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Maturation of ovarian steroid biosynthetic pathways in puberty and their hormonal control

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Summary. In rats, mice and rhesus monkeys of different ages, radioactive (^{14}C or ^{3}H) progesterone, ^{3}H-5α-pregnane-3,20-dione and/or ^{34}C-testosterone were incubated with ovarian homogenate or directly injected into the ovary. After treatments, radioactive products in the incubation mixture and in the ovary or ovarian vein blood were extracted isolated, measured and identified.

All ovaries from rats, mice and monkeys of different ages converted significant amounts of progesterone to 17-hydroxyprogesterone, androstenedione and/or testosterone. Ovaries from immature rats and immature mice synthesized a significant quantity of 5α-reduced C_{21}-17-OH- and C_{19}-steroids such as 3α, 17α-dihydroxy-5α-pregnan-20-one, androsterone and 5α-androstane-3α, 17β-diol, whereas ovaries from suckling and adult rats and mice, and those from monkeys in all ages formed very little or no 5α-reduced C_{21}-17-OH- and C_{19}-steroids. These 5α-androgens produced by immature rodent ovaries were shown to be synthesized mainly by a pathway through 5α-reduced C_{21}-stéroids, which was regulated by LH but not by FSH or sex steroids.

Introduction.

Previous studies of androgen biosynthesis have demonstrated that rat and mouse testes of suckling and adult animals are capable of a high rate of progesterone conversion to testosterone. On the other hand, testes of immature rats and immature mice yield 5α-reduced C_{19}-steroids (5α-androgens) such as androsterone and 5α-androstane-3α, 17β-diol as major products (Inano *et al.*, 1967 ; Coffey *et al.*, 1971 ; Ficher and Steinberger, 1971 ; Tsujimura and Matsumoto, 1974 ; Yamada *et al.*, 1976). We also found by *in vitro* and *in vivo* studies that progesterone was converted to these 5α-androgens primarily by a pathway through 5α-reduced C_{21}-steroids (C_{21}-5α-steroids) in these immature testes (Yamada and Matsumoto, 1974 ; Tsujimura *et al.*, 1975 ; Yamada *et al.*, 1976).

Previous studies have demonstrated that immature rat ovary contains high 5α-reductase activity (Mason, 1970) and that immature rat ovary forms a large quantity

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of 5α-androgens such as 5α-androstane-3α, 17β-diol, while adult rat ovary is unable to produce a significant quantity of 5α-androgens (Springer and Eckstein, 1971 ; Eckstein and Ravid, 1974). These 5α-androgens which can not be converted to œstrogen, have been shown to exert a negative feedback on gonadotrophin release and to induce precocious ovulation in rats (Eckstein and Ravid, 1974 ; Eckstein, 1975), Furthermore, ovariectomy of immature rats resulted in a marked increase in gonadotrophin level though plasma œstradiol-17β levels in the immature female rats were always the lowest (almost undetectable) (Meijs-Roelofs *et al.*, 1973). Since these findings indicate that the formation of 5α-androgens in immature rat ovary seems to have a biological significance, the age-dependent patterns of 5α-androgen formation which have been shown to be present in rat and mouse testes, should be clarified systematically in rat and mouse ovaries. In addition, a pathway leading to formation of these 5α-androgens and hormonal regulation of 5α-reductase activity in rodent ovary are also reported in this paper. Some findings on rat ovaries presented in this paper have already been reported in our previous papers (Karakawa *et al.*, 1976 ; Terakawa *et al.*, 1978).

In immature humans and immature monkeys, limited or no rise in gonadotrophin levels following castration has been reported (Odell and Swerdloff, 1976) and the formation of no or very small amounts of testicular 5α-androgens has been found (Mizutani *et al.*, 1977). We are also reporting on the age-dependent pattern of 5α-androgen formation in rhesus monkey ovaries.

Materials and methods.

Animals. — Female rats of the Sprague-Dawley strain, female mice of the d. d. strain and female rhesus monkeys of different ages (suckling, immature, pubertal and adult animals) were used. In some experiments, female rats were hypophysectomized at 21 days of age, and treatment was started 3 days later. Rats were injected daily with 1-90 μg of NIH-LH-S19, 10 or 50 μg of NIAMD-Rat-FSH-B-1, 1 mg of 5α-androstane-3α, 17β-diol, 1 mg of testosterone or 20 μg of œstradiol-17β for 3 days and were killed at 27 days of age.

Incubation procedure. — Homogenates of ovaries (1-90 mg) of different ages were incubated with radioactive substrates (1-6.6 nmol/ml) and NADPH at 37 °C in 1 ml medium as previously described (Karakawa *et al.*, 1976 ; Terakawa *et al.*, 1978).

Injection of radioactive steroids. — Radioactive progesterone and/or ^{3}H-5α-pregnane-3,20-dione, each suspended in 5 μl of saline solution were injected directly into rat ovary. The ovary and ovarian vein blood were obtained after the injection, and radioactive products were extracted.

Analysis and identification of steroids. — To the incubation mixtures and the extracts, 2-50 μg quantities of progesterone, 16α-hydroxypregn-4-ene-3,20-dione, 20α-hydroxypregn-4-en-3-one, 5α-pregnane-3,20-dione, 3$^{\alpha}_{\beta}$-hydroxy-5α-pregnan-20-one (5α-pregnanolones), 17α-hydroxypregn-4-ene-3,20-dione (17-hydroxyprogesterone), 17α-hydroxy-5α-pregnane-3,20-dione, 3α, 17α-dihydroxy-5α-pregnan-20-one, 3β, 17α-dihydroxy-5α-pregnan-20-one, androstenedione, testosterone, 5α-androstane-3,17-

dione (androstanedione), androsterone, 3β-hydroxy-5α-androstan-17-one (epiandrosterone), 17β-hydroxy-5α-androstan-3-one (dihydrotestosterone), 5α-androstane-3α, 17β-diol (androstane-3α, 17β-diol), 5α-androstane-3β, 17β-diol, œstradiol-17β and/or œstrone were added as nonradioactive carriers. The analysis of these steroids by paper (Zaffaroni and Burton, 1951) and column (Seki and Matsumoto, 1967) chromatography with acetylation of steroids, identification or tentative identification of metabolites by recrystallization to constant specific activity and the calculation of metabolite found in each steroid fraction were the same as previously described (Yamada *et al.*, 1973 ; Karakawa *et al.*, 1976). 5α-Reductase activity was estimated as previously described (Terakawa *et al.*, 1978).

Results.

1. *Metabolism of* ^{3}H-*progesterone in rat ovaries of different ages.*

The percentage formation of ^{3}H-4-ene-3-ketosteroids and ^{3}H-5α-steroids from ^{3}H-progesterone (1 nmol) by 50 mg of rat ovarian homogenate is shown in figure 1. At 7 (suckling) and 70 (adult) days of age, the major C_{19}-steroids formed from progesterone were androstenedione and testosterone. At 20 and 30 days of age (immature), however, no accumulation of these C_{19}-4-ene-3-ketosteroids was found, at which

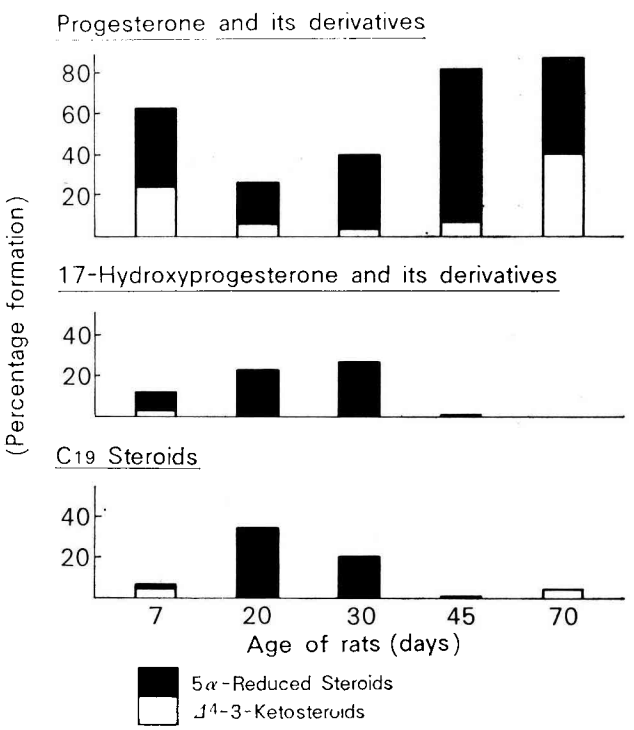

FIG. 1. — *Percentage formation of* ^{3}H-*steroids from* ^{3}H-*progesterone by rat ovarian homogenates.* Fifty mg tissue was incubated with ^{3}H-progesterone (1 nmol : 1 μCi per tube) at 37 °C for 30 min in 1 ml.

time the conversion of progesterone to C_{19}-5α-steroids, such as androsterone and 5α-androstane-3α, 17β-diol, reached 30 p. 100. In these immature ovaries, the major C_{21}-steroids formed from progesterone were 3α-hydroxy-5α-pregnan-20-one and 3α, 17α-dihydroxy-5α-pregnan-20-one.

Radioactivity of ^{3}H-4-ene-3-ketosteroids and ^{3}H-5α-steroids found in ovary and ovarian vein blood following injection of ^{3}H-progesterone into rat ovary is shown in figure 2. In immature rats, major 17-OH-C_{21}- and C_{19}-metabolites of progesterone were 5α-steroids such as 3α, 17α-dihydroxy-5α-pregnan-20-one and androsterone. A significant but small quantity of 5α-androstane-3α, 17β-diol was also found in the ovarian vein blood as well as in the ovary of immature rat. In adult rats, however, only small amounts of these 5α-steroids were found, and 20α-hydroxypregn-4-en-3-one was the major metabolite. Oestradiol-17β could be isolated in very small amounts only in adult ovaries.

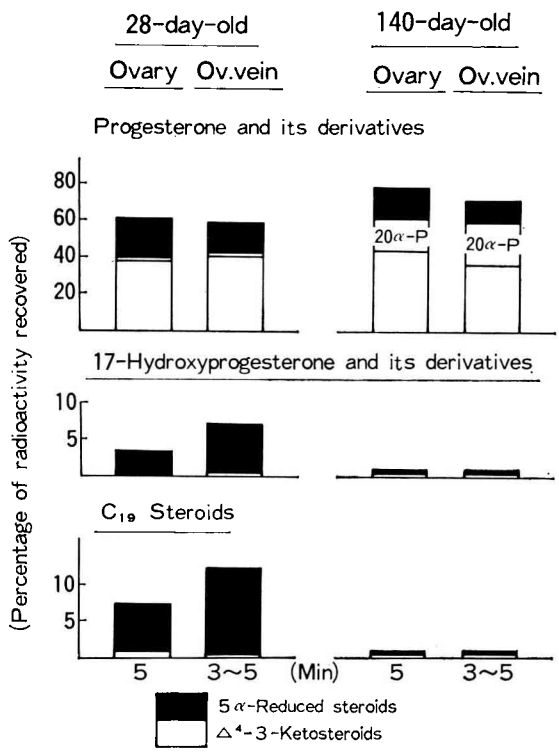

FIG. 2. — *Radioactivity of ^{3}H-steroids in ovary and ovarian vein blood following injection of ^{3}H-progesterone* (0.6 nmol/30 μCi per 5 μl) *into rat ovary.*

2. *Formation of C_{19}-5α-steroids from progesterone by a pathway through C_{21}-5α-steroids in immature rat ovary.*

Since findings shown in figures 1 and 2 indicate that significant amounts of 5α-androgens are synthesized in immature rat ovaries but not in ovaries of suckling and

adult rats, pathways leading to formation of these 5α-androgens in the immature rat ovary have been investigated. Figure 3 shows the results when ^{3}H-progesterone was incubated with homogenate from immature rat ovaries for varying periods. About 95 p. 100 of the precursor progesterone was converted to metabolites within the first 5 min. A rapid increase in 3α-hydroxy-5α-pregnan-20-one coccurred followed by declining amounts after 5 min. 17-OH-C_{21}-5α-steroids such as 3α,17α-dihydroxy-5α-pregnan-20-one accumulated between 15 and 45 min and then gradually decreased. 5α-Androgens such as androsterone and 5α-androstane-3α, 17β-diol increased steadily and reached 70 p. 100 of the total radioactivity. 17-Hydroxyprogesterone, androstenedione and testosterone were almost undetectable throughout the entire incubation time.

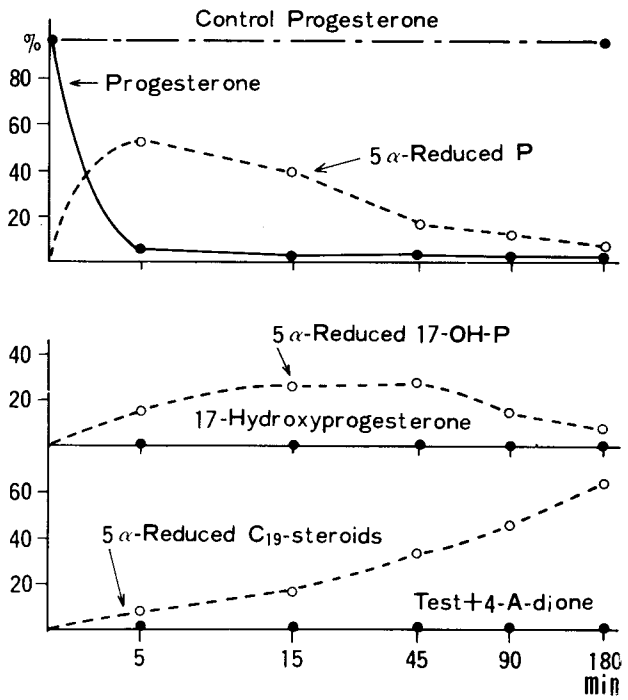

FIG. 3. — *Percentage formation of* ^{3}H-*steroids, at different time intervals following incubation of* 50 mg *ovarian homogenate from* 28-*day old rats with* 1 nmol *of* ^{3}H-*progesterone in* 1 ml *at* 37 °C. P : progesterone, 17-OH-P : 17-hydroxyprogesterone, Test : testosterone, 4-A-dione : androstenedione.

Experiments were attempted to determine ^{3}H/^{14}C ratios in 5α-products following incubation of immature rat ovaries with ^{3}H-5α-pregnane-3,20-dione plus ^{14}C-progesterone or after injection of these two radioactive substrates into the immature rat ovary (table 1). Significant augmentation of progesterone isotope was observed in 3α, 17α-dihydroxy-5α-pregnan-20-one compared with 3α-hydroxy-5α-pregnan-20-one, indicating that 3α, 17α-dihydroxy-5α-pregnan-20-one was formed from 3α-hydroxy-5α-pregnan-20-one and 17-hydroxyprogesterone. The ratios in 3α, 17α-dihydroxy-

5α-pregnan-20-one and 5α-androgens showed a slight difference indicating that 5α-androgens were mainly formed from 17-OH-C_{21}-5α-steroids.

TABLE 1

Ratios of ^{3}H-5α-pregnane-3,20-dione to ^{14}C-progesterone as precursors of metabolites in immature rat ovary

Condition	*In vitro*		*In vivo*
Reaction time (min)	30	30	2
^{3}H-5α-pregnane-3,20-dione (nmol)	1.0	6.6	0.5
^{14}C-progesterone (nmol)	6.6	6.6	6.6
Original mixture	1.00	1.00	1.00
3α-Hydroxy-5α-pregnan-20-one	1.34	1.17	5.93
3α,17α-Dihydroxy-5α-pregnan-20-one	0.82	0.41	0.95
Androsterone	0.71	0.33	1.04
5α-Androstane-3α,17β-diol	0.44	0.24	0.80

Ovarian homogenate (50 mg) was incubated with ^{3}H- and ^{14}C-substrates for 30 min at 37 °C in 1 ml. In *in vivo* experiment, ^{3}H- and ^{14}C-substrates suspended in 5 μl of saline solution were directly injected into ovary and metabolites in the ovary were analyzed.

In ovaries of immature rats, the results obtained by *in vitro* and *in vivo* studies indicate two biosynthetic pathways leading to 5α-androgens, one from progesterone via C_{21}-5α-steroids (major pathway), and the second via androstenedione and testosterone (fig. 4).

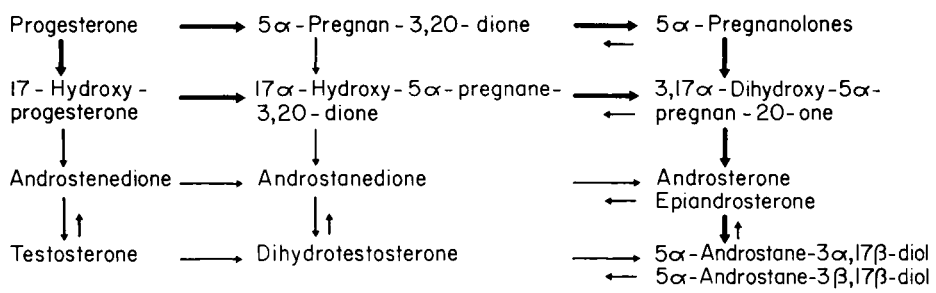

FIG. 4. — *Metabolic pathways leading to formation of 5α-androgens from progesterone in immature rat ovary.*

3. *Hormonal regulation of 5α-reductase activity in immature rat ovary.*

The above observations in rats clearly show that efficient formation of 5α-androgens by the 5α-reduced pathway is present only in immature ovary. Since the formation of 5α-androgens is stimulated by the presence of high 5α-reductase activity in the

immature rat ovary, hormonal regulation of the 5α-reductase activity has been investigated.

Female rats were hypophysectomized at 21 days of age, and after a lapse of 3 days the hypophysectomized rats were injected daily with LH, FSH or sex steroids. At 27 days of age, 5α-reductase activity in ovaries was measured. The 5α-reductase activity (nmol/g/hr) decreased significantly 6 days following hypophysectomy. A distinct response to LH in 5α-reductase activity of the hypophysectomized rat ovaries was found, with a dose-response in activity from 260 ± 94 (SD) in the hypophysectomized control to the maximum level of 1960 ± 177 (SD) nmol/g/hr, using doses from 3 to 90 μg per day. In contrast, FSH was not effective, for it had little effect on5α-reductase activity even when 10 or 50 μg was given each day (fig. 5). No stimulation of 5α-re-

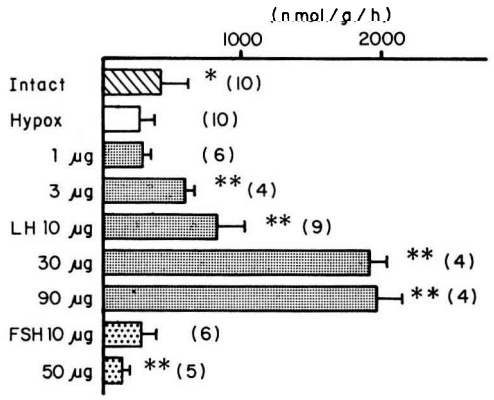

FIG. 5. — *Effect of* LH *treatment and* FSH *treatment on 5α-reductase activity* (nmol/g/hr) *in ovaries of immature hypophysectomized rats.* Rats were hypophysectomized at 21 days of age, and treatment was started 3 days later. The rats were injected daily with 1-90 μg of NIH-LH-S19 or 10-50 μg of NIAMD-Rat-FSH-B-1 for 3 days and were killed at 27 days of age. ( ) : No. of rats used. Differences from Hypox (hypophysectomized) control (P) : * < 0.05, ** < 0.01 (A t-test was used).

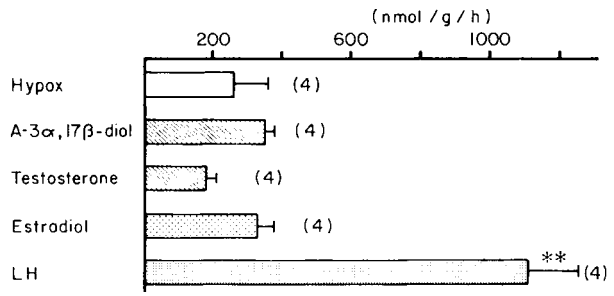

FIG. 6. — *Effect of sex steroids and* LH *on 5α-reductase activity in ovaries of immature hypophysectomized rats.* Treatments are described in legend of figure 5. The hypophysectomized rats were injected daily with 1 mg of 5α-androstane-3α, 17β-diol (A-3α, 17β-diol), 1 mg of testosterone, 20 μg of œstradiol-17β (Estradiol) or 10 μg of NIH-LH-S19 for 3 days. ( ) : No. of rats used. Difference from Hypox control (P) : ** < 0.01 (A t-test was used).

ductase activity was observed by injection of androgens and œstradiol-17β in large doses, showing that the effect of LH is not mediated by sex steroids. (fig. 6). The weight of ovaries increased from the hypophysectomized control by injection of FSH (approximately twice) but not by LH or sex steroids. These results show that 5α-reductase activity in immature rat ovaries is regulated by LH.

4. *Metabolism of* ^{3}H-*progesterone in mouse and monkey ovaries of different ages.*

The age-dependent pattern of progesterone metabolism has also been examined in ovaries of rhesus monkeys and mice. All ovarian homogenates from monkeys and mice of different ages converted 10-80 p. 100 of progesterone to 17-hydroxyprogesterone, androstenedione, testosterone and/or 20α-hydroxypregn-4-en-3-one (figs. 7, 8). In monkeys, however, all ovaries of different ages (immature, pubertal and adult) formed no 5α-metabolites of these 4-ene-3-ketosteroids (fig. 7). In mice, ovaries at 10 (suckling), 35 (pubertal) and 60 (adult) days of age were unable to form C_{21}-17-OH-5α-steroids and C_{19}-5α-steroids whereas those at 21 and 28 days of age (immature) formed significant amounts of 3α, 17α-dihydroxy-5α-pregnan-20-one, androsterone and 5α-androstane-3α, 17β-diol (fig. 8).

Discussion.

In rats, the formation of a large quantity of 5α-androgens by the pathways through C_{21}-5α-steroids and C_{19}-4-ene-3-ketosteroids is present in immature ovaries but not in ovaries of suckling and adult animals (figs. 1-4, table 1). In contrast to immature rats, ovaries of immature as well as adult monkeys do not form a significant quantity of 5α-androgens (fig. 7). Although ovaries of immature mice synthesize 5α-androgens from progesterone, this age-dependent formation of 5α-steroids is less active than that in rats (fig. 8). Ovariectomy of immature animals in which the uterus shows little stimulated effects by estrogens, results in a significant increase in gonadotrophin level in rats (Meijs-Roelofs *et al.*, 1973) but not in monkeys (Odell and Swerdloff, 1976). These observations suggest that the formation of 5α-androgens in ovaries may be of biological significance in immature rodents. Although the 5α-reductase in immature rat ovaries is shown to be maintained by LH (figs. 5 and 6), hormonal regulation of the age-dependent formation of 5α-androgens in rodent ovaries hat not been satisfactorily clarified. Hormonal regulation of 5α-reductase in ovaries of suckling and adult rats should be examined in future studies.

Efficient formation of C_{21}-17-OH-5α-steroids and C_{19}-5α-steroids which is similar to that by immature rat ovaries (figs. 1-4) is present in immature rat testes (Inano *et al.*, 1967 ; Coffey *et al.*, 1971 ; Ficher and Steinberger, 1971 ; Yamada *et al.*, 1976), and is present weakly in immature mouse testes (Tsujimura and Matsumoto, 1974), but is absent in immature monkey and human testes (Mizutani *et al.*, 1977). The previous findings and the present results seem to suggest that testes and ovaries show similar prepubertal 5α-androgen synthesis in each species and that the formation of a significant quantity of 5α-androgens may be absent in ovaries of immature humans. Prepubertal changes of gonadal 5α-androgen biosynthesis seem to be variable in different species of animals.

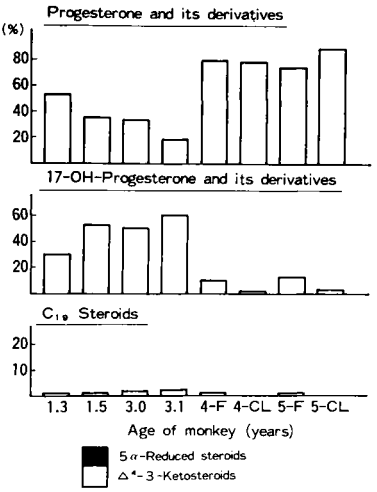

FIG. 7. — *Percentage formation of* ^{3}H-*steroids from* ^{3}H-*progesterone by ovarian homogenates from rhesus monkeys.* Ninety mg tissue was incubated with ^{3}H-progesterone (1 nmol : 1 μCi per tube) at 37 °C for 2 hr in 1 ml. F : follicles and interstitial cells. L : Corpora lutea.

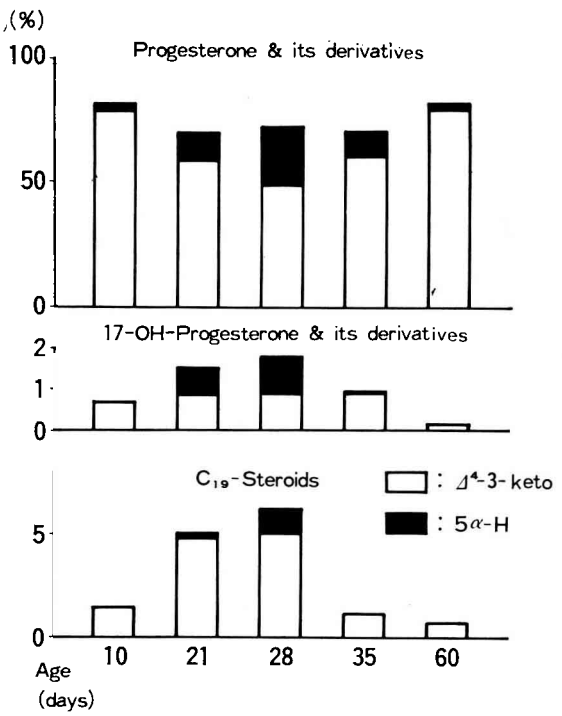

FIG. 8. — *Percentage formation of* ^{3}H-*steroids from* ^{3}H-*progesterone by mouse ovarian homogenates.* Eighty mg tissue was incubated with ^{3}H-progesterone (2 nmol : 1 μCi per tube) at 37 °C for 1 hr in 1 ml.

Conclusion.

In rats and mice, significant amounts of 5α-androgens are synthesized in immature ovaries but not in suckling and adult ovaries. These 5α-androgens are formed mainly by the pathway through C_{21}-5α-steroids which is regulated by LH. In monkey ovaries, the age dependent pattern of 5α-androgen formation is not found.

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Résumé. De la progestérone (^{14}C ou ^{3}H), de la ^{3}H-5α-pregnane-3,20-dione et/ou de la ^{14}C testostérone ont été, soit incubées avec des fragments d'ovaire, soit injectées directement dans les ovaires de ratte, de souris ou de macaque rhésus de différents âges. Après ces traitements, les dérivés radioactifs ont été extraits, isolés, identifiés et mesurés soit à partir de l'incubation, soit dans le sang de la veine ovarienne.

Les ovaires des trois espèces, quel que soit l'âge, convertissent la progestérone en 17α-OH progestérone et en androstènedione et/ou testostérone. Les ovaires de ratte et de souris immatures synthétisent des dérivés 5α réduits en C_{21} ou C_{19} tels que le 3α,17α-dihydroxy-5α-pregnane-20-one, l'androstérone et le 5α-androstane-3α, 17β-diol, tandis que les ovaires de rattes ou de souris allaitantes, ainsi que ceux du Rhésus de tout âge, forment peu ou pas du tout de dérivés 5α réduits. Les androgènes 5α réduits produits par les ovaires de rongeurs immatures proviennent principalement de la voie passant par les stéroïdes en C_{21},5α réduits, qui est régulée pa LH, mais ni par FSH, ni par les stéroïdes sexuels.

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