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Δ^5, 3β Hydroxysteroid Dehydrogenase Activity in Cat Placental Labyrinth: Evolution during Pregnancy, Subcellular Distribution

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ABSTRACT

The capability of the cat placenta to synthesize progesterone was studied by measurement of the biochemical activity of Δ^5-3β-hydroxysteroid dehydrogenase (Δ^5, 3β HSD). This activity was assessed by measuring of the rate of [^{3}H]-progesterone formation from [^{3}H]-pregnenolone through use of a double isotope dilution method. The enzymatic activity was distributed equally between mitochondrial and microsomal fractions, although the specific activity was higher in the microsomal fraction as compared to the mitochondrial fraction. The specific activity in the homogenate was 28.2 ± 1.16 nmol/min/mg protein between Days 28–32 of gestation; it increased to 49.3 ± 1 nmol/min/mg protein between Days 50–62. The total enzymatic activity increased greatly in the last stage of gestation due to the increase in placental weight. These results indicate that placental production of progesterone increases significantly during the latter part of gestation and explain the fact that ovariectomy after Day 49 of pregnancy does not alter the duration of pregnancy.

INTRODUCTION

In the cat, nidation occurs near the end of the second week of pregnancy and the gestation period is ∿65 days (Courrier and Gros, 1935; Scott, 1970). The fact that ovariectomy after Day 45 (Scott, 1970) or Day 49 (Courrier and Gros, 1935) does not interrupt gestation suggests the possibility of steroid synthesis in the placenta which can replace ovarian production of progesterone. This period of placental activity is correlated with the maximal activity of steroidogenic enzymes as detected histochemically. These enzymes have been shown to be localized in the syncytiotrophoblast labyrinth (Malassiné and Juillard, 1973).

Progesterone is well known to be essential for the maintenance of pregnancy. In the context of cat placental endocrine function, we particularly studied the conversion of pregnenolone to progesterone through the evolution of the enzyme system (Δ^5, 3β hydroxysteroid dehydrogenase (E.C. 1.1.1.5.1), Δ^{4-5},3 ketosteroid isomerase (E.C. 5.3.3.1.) during gestation. Subcellular localization of the enzyme was also studied in cat placental labyrinth. The enzyme Δ^5, 3β hydroxysteroid dehydrogenase (Δ^5, 3β HSD), catalyses the rate-limiting dehydrogenation step in the conversion of pregnenolone to progesterone. While the enzyme has been classically regarded as microsomal membrane-bound enzyme, Δ^5, 3β HSD is also localized in mitochondrial fractions of some organs, especially in human placenta (Koide and Torres, 1965; Ferré et al., 1975). This distribution problem is more acute in the case of cat endotheliochorial placenta. A previous study showed that the classical ultrastructural features of the steroid secreting cells are not all observed in the syncytiotrophoblast where steroidogenesis occurs (Malassiné, 1974). Human syncytiotrophoblast, however, exhibits all these ultrastructural features (Kaufmann and Stegner, 1972).

MATERIALS AND METHODS

Radioactive steroids, pregnenolone-[7-^{3}H] (specific activity: 16 Ci/mM), progesterone-[4-^{14}C] (specific activity: 29 mCi/mM) were purchased from Radiochemical Center, Amersham, England or CEA, Saclay, France. These labeled steroids were repurified by

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thin-layer chromatography prior to use. Nonradioactive steroids and nicotinamide adenine dinucleotide (NAD^+) were obtained from Sigma Chemical Co. All mineral reagents, sucrose and organic solvents were analytical grade from Merck.

Cat placentae were obtained by hysterectomy from animals anesthetized with Nembutal. The gestation period was determined by the stage of fetal development (Sainmont, 1906; Scott, 1970) or, in some cases, by the day of copulation.

Small pieces of labyrinth were quickly collected, washed with a pH 7.4 buffer solution (0.01 M Tris HCl, 0.25 M sucrose) at 4°C and then homogenized in a similar solution with a glass Potter-Elvehjem apparatus fitted with a teflon pestle (0.5 or 1 g of placental tissue in 5 ml buffer solution). Subcellular fractionation was carried out at 4°C as described in Ferre et al. (1975). Homogenates and fractions were immediately frozen in liquid nitrogen and stored at −80°C. These storage conditions (maximum 8 weeks) do not significantly alter enzyme activity.

Activity of Δ^5, 3β hydroxysteroid dehydrogenase (Δ^5, 3β HSD) was determined by measuring the rate of formation of [^{3}H]-progesterone from [^{3}H]-pregnenolone at 37°C with a double isotope dilution method (Ferré et al., 1975). The incubation mixture employed contained 2×10^{-2} M Tris HCl (pH 7.4), 1.1×10^{-2} M NaCl, 1.5×10^{-2} M KCl, 2×10^{-2} M sucrose, 5×10^{-4} M NAD^+, 5×10^{-5} M (0.05 μCi) [7-^{3}H]-pregnenolone and enzymatic preparation in a total volume of 10 ml. One milliliter of the incubation medium was pipetted at various times. The enzymatic reaction was stopped by placing the samples in an ice bath and the products were immediately extracted with 2 ml ether (3 times). At this time, 5×10^{-9} μCi of [4-^{14}C]-progesterone was added as radioactive tracer.

Isolation of progesterone was done by thin-layer chromatography (Silica gel G 60) in the system chloroform/acetone (185:15; v/v). Progesterone was eluted after UV visualization and ^{14}C and ^{3}H radioactivities were counted by liquid scintillation. The ^{3}H/^{14}C ratio did not change either after several thin-layer chromatographies or reduction into 20β-hydroxypregn-4-en-3-one.

The presence of progesterone metabolites was investigated after extraction (ether/chloroform, 3:1; v/v) by paper chromatography [(cyclohexane/benzene, 1:1; v/v) (formamide/methanol, 1:1; v/v)] during a 5 h migration period. After detection of the steroids, a radiochromatogram was analyzed by a Scanner radiochromatograph and eluate radioactivities were counted by liquid-scintillation (Guichard et al., 1973). The purity of mitochondrial and microsomal fractions was determined by electron microscope examination. Protein concentrations were determined by the method of Lowry et al. (1951).

Specific Δ^5, 3β HSD activities ± SEM are reported. Significant differences were estimated by Student's t test.

RESULTS

Assay Conditions for Δ^5, 3β HSD Activity in Homogenates

The substrate concentration (5×10^{-5} M) was not limiting and homogenate enzyme was not inhibited by excess of substrate. Enzyme activity was measured under linearity conditions both of time (Fig. 1) and of protein concentration (Fig. 2). As to protein concentrations, enzyme specific activity remained constant irrespective of the concentration (Fig. 2).

The kinetic of enzymatic oxidation of pregnenolone to progesterone was linear for only some minutes (Fig. 1). This phenomenon was observed not only during homogenate incubations but also during microsomal or mitochondrial incubations. Since our estimates of Δ^5, 3β HSD depended upon measurement of progesterone only, it was necessary to ascertain that the progesterone formed was not being metabolized. When homogenates (200 μg of protein/ml) were incubated for 30 min, more than 97% of pregnenolone tritium radioactivity was recovered in progesterone eluate after thin-layer chromatography. After paper chromatography

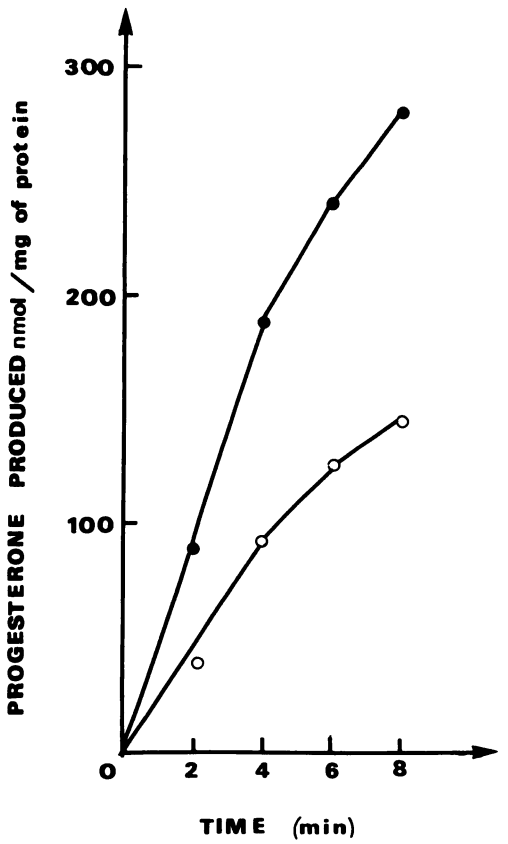

FIG. 1. Kinetics of conversion of pregnenolone to progesterone by placental labyrinth homogenates. o—o, Day 30 homogenate; •—•, Day 53 homogenate.

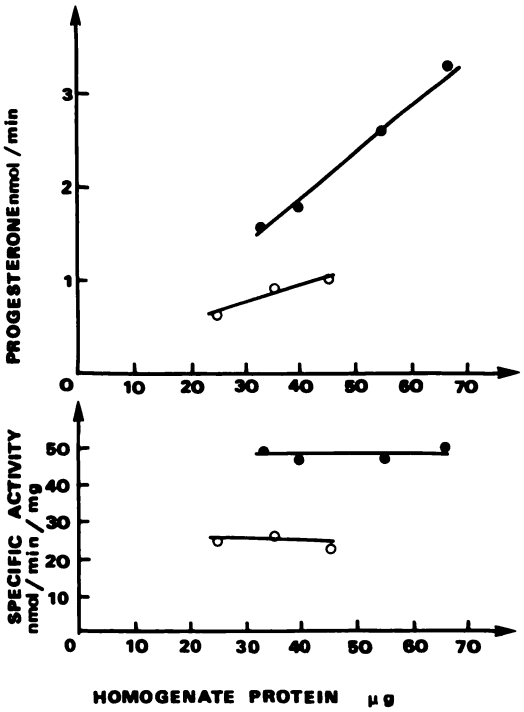

FIG. 2. Relationships between protein content of homogenate, progesterone formation (nmol/min) and placental Δ^5, 3β HSD specific activity (nmol progesterone produced/min/mg homogenate protein). o—o, Day 30 homogenate; •—•, Day 53 homogenate.

of incubation extracts, radiochromatograph analysis showed only 1 peak corresponding to progesterone. Thus, in our experimental conditions, progesterone does not seem to have been metabolized. Paraplacenta homogenates, from lateral placental area specialized in erythrophagocytosis, did not exhibit any significant enzymatic activity (1.5 nmol/min/mg protein).

Development of Δ^5, 3β HSD Activity in Labyrinth during Gestation

At least 4 "trials" were made from the same placenta under the following conditions: incubation time ≤4 min, protein concentration between 30–60 µg/ml, temperature 37°C.

Table 1 and Fig. 3 show the evolution of the specific enzyme activity during gestation with a very significant increase ($P<0.001$) observed between Days 28–32 (28.2 ± 1.16 nmol/min/mg protein) and Days 50–62 of gestation (49.3 ± 1.1 nmol/min/mg protein). Placental protein concentration was relatively constant

TABLE 1. Evolution of fetal and placental wet weight and of specific and total Δ^5, 3β HSD activity during gestation. Mean ± SEM; (n).

Gestational age (days)	Weight of fetus (g)	Weight of placentae (g)	Protein mg/g placenta	Specific activity nmol/min/mg	Total enzyme activity µmol/min/placenta
28 ± 1	1.56 ± 0.12 (3)	5.80 ± 0.25 (3)	105	28.75 ± 1.10 (4)	16.9 ± 0.65 (4)
30 ± 1	2.05 ± 0.08 (4)	9.32 ± 0.34 (4)	115	23.80 ± 0.47 (4)	24.4 ± 0.50 (6)
32 ± 2	3.82 ± 0.11 (4)	10.75 ± 0.47 (4)	108	33.00 ± 1.30 (5)	35.6 ± 1.40 (5)
45 ± 2	20.75 ± 0.32 (4)	16.50 ± 0.48 (4)	105	33.60 ± 1.48 (4)	59.9 ± 2.60 (4)
50 ± 2	45.60 ± 1.40 (5)	16.44 ± 0.92 (5)	102	50.20 ± 1.28 (5)	84.5 ± 2.10 (5)
52 ± 1	51.50 ± 1.30 (4)	11.00 ± 0.20 (4)	110	40.50 ± 1.32 (4)	62.3 ± 1.60 (4)
53 ± 1	54.50 ± 0.86 (4)	17.25 ± 0.14 (4)	104	47.30 ± 0.66 (4)	83.9 ± 1.15 (4)
54 ± 1	63.50 ± 3.30 (3)	17.33 ± 0.16 (3)	105	56.25 ± 0.85 (4)	103.3 ± 1.60 (4)
58 ± 2	115.75 ± 2.30 (5)	20.25 ± 0.47 (5)	110	47.75 ± 0.85 (4)	105.0 ± 1.80 (4)
62 ± 2	179.00 ± 2.00 (3)	14.30 ± 0.15 (3)	102	57.20 ± 0.47 (4)	80.3 ± 0.70 (4)

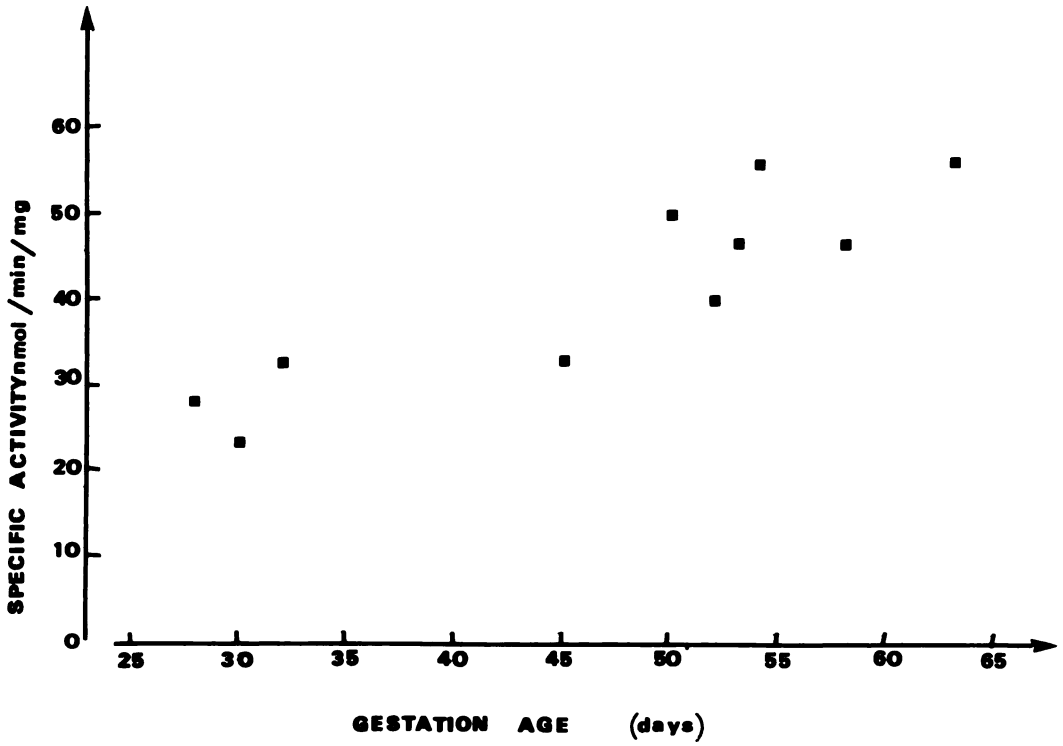

FIG. 3. Specific Δ^5, 3β HSD activities of placental homogenates during gestation. Each value represents the mean of at least 4 "trials" made from the same placenta (Table 1). Gestation age (± 1 or 2 days) was determined by the stage of fetal development.

during gestation (Table 1). However, placental weight increased between Day 28 and Days 58–60 of gestation and decreased only during the last 3 days before parturition. Thus, total placental enzyme activity increased greatly during the latter part of gestation (Fig. 4). The lower total enzyme activity observed at Day 62 is related to decreased placental weight.

Subcellular Distribution of Δ^5, 3β HSD Activity

Ultrastructural examination of 105,000 g pellets revealed a satisfactory purity of the microsomal fraction. The mitochondrial population was not homogenous in that some lysosomal and microsomal contamination was present. Ultrastructural analysis of the placental labyrinth (endotheliochorial formation) enabled a differentiation to be made between trophoblastic mitochondria (smaller size) and maternal mitochondria (larger size). Enzymatic activity was essentially located in particulate fractions (Table 2). Activity of Δ^5, 3β HSD in the 1500 g fraction probably resulted from inadequate tissue grinding, due to tissue heterogeneity and the necessity of avoiding enzymatic denaturation. It is possible that contamination of mitochondria with some microsomes interfered with the specific activity measurement of this fraction. However, contamination alone cannot account for the activity that was observed.

DISCUSSION

The increase of total enzymatic activity during the second half of the gestation period supports the role of the placenta of the cat in progesterone biosynthesis during this period. It must be emphasised that the activities of enzymes in vivo are dependent not only on the amount of enzyme protein present but also on the concentration of other factors, such as substrates, cofactors, inhibitors and possible allosteric effectors. The absence of progesterone metabolism and its relatively high intraplacental concentration (≅600 ng/g wet weight tissue, current study) suggest that progesterone synthesis regulation results from a

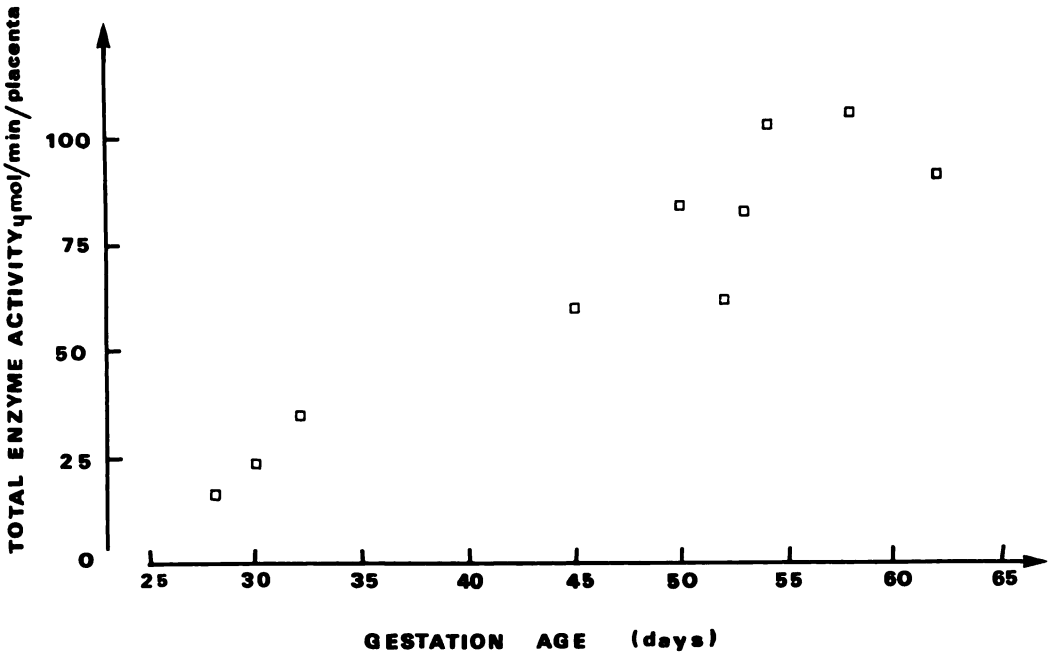

FIG. 4. Total Δ^5, 3β HSD activity in placenta during gestation. Each value represents the mean of at least 4 "trials" made from the same placenta (Table 1).

partial inhibition of Δ^5, 3β HSD activity by progesterone itself.

Another study indirectly suggests a placental source of progesterone during the second half of gestation period. In the cat, the luteal phase of pseudopregnancy is ∿36 days, with luteal regression beginning ∿21 days after ovulation (Paape et al., 1975). Significant differences between plasma progesterone levels in pregnant and pseudopregnant animals are observed 28 days after ovulation (Verhage et al., 1976), suggesting an extragonadal source for progesterone. These differences do not occur in the ferret (Heap and Hammond, 1974), dog (Concannon et al., 1975) or blue fox (Møller, 1973), animals whose ovaries are needed during the entire period of gestation.

The distribution of Δ^5, 3β HSD activity between microsomes and mitochondria is in agreement with those described in the human placenta (Koide and Torres, 1965; Ferré et al., 1975). Mitochondrial Δ^5, 3β HSD has also been described in the testis (Sulimovici et al., 1973) and ovary (Jackanicz et al., 1970). Its localization in the adrenal is still uncertain (McCune et al., 1970; Moustafa and Koritz, 1975; Kream and Sauer, 1976). It must be noted that the trophoblastic mitochondria of cat placental labyrinth do not exhibit ultrastructural features (tubular or vesicular cristae) of steroid secreting cells. This peculiarity plus the lack of typical smooth reticulum, could result from specific variations, or from the fact that the placenta is an incomplete endocrine organ (Malassiné, 1974).

The absence of progesterone metabolites in our incubations is in agreement with similar studies in rat placenta (Wiener, 1974) and in rat (Marcal et al., 1975) or mouse (Chew and Sherman, 1975) trophoblast. It should be emphasized that experimental conditions (NAD^+ concentration in excess) discourage conversion of progesterone to reduced metabolites on A ring or C_{20}. During perfusion of the human placenta, placental metabolism of progesterone is also limited (Palmer et al., 1966). However, incubations in the presence of NADPH have shown a placental progesterone metabolization in the cow and ewe (Ainsworth and Ryan, 1967), rat (Townsend and Ryan, 1970; Chan and Leathem, 1975; Sanyal and Villee, 1976) and rabbit (Marchut, 1976). The in vivo metabolization of progesterone probably depends on the nature and availability of cofactors.

The specific activities remained constant at the enzyme concentrations used in this study. This is consistent with a rat trophoblast study

TABLE 2. Subcellular distribution of Δ^5, 3β HSD activity in the cat placental labyrinth. Activities/fraction are given as mean ± SEM, (n). Placentae from Day 52 pregnancy.

	Whole homogenate	Nuclear fraction	Mitochondria-		Microsomes	Cytosol	Recovery
			10,000–12,000 g	12,000–15,000 g	105,000 g		
Activity (nmol/min/fraction)	2981 ± 45 (4)	701 ± 6.1 (4)	830 ± 8.1 (4)	95.3 ± 1.9 (4)	910.7 ± 5.2 (4)	44 ± 1.67 (4)	86.6
% recovered activity		27.1	32.1	3.7	35	2.75	
Protein (mg/fraction)	53	19.1	7.7	2.5	5	16	94.9
Specific activity (nmol/min/mg)	56.2	36.7	107	38.1	182	2.75	

(Marcal et al., 1975). Yet these observations contradict the findings of Wiener (1974, 1976, 1978) and Wiener and Reiner (1978) who observed an effect of dilution upon specific activity in a system using much higher protein concentrations. This was explained by the presence of a hypothetical endogenous inhibitor.

The lack of linearity of enzyme kinetics is also described in the placentae of humans and other species (Wiener, 1976; Chew and Sherman, 1975). This suggests that the Δ^5, 3β HSD activity in the cat is subject to retroinhibition by the product of the reaction as proposed for human placenta (Goldman and Sheth, 1973; Townsley, 1975; Ferré et al., 1975; Saure et al., 1977).

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