J. steroid Biochem. Vol. 23, No. 4, pp. 517–522, 1985
Printed in Great Britain.

0022-4731/85 $3.00 + 0.00

THE EFFECTS OF STEROID HORMONES AND GONADOTROPINS ON *IN VITRO* PLACENTAL CONVERSION OF PREGNENOLONE TO PROGESTERONE

NAGARATNAM P. DAS*, FIRYAL S. KHAN-DAWOOD and M. YUSOFF DAWOOD†
The Division of Reproductive Endocrinology, Department of Obstetrics and Gynecology, University of Illinois College of Medicine, Chicago, IL 60612, U.S.A.

(*Received* 18 *June* 1984)

Summary—Using human term placental mitochondrial preparations, optimal conversion of [^{3}H]pregnenolone to [^{3}H]progesterone was obtained at 30 min incubation and with a mitochondrial protein content of 2.5–3.5 mg/ml. Estradiol, estrone, progesterone and testosterone in a dose range of 0.03–8.66 μmol inhibited the *in vitro* conversion of [^{3}H]pregnenolone to [^{3}H]progesterone by placental homogenates. All four steroids inhibited the pregnenolone to progesterone conversion in a dose-dependent manner. The ID50 (dose required to inhibit conversion of pregnenolone to progesterone by 50%) was 0.04 μmol for estradiol, 0.13 μmol for testosterone, 0.3 μmol for progesterone and 1.0 μmol for estriol. Neither gonadotropin releasing hormone (50–1000 ng) nor human chorionic gonadotropin (5–500 IU) affected the placental basal conversion rate of pregnenolone to progesterone *in vitro*. Our findings indicate that steroid hormones such as estradiol, estrone, testosterone and progesterone can inhibit local placental progesterone biosynthesis through inhibition of the enzyme complex 5-ene-3β-hydroxysteroid dehydrogenase.

INTRODUCTION

The placenta is the major source of progesterone in late human pregnancy since the human fetus is deficient in the enzyme complex 5-ene-3β-hydroxysteroid dehydrogenase and 5-ene-4-ene isomerase. This enzyme complex is present in both the mitochondria and microsomes of the trophoblast and converts pregnenolone to progesterone. Although a decline in circulating progesterone at the approach of the onset of labor has been found in some species [1–3], such a change has not been convincingly demonstrated in women. Nevertheless there could be a change in the local placental production or metabolism of progesterone in women at the approach of or during labor. Because local placental progesterone metabolism could potentially modulate uterine activity and therefore labor, studies on placental progesterone synthesizing reserve capacity might yield useful information towards a better understanding of such a function. Therefore, investigations were undertaken to examine the pregnenolone–progesterone converting activities of the placenta and specifically the placental steroidogenic enzyme 5-ene-3β-hydroxysteroid dehydrogenase. We report here the results of our study examining the effects of some steroid hormones and gonadotropins on the *in vitro* conversion of pregnenolone to progesterone in cell-free freshly prepared placental mitochondria obtained from term placentae in women who were not in labor.

EXPERIMENTAL

Materials and methods

Chemicals. The steroids and all common standard compounds used were purchased from Sigma Chemical Co., St Louis, Missouri. The chorionic gonadotropin (5000 U.S.P. units) was obtained from Ayerst Lab. Inc., New York and the gonadorelin–HCl (synthetic gonadotropin releasing hormone) from Warner-Lambert Company, Ann Arbor, Michigan. The labelled steroids namely [4-^{14}C]progesterone (sp. act 57.2 mCi/mmol) and [7-^{3}H(*N*)]pregnenolone (sp. act. 19.3 Ci/mmol) were purchased from New England Nuclear, Boston, Massachusetts. The steroids were found to be pure by thin-layer chromatography. Flexible thin layer sheets (20 × 20 cm) coated with silica gel IB2-F were purchased from J. T. Baker Chemical Co., Phillipsburg, N.J.

Preparation of tissue. Fresh tissues from 28 placentae with membranes attached were collected immediately at the time of elective repeat Cesarean section performed at 39–40 weeks gestation on otherwise uncomplicated pregnancies. Each placenta was immediately placed in crushed ice in the operating room and rapidly transported to the laboratory. All manip-

*On sabbatical leave from: The National University of Singapore.
Present address: Department of Biochemistry, Faculty of Medicine, National University of Singapore, Singapore.
†Address for correspondence: Department of Obstetrics and Gynecology, University of Illinois College of Medicine, 840 South Wood Street, Chicago, IL 60612, U.S.A.

ulations were carried out at 4°C. Each placenta was dissected and samples (2 g each) of cotyledons were removed, washed in ice-cold 0.9% NaCl solution to remove the blood. The washed cotyledons were either homogenized immediately or dried over filter papers and kept frozen at −75°C until ready for analysis.

Each placental cotyledon tissue (2 g) was further washed with Buffer A (0.25 M sucrose + 10 mM Tris–HCl, pH 7.4). The tissue was then minced with scissors and homogenized in 10 ml of Buffer A using the polytron homogenizer at speed 5–6 for 6 sec. The homogenates were centrifuged for 10 min at 1000 *g*. The supernatant was taken and recentrifuged for 20 min at 10,000 *g* to obtain the mitochondrial pellets. The supernatant was discarded and the pellets were resuspended in 5 ml of Buffer B (20 mM Tris–HCl + 11 mM NaCl + 15 mM KCl + 20 mM sucrose, pH 7.4). Samples of the pellets were verified by electron microscopy to contain mitochondria.

Incubation mixture. Aliquots of 0.05 μCi of [7-^{3}H(*N*)]pregnenolone dissolved in ethanol were placed in test tubes. The ethanol was evaporated off and 30 μl propylene glycol was added into each tube. These tubes served as controls. For testing the effects of some hormones on the 5-ene-hydroxysteroid dehydrogenase activity present in the mitochondrial fractions, varying amounts of the test compounds were added to the tubes containing the [7-^{3}H(*N*)] pregnenolone and propylene glycol. The pH of the incubation mixture did not change upon addition of the various hormones in the doses used. All the test tubes were preincubated in a shaking water bath at 37°C for 15 min before the addition of 1 ml of the mitochondrial fraction. All the test tubes containing the reaction mixtures were then allowed to incubate for 30 min with constant shaking at 37°C. At the end of the incubation time, all the tubes were removed from the water bath and placed in an ice bath. To each tube was added 2 ml of the extracting solvent (diethyl ether–ethyl acetate, 1:1, v/v) followed by 0.03 μmol each of unlabelled progesterone and pregnenolone dissolved in ethanol. An aliquot amount of [^{14}C]labelled progesterone (the equivalent of 10,000 cpm) was also added into each of the tubes. Each tube was Vortex mixed for 1 min and allowed to stand for 5 min in ice. The supernatant organic layer was collected. The aqueous reaction mixture was further extracted twice with 2 × 1 ml extracting solvent and the organic layer was removed and pooled together. The organic extract was evaporated to dryness over a blowing air trap. The residue in each tube was redissolved in 20 μl of ethanol solution. Samples of 10 μl solution were used for spotting on 20 × 20 cm flexible thin layer sheets coated with silica gel 1B 2-F. The thin layer sheets were developed in chloroform–acetone–ethanol (84:15:1, by vol). Unlabelled progesterone and pregnenolone (0.03 μmol) were also spotted on each of the plates as reference standards. The progesterone spot ($R_f = 0.51$) was visualized under ultraviolet light (short wavelength) as a dark absorbed spot while the pregnenolone spot ($R_f = 0.38$) showed up as a bluish red colored spot after spraying with 10% alcoholic phosphomolybdic acid and heated for 1 min at 100°C. The spots corresponding to progesterone and pregnenolone from the extracts were marked and cut out and placed into several plastic scintillation vials. To each vial was added 8 ml of the scintillation fluid (prepared by mixing 1 Gel-Pak of "Omnifluor" purchased from New England Nuclear to 2 l of toluene and 1 l of Triton X-100) and counted for 5 min in a Beckman scintillation counter model LS 7500 which has a counting efficiency of 44.5 and 78.6% for tritium and carbon-14 labelled standards respectively. Separate counting vials containing 0.05 μCi of [7-^{3}H(*N*)]pregnenolone and the equivalent amount of ^{14}C-labelled progesterone that was originally added into each of the reaction mixture before extraction were also prepared and counted. It was noted that with the authentic unlabelled progesterone added as carrier, about 92–98% of the radioactivity was isolated in association with the carrier. Saure[4] has reported on similar analyses that only progesterone in radiochemically homogenous form can be eluted from the respective thin layer chromatography fractions and thus more specific methods for the identification of the progesterone formed in the present incubations were unnecessary.

The enzymatic activity was calculated from the conversion of tritiated pregnenolone into tritiated progesterone, corrected for the methodological losses and subtraction of the blank value. The recovery of the [4-^{14}C]progesterone was found to be 88.0–92.5% after incubations with boiled mitochondrial fractions.

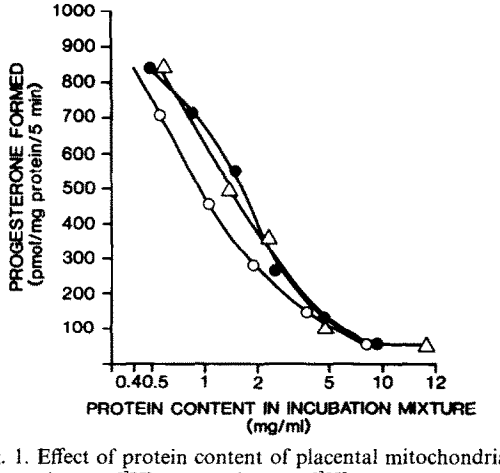

Fig. 1. Effect of protein content of placental mitochondrial preparation on [^{3}H]pregnenolone to [^{3}H]progesterone conversion *in vitro*. The quantity of progesterone formed per mg of protein per 5 min incubation appears to be inversely related to the amount of protein content in the incubation mixture. However, the total amount of progesterone formed per 5 min incubation reaches a maximum and remains at that amount after 2.5–3.5 mg of mitochondrial protein/ml are present in the incubation mixture. Each symbol represents preparation from a term placenta. Three term placentas were used individually.

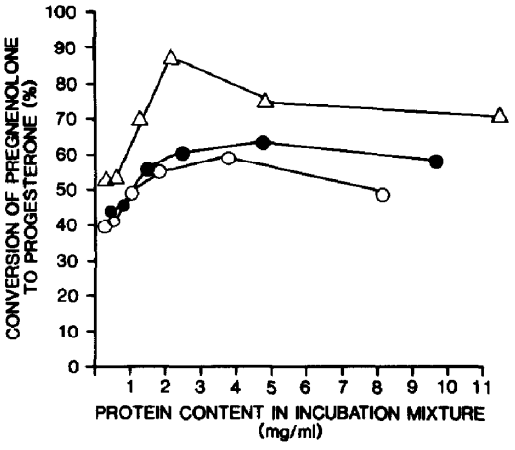

Fig. 2. Effect of protein content of placental mitochondrial preparation on per cent conversion of [^{3}H]pregnenolone to [^{3}H]progesterone *in vitro*. The percentage conversion of [^{3}H]pregnenolone to [^{3}H]progesterone reached a maximum at about 2.5–3.5 mg of placental mitochondrial protein/ml in the incubation mixture.

Protein content. The protein content of each fraction was determined as described by Lowry *et al.*[5] with bovine serum albumin as the protein standard. In most of the experiments, the mitochondrial fraction isolated was diluted so that each incubation mixture would contain a protein content in the range of 2.5–3.4 mg protein/ml.

RESULTS

When the protein content of the placental mitochondrial preparation was examined, the quantity of progesterone formed per mg of placental mitochondrial protein per 5 min incubation was inversely related to the amount of placental mitochondrial protein present in the incubation mixture (Fig. 1). However, the percentage of [^{3}H]pregnenolone converted to [^{3}H]progesterone reached a maximum at about 2.5–3.5 mg protein/ml (Fig. 2.). Thus a placental mitochondrial protein content of 2.5–3.5 mg protein/ml was used for all the subsequent experiments.

Figure 3 shows the effect of varying incubation times on the percentage conversion of pregnenolone to progesterone *in vitro* there is a rapid conversion of $68.4 \pm 4.8\%$ (mean $\pm$ SE) of pregnenolone to progesterone within 5 min of incubation. At 30 and 60 min of incubation time, the percentage of pregnenolone to progesterone conversion increased significantly to $83.1 \pm 4.6\%$ ($P = <0.01$) and $94.7 \pm 7.4\%$ ($P = <0.05$) compared to the conversion rate at 5 min. However, there was no significant increase in the conversion rate of pregnenolone to progesterone from 30 to 60 min incubation time. Thus, we have chosen to use an incubation time of 30 min for all other compartments.

The effects of estradiol, estrone, progesterone and testosterone on the *in vitro* conversion of pregnenolone to progesterone by term placental homogenates are shown in Fig. 4. All the four steroids with the dose range of 0.03–8.66 μmol, inhibited the conversion of pregnenolone to progesterone in a dose-dependent manner. On a weight for weight basis, the order of potency for this inhibition was estradiol, testosterone, progesterone and estriol. The i.d. 50 (dose needed to inhibit the conversion of pregnenolone to progesterone by 50%) dose for estradiol

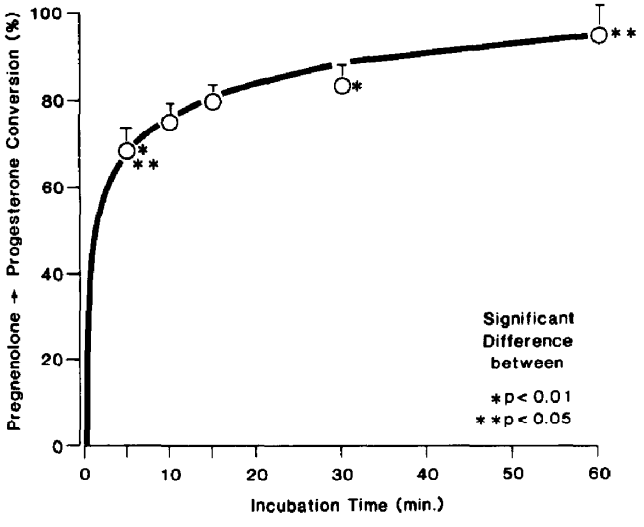

Fig. 3. Effect of varying incubation time on the *in vitro* conversion of [7-^{3}H(N)]pregnenolone to progesterone by human term placental villus tissue. 0.05 μCi of [7-^{3}H(*N*)]pregnenolone was used for each incubation. At 5 min there was $68.4 \pm 4.8\%$ of [7-^{3}H(*N*)]pregnenolone converted to progesterone. There was a further significant increase at 30 min ($P = <0.05$) and 60 min ($P = <0.01$) of incubation compared to the percentage conversion at 5 min. For each incubation time, experiments were carried out on 8 different placentas.

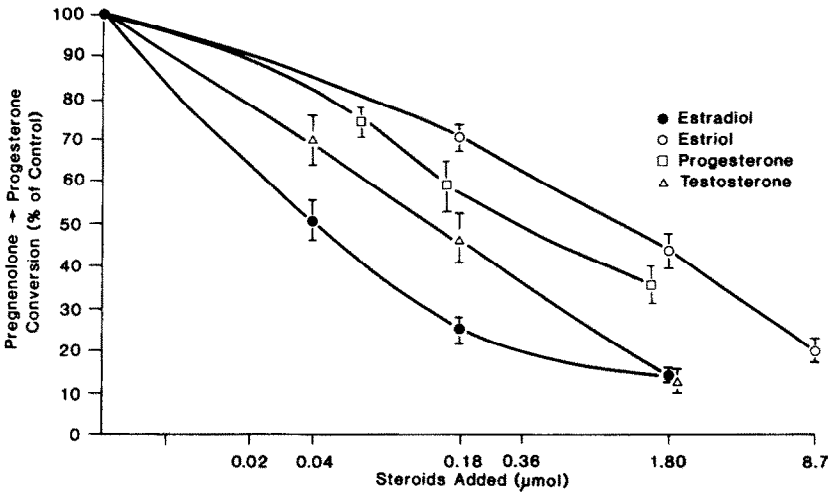

Fig. 4. Effect of various steroid hormones on the *in vitro* conversion of [7-^{3}H(*N*)]pregnenolone (0.05 μCi per incubation) to progesterone by human term placental villus tissue obtained at elective repeat Cesarean section. The incubation time was 30 min for all experiments. For each of the dose of steroid hormone tested, experiments were carried out on 8 different placentas. Each of the values on the graph represents the mean ± SEM and the values are given as the conversion of pregnenolone to progesterone expressed as a percentage of the conversion rate in control tube (100%) where no test steroid was added.

was 0.4 μmol, testosterone 0.13 μmol, progesterone 0.3 μmol and estriol 1 μmol (Table 1).

Both gonadotropin releasing hormone [GnRH] (50–1000 ng) and human chorionic gonadotropin [hCG] (5 to 500 i.u.) did not affect the basal conversion rate of pregnenolone to progesterone by placental homogenates *in vitro* (Table 2).

DISCUSSION

Using placental mitochondrial preparations obtained from term placental homogenates, we find that pregnenolone can be converted to progesterone *in vitro* indicating that the enzyme 5-ene-3β-hydroxysteroid dehydrogenase is present in the placental mitochondria. The *in vitro* conversion rate of pregnenolone to progesterone is rapidly achieved with 5 min of incubation and increases further to reach a nadir at 30 min. The maximal conversion rate of pregnenolone to progesterone is achieved with a protein content of 2.5–3.5 mg/ml of the mitochondrial preparation. Thus we have standardized and employed a protein content of 2.5–3.5 ng/ml in our mitochondrial preparation and an incubation time of 30 min for the enzymatic conversion of pregnenolone to progesterone *in vitro*.

Table 1. ID_{50}* of various steroids on placental conversion of pregnenolone to progesterone *in vitro*

Steroid	ID_{50}
Estradiol	0.036 μmol
Estriol	1.0 μmol
Testosterone	0.132 μmol
Progesterone	0.302 μmol

*ID_{50} is the dose required to inhibit basal conversion by 50%.

Our findings demonstrate that estradiol, estriol, progesterone and testosterone can inhibit the conversion of pregnenolone to progesterone *in vitro* by mitochondrial preparations of the placental villus, presumably by inhibiting the placental enzyme complex 5-ene-3β-hydroxysteroid dehydrogenase and 5-ene-4-ene isomerase which catalyze the conversion of pregnenolone to progesterone. These observations imply a feedback regulation of placental conversion of pregnenolone to progesterone by steroid hormones, which are either the end product of placental biosynthesis or intermediates in placental steroid metabolism. Estradiol, estriol and progesterone are the final products while testosterone is an intermediate in placental steroid biosynthesis. Thus accumulation of progesterone could physiologically autoregulate the rate of further conversion of pregnenolone to progesterone through a feedback inhibition on the enzyme 5-ene-3β-hydroxysteroid

Table 2. Effect of GnRH and hCG on the *in vitro* conversion of [7-^{3}H(*N*)]pregnenolone to progesterone by human term placental villus tissue

Hormone	Dose	Conversion of pregnenolone to progesterone* Mean ± SEM (as % of control) (n = 3)
Control	—	100*
GnRH	50 ng	106.9 ± 4.0
	250 ng	90.3 ± 8.2
	750 ng	105.1 ± 5.6
	1000 ng	100.7 ± 1.9
hCG	5 IU	97.5 ± 2.8
	50 IU	99.5 ± 3.7
	250 IU	104.8 ± 4.2
	500 IU	103.8 ± 3.3

*Incubation time was 30 min.

dehydrogenase. Similarly estradiol and estriol could regulate their biosynthesis from dehydroepiandrosterone sulfate through retroinhibition of 3β-hydroxysteroid dehydrogenase. Our findings on the inhibitory effect of estradiol on placental conversion of pregnenolone to progesterone confirm some previous observations [6–10].

With placental slices, physiologic doses (0.09–0.9 μmol) were stimulatory but larger doses (4 μmol) of estradiol were inhibitory on placental conversion of pregnenolone to progesterone [10]. In contrast, employing a cell free system with the mitochondrial enzyme readily accessible, we found that even as low as 0.04 μmol estradiol was inhibitory on the *in vitro* conversion of pregnenolone to progesterone. Our observations are more relevant to the intracellular mechanisms governing the bioconversion of pregnenolone to progesterone since steroids such as estradiol are produced in the trophoblast cells and are therefore closer to the mitochondria than when placed in an incubation media outside intact tissue slices. During pregnancy, both the placental production and secretion of estradiol and estriol increase until term. Therefore, it is tempting to hypothesize that the increase in placental estriol and estradiol contributes to a feedback inhibition on the enzyme 5-ene-3β-hydroxysteroid dehydrogenase and consequently a reduction in placental production of progesterone from pregnenolone. This could lead to a high estrogen and declining progesterone milieu around the placenta and the uterus and thus alter the sensitivity of the myometrium to oxytocin. Free fatty acids including arachidonic acid can inhibit both microsomal and mitochondrial 5-ene-3β-hydroxysteroid dehydrogenase [8]. Thus a rising production of placental estrogens not only inhibits placental pregnenolone–progesterone conversion but can also stimulate prostaglandin generation and arachidonic acid release which in turn further inhibits placental pregnenolone–progesterone conversion.

The mechanism by which steroids inhibit the enzyme 5-ene-3β-hydroxysteroid dehydrogenase is unclear but the dose-dependent inhibition by estradiol, estriol, progesterone and testosterone suggests competitive inhibition. Dehydroepiandrosterone and estrone inhibit placental 5-ene-3β-hydroxysteroid dehydrogenase competitively but the 4-ene-3-ketones inhibit the conversion of pregnenolone–progesterone in a noncompetitive manner [13]. In contrast, testosterone inhibits 5-ene-3β-hydroxysteroid dehydrogenase independent of its aromatization [9].

We found that hCG did not affect the conversion of pregnenolone to progesterone by placental mitochondrial preparations. Using cultures of syncytiotrophoblast, Paul *et al.*[14] also found no effect of hCG on progesterone production. Thus, unlike its stimulatory effect on corpus luteum progesterone production, hCG does not appear to have a significant effect on placental production of progesterone from pregnenolone. Since the placenta has GnRH [15], this decapeptide may play a role in placental steroidogenesis. With placental cultures, high concentrations of GnRH were found to inhibit progesterone production [16]. However, we were unable to observe any effect of GnRH on the *in vitro* conversion of pregnenolone to progesterone by the placental mitochondrial enzyme 5-ene-3β-hydroxysteroid dehydrogenase.

In conclusion, our studies have shown that mitochondrial preparations from fresh term placental homogenates contain the enzyme complex 5-ene-3β-hydroxysteroid dehydrogenase and 5-ene-4-ene isomerase and are able to convert pregnenolone to progesterone. This conversion is inhibited by estradiol, estriol, progesterone and testosterone suggesting retroinhibition by end product steroids produced in the placenta. Both GnRH and hCG did not have any significant effect on placental conversion of pregnenolone to progesterone.

REFERENCES

1. Bedford C. A., Challis J. R. C., Harrison F. A. and Heap R. B.: The role of oestrogens and progesterone in the onset of parturition in various species. *J. reprod. Fert.* (Suppl. 16) (1972) 1–24.
2. Thornburn G. D., Nicol D. H., Bassett J. M., Shutt D. A. and Cox R. I.: Parturition in the goat and sheep: changes in corticosteroids, progesterone, oestrogen and prostaglandin F. *J. reprod. Fert.* (Suppl. 16) (1972) 61–84.
3. Ash R. W. and Heap R. B.: Oestrogen, progesterone and corticosteroid concentrations in peripheral plasma of sows during pregnancy, parturition, lactation and after wearing. *J. Endocr.* **64** (1975) 141–154.
4. Saure A.: The biosynthesis and metabolism of steroids in the human placenta of 9–14 weeks *in vitro*. *Ann. Acad. Sci Fenn.* (Sect V) *Medica* **159** (1973) 1–131.
5. Lowry O. H., Rosenbrough N. J., Farr A. L. and Randall R. J.: Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193** (1951) 265–275.
6. Wiener M. and Allen S. H. G.: Inhibition of placental steroid synthesis by steroid metabolites II: Inhibitor specificity. *Steroids* **12** (1968) 261–273.
7. Goldman A. S. and Sheth K.: Inhibitors of human placental C_{19} and C_{21} 3β-hydroxysteroid dehydrogenases. *Biochim. biophys. Acta* **315** (1973) 233–249.
8. Ferre F., Breuiller M., Cedard L., Duchesne M. J., Saintot M., Descomps B. and Crastes de Paulet A.: Human placenta ^{5}-3β hydroxysteroid dehydrogenase activity (^{5}-3β HSDH): Intracellular distribution, kinetic properties, retroinhibition and influence of membrane dilipidation. *Steroids* **26** (1975) 551–570.
9. Grimshaw R. N., Mitchell B. F. and Challis J. R. G.: Steroid modulation of pregnenolone to progesterone conversion by human placental cells *in vitro*. *Am. J. Obstet. Gynec.* **145** (1983) 234–238.
10. Genti-Raimondi S., Patrito L. C. and Flury A.: Effect of estradiol-17β on the conversion of pregnenolone to progesterone in human term placenta incubated *in vitro*. *Steroids* **41** (1983) 467–474.
11. Gibb W.: Substrate and nucleotide specificity of placental microsomal 3β-hydroxysteroid dehydrogenase. *Steroids* **37** (1982) 23–31.
12. Power S. G. A. and Collins J. R. G.: Activity of 3β-hydroxysteroid dehydrogenase-5,4-ene-isomerase in chorioallantois and amnion from pregnant sheep. *J. Endocr.* **97** (1983) 347–356.

13. Townsley J. D.: Inhibition of placental 3β-hydroxysteroid dehydrogenase by naturally occurring steroid. A potential mechanism regulating oestrogen synthesis from conjugated precursors. *Acta endocr., Copenh.* **79** (1975) 740–748.
14. Paul S., Das C., Jailkhaui B. L. and Talwar G. P.: Progesterone synthesis by human placental syncytiotrophoblast *in vitro*-preferred precursor and effect of human chorionic gonadotropin. *J. steroid Biochem.* **14** (1981) 311–313.
15. Silver-Khodr T. M. and Khodr G.S.: Content of luteinizing hormone releasing factor in the human placenta. *Am. J. Obstet. Gynec.* **130** (1978) 216–219.
16. Branchaud C. L., Goodyer C. G. and Lipowski L. S.: Progesterone and estrogen production by placental monolayer cultures: effect of dehydroepi-androsterone and luteinizing hormone-release hormone. *J. clin. Endocr. Metab.* **56** (1983) 761–766.