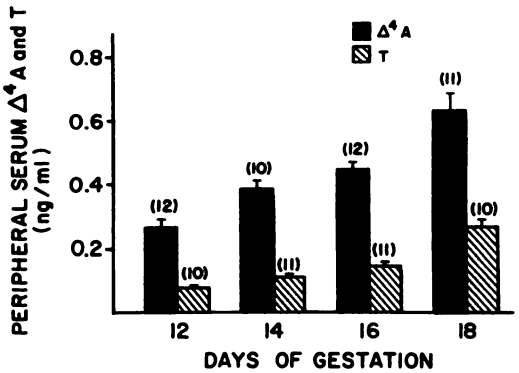

FIG. 3. Peripheral serum Δ^4A and T concentrations (ng/ml) on Days 12, 14, 16, and 18 of rat gestation. Each bar represents the mean ± SEM and the number of animals in each group is shown in parentheses.

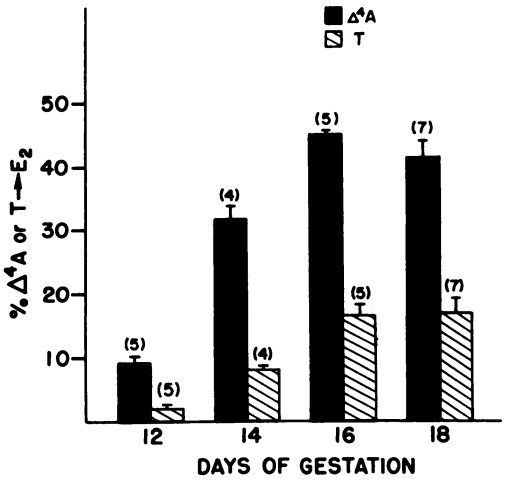

FIG. 4. Ovarian conversions of [^{3}H]Δ^4A or [^{3}H]T to [^{3}H]E_2 (mean ± SEM percent of disintegrations/min of precursor converted to disintegrations of $E_2 \cdot min^{-1} \cdot 20$ mg tissue) on Days 12, 14, 16, and 18 of rat gestation. The number of rats in each group is shown in parentheses.

DISCUSSION

The present study demonstrates that during the second half of rat pregnancy the placenta has the capacity to form more Δ^4A compared to T from P_5 substrate in vitro. This suggests that the placenta may preferentially produce more Δ^4A than T for secretion into the circulation. Our results further demonstrate that, indeed, the uterine vein and peripheral serum concentrations of Δ^4A were 2- and 3-fold greater, respectively, than the concentrations of T. Moreover, the current study shows that the ovaries preferentially utilized Δ^4A over T for the formation of E_2 in vitro. It has previously been suggested that the placenta may provide androgen as substrate for estrogen formation within the ovary during the second half of rat pregnancy (Rembiesa et al., 1972). Testosterone has generally been considered the androgen of importance. However, our data suggest that during the second half of rat pregnancy Δ^4A, and not T, may be the predominant substrate of placental origin for E_2 production by the ovary.

The present study also demonstrates that there is a shift in placental steroidogenesis during the second half of rat pregnancy from the formation of P_4 at midgestation to androgen, predominantly Δ^4A, by Day 14 of gestation. This very likely reflects the development of the 17-hydroxylase and/or 17,20-lyase enzymes essential for Δ^4A and T formation. The elevated conversion of P_5 to P_4 by the placenta at midgestation correlates well with the peaks in Δ^5-3β-hydroxysteroid dehydrogenase (3β-HSD) enzyme activity in trophoblast cells (Marcal et al., 1975) and in vitro placental P_4 production (Matt and Macdonald, 1984) previously reported on Day 12 of rat gestation.

Although there was preferential production of Δ^4A compared to T by the placenta on Days 12 to 16, there was an increased ability of the placenta to form T from P_5 substrate late in gestation. The in vitro placental production and peripheral serum concentration of T increase throughout the second half of rat pregnancy, with a peak in T on Day 18 of gestation (Macdonald and Matt, 1984; Matt and Macdonald, 1984). The present study demonstrates that on Day 18 there is an increased conversion of P_5 substrate to T and simultaneously a slight decline in Δ^4A formation. The latter events are then associated with an increase in the peripheral and uterine vein serum concentrations of T. This may reflect a change in placental 17β-HSD enzyme activity very late in rat gestation, so that the relative ability of the placenta to form T develops later in comparison to Δ^4A.

Our data show that the ovary develops an increasing ability to aromatize both Δ^4A and T during the second half of rat pregnancy. It has previously been reported that the capacity of

TABLE 1. Ovarian concentrations of Δ^4A and T (mean ng/10 mg tissue ± SEM) on Days 12, 14, 16, and 18 of rat gestation.

Steroid	Ovarian concentration (ng/10 mg tissue)			
	Day 12	Day 14	Day 16	Day 18
Δ^4A	1.65 ± 0.08	1.75 ± 0.50	0.96 ± 0.05	0.64 ± 0.04
T	0.19 ± 0.01	0.20 ± 0.04	0.12 ± 0.02	0.12 ± 0.01

luteal tissue to aromatize T increases during the second half of gestation (Gibori et al., 1982). Additionally, however, our study demonstrates that the ovary has a much higher concentration of endogenous Δ^4A compared to T and preferentially uses Δ^4A as the androgen substrate for estrogen formation, at the same time that there is an increase in the ability of the placenta to produce Δ^4A. This may explain the increase in peripheral serum concentration and corpora lutea content of E_2 during the second half of rat pregnancy (Taya and Greenwald, 1981). There was no further increase in the ability of the ovary to aromatize androgens on Day 18 of gestation, which correlates with a lack of change in corpora lutea content of E_2 after Day 16 (Taya and Greenwald, 1981).

During the second half of gestation the concentrations of Δ^4A and T were 4- to 12-fold greater in the uterine venous blood than in the peripheral circulation, which has previously been shown for T (Sridaran et al., 1981). This further supports the suggestion of a placental source of androgen during the second half of rat pregnancy. In the present study, there was no increase in the serum concentrations of Δ^4A and T in the uterine vein between Days 12 and 16, despite the increase in capacity of the placenta to form androgens, in placental weight, and in Δ^4A and T concentrations in the peripheral serum. However, there is an increase in uterine blood flow (Csepli et al., 1968) and thus in perfusion of the uterofetoplacental unit with advancing gestation. This may cause a dilution of placental steroids in the uterine vein blood. Therefore, it is possible that there indeed is enhanced placental androgen secretion in vivo and delivery of increasing amounts of androgen to the peripheral circulation during the second half of rat pregnancy.

Prior studies have shown that the peripheral and uterine vein serum concentrations of Δ^4A were greater than T during very late rat (Legrand et al., 1984) and mouse (Soares and Talamantes, 1983) pregnancy. The peripheral serum concentrations of T in the present study were lower than values reported by Bridges et al. (1982) and Sridaran et al. (1981). The peripheral serum Δ^4A levels reported by Wilke et al. (1982) were greater than our values, but showed the same overall rise throughout the second half of rat pregnancy. We probably obtained lower T and Δ^4A concentrations because all serum samples were purified by column chromatography prior to analysis by RIA. Apparently, this eliminated other steroids that cross-reacted significantly with the Δ^4A and T antisera.

In summary, the present study shows that during the second half of rat pregnancy: 1) the placenta converts a much greater percentage of P_5 substrate to Δ^4A than to T in vitro; 2) the concentrations of Δ^4A are much greater than T in the uterine vein and peripheral sera; and 3) the ovary preferentially uses Δ^4A over T for E_2 production in vitro. We suggest, therefore, that placental Δ^4A is the predominant androgen utilized in the formation of E_2, a steroid hormone that in turn maintains luteal function during the second half of rat pregnancy.

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