PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **184,** 14–18 (1987)

Quantitative Changes in Ovarian 17α-Hydroxylase/$C_{17,20}$-Lyase and Aromatase Activities during the Estrous Cycle of the Hamster (42439)

DONALD C. JOHNSON

Departments of Gynecology & Obstetrics and Physiology, Ralph L. Smith Research Center, University of Kansas Medical Center, Kansas City, Kansas 66103

Abstract. Aromatase activity of the microsomal fraction of ovarian homogenates, measured by a tritium exchange assay using androstenedione (A-dione) as substrate, did not change during the 4-day estrous cycle of the hamster. In contrast, 17α-hydroxylase, measured by a tritium exchange assay with progesterone as substrate, and particularly C17,20-lyase activity, evaluated by acetic acid production from progesterone, drastically decreased on the afternoon of proestrus (Day 4). The latter two activities remained low on Days 1 and 2 but increased dramatically on Day 3 and the morning of Day 4. The serum concentration of A-dione and 17-hydroxyprogesterone reached a peak at 1600 hr of proestrus and then decreased rapidly as the enzyme activities decreased. A-dione levels were undetectable on Day 1 but 17-hydroxyprogesterone levels remained elevated through Day 2. The results are consistent with the view that 17α-hydroxylase and C17,20-lyase are activities of a single cytochrome *P*-450, as has been shown for testis and adrenal. Increases in hydroxylase and lyase activities occur concomitantly with decreases in the serum concentrations of progesterone, suggesting that the latter steroid may play a role in controlling these enzymes.

Estrogen synthesis by the ovary is a cyclical process reaching a peak on the day of proestrus in the hamster (1) and in the rat (2). The large increase in luteinizing hormone (LH) output from the pituitary that is responsible for ovulation occurs on the afternoon of proestrus and induces a precipitous decrease in estrogen concomitant with an increase in progesterone synthesis by the ovaries (3). Early studies using rats (4) suggested that loss of estrogen production was secondary to inhibition of aromatase activity. Subsequent investigations, which have been extensively reviewed recently (5), demonstrated that a shortage of androgen precursor for the latter enzyme was the principal factor for loss of estrogen synthesis. A similar rationale applies for the hamster (3). That is, in the hamster the serum levels of testosterone, and estradiol, fall following the LH surge (6–8). However, the concentration of 17-hydroxyprogesterone in the serum increases. This has led to the suggestion that a consequence of the LH surge, which occurs between 1400 and 1600 hr on proestrus (9), is a loss of C17,20-lyase but not 17α-hydroxylase activity in the ovary (8). This interpretation is inconsistent with the current view that the two enzymes are activities of a single cytochrome *P*-450. Although both activities have been found for a presumably pure protein isolated from the microsomal fractions of testis and adrenals (reviewed in (10)) no purified material of ovarian origin has been studied. However, parallel changes in 17α-hydroxylase and C17,20-lyase in the ovaries of hypophysectomized immature rats treated with gonadotropin support the view that a single entity is also involved in this tissue (11, 12). In order to obtain direct evidence of changes in steroidogenic enzymes during the estrous cycle in the hamster a comparative study of the aromatase, 17α-hydroxylase, and C17,20-lyase activities was undertaken with particular attention given to changes on the day of proestrus.

Materials and Methods. Mature adult hamsters (*Mesocricetus auratus*) were obtained from Sasco, Inc. (Omaha, Nebr.). The body weights of these animals, which are presumably viral antibody free, varied between 130 and 220 g with a mean weight of 160.3 ± 2.5 g (N = 95). All animals exhibited at least 2, but the majority had shown more than 10, 4-day estrous cycles in our animal facility; they were maintained on a 14-hr light (0600–2000 hr):10-hr dark schedule. Groups of 5 to 7 animals were decapitated at various times during the cycle (Day 1 = estrus), with the blood from the trunk collected in 12 × 75-mm culture tubes. After clotting at room temperature the blood was stored at 4°C overnight. The serum

0037-9727/87 $1.50

was separated after centrifugation at 2200*g* for 15 min and then stored at −20°C until assayed.

The ovaries were removed as quickly as possible and placed in ice-cold incubation medium (RPMI 1640) containing 100 μM NADPH (Sigma Chemical Co., St. Louis, Mo.). After removal of the oviducts and fat the ovaries were weighed on a torsion balance and frozen in 50 mM EDTA containing 100 μM NADPH (pH 7.4). The ovaries were thawed and homogenized (glass–Teflon homogenizer) in 1.15% KCl containing 10 mM EDTA. The homogenate was centrifuged (4°C) at 10,000*g* for 20 min; the supernatant fluid was then centrifuged at 105,000*g* for 60 min. The pellet (microsomal fraction) was resuspended in 0.05 M Hepes buffer (pH 7.4) and immediately assayed for 17α-hydroxylase, C17,20-lyase, and aromatase activities.

The details of the enzyme assays have been published (11). Briefly, a tritium exchange assay was used for 17α-hydroxylase and aromatase. [17α-^{3}H]Progesterone (1.21 mCi/mmole) was prepared by Dr. P. Kremers (University of Liege, Belgium) and used (100 μM) for the hydroxylase assay without further dilution. Preliminary studies indicated that progesterone was preferred over pregnenolone as a substrate for hamster ovarian microsomal 17α-hydroxylase. [1,2-^{3}H]Androst-4-ene-3,17-dione (48.5 Ci/mmole; 45% of activity at 1β, 10% at 1α, 35% at 2β, and 10% at 2α) was purchased from New England Nuclear Corporation (Boston, Mass.). The labile 2β^{3}H was removed by enolization in hot alcoholic KOH (13) followed by purification on Sephadex LH-20. The purified label was diluted to 37.1 mCi/mmole with unlabeled androstenedione and used at a concentration of 10 μM for the assay. The assay was run in a 12 × 75-mm culture tube with a total volume of 200 μl containing NADPH, NADP, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase (Sigma) in addition to the microsomal fraction of the ovarian homogenate; quadruplicate samples for each homogenate were tested. The reaction was stopped after 30 min by the addition of 400 μl of a 10 mg/ml suspension of charcoal. Following removal of charcoal by centrifugation the tritiated water was distilled under reduced pressure.

[21-^{14}C]Progesterone (New England Nuclear Corp.), diluted to 13 mCi/mmole with unlabeled progesterone, was used at 40 μM as the substrate of C17,20-lyase. Preliminary studies demonstrated that hamster ovarian microsomal C17,20-lyase prefers progesterone over 17-hydroxyprogesterone as a substrate, as does the rat enzyme (11). The assay solution was the same as that used for hydroxylase and aromatase but the reaction was stopped with 0.1 N HCl. The labeled acetic acid produced by the action of lyase on progesterone was removed by distillation under reduced pressure. The radioactivity in the distillates was determined by a Packard Model 2425 liquid scintillation spectrometer with a 48% efficiency for ^{3}H and a 92% efficiency for ^{14}C. The results of each assay were expressed as nanomoles of substrate converted per hour per milligram of microsomal protein; the concentration of the latter was determined by the Bradford method (14).

Unextracted sera were assayed for 17-hydroxyprogesterone content by radioimmunoassay using a kit purchased from Diagnostic Products, Inc. (Los Angeles, Calif., Coat-A-Count) The intraassay coefficient of variation was 3% with a minimal detectable level of 50 pg/ml using 50 μl of serum. Details of the radioimmunoassays using tritiated steroids have been reported (15). Androstenedione was assayed following extraction of 100 μl of serum with 2 ml of freshly opened anhydrous diethyl ether; androstenedione standards (5–500 pg) (Steraloids, Wilton, N.H.) were extracted from 100 μl of sera obtained from ovariectomized + adrenalectomized adult female rats. The antiserum, obtained from Dr. John Resko (Department of Physiology, University of Oregon Health Sciences Center, Portland, Oreg.), cross-reacts 50% with 5α-androstane-3,17-dione, 4% with dehydroepiandrosterone, 0.3% with testosterone, 1% with cortisol, and 0.07% with progesterone. The intraassay coefficient of variation was 6.6% with a minimal detectable concentration of 50 pg/ml. Estradiol was assayed using a specific antiserum obtained from Dr. D. C. Collins (Emory University, Atlanta, Ga.). The intraassay coefficient of variation was 4% and the minimal detectable concentration was 25 pg/ml using 300 μl of serum for both the experimental samples and the standards.

Student's t test was used for comparison of means obtained in the same assay; differences with a $P < 0.05$ were considered significant.

Results. *Enzyme assays.* Changes in 17α-hydroxylase activity during the estrous cycle are shown in Fig. 1. After a minimal value at 0900 hr of Day 1, activity increased slightly by 0900 hr, but remained at about the same level ($P > 0.05$) at 2100 hr of Day 2. A profound increase in activity ($P < 0.001$) occurred between 2100 hr of Day 2 and 0900 hr of Day 3. Furthermore, during Day 3 activity increased rapidly and remained elevated until the afternoon of Day 4. By 1700 hr of Day 4 activity had decreased drastically and it continued to decline until 0900 hr of Day 1; however, the majority of the decrease had occurred by 2100 hr of Day 4. A similar pattern of change was seen with C17,20-lyase activity (Fig. 2), but the variability from one series to another was greater than for hydroxylase even though all of the homogenates were treated in identical fashion. However, a large decrease was seen on Day 4, similar to that seen with hydroxylase activity. The activity of lyase was considerably less than that of hydroxylase at all times. The mean hydroxylase/lyase ratio, obtained from activities of the same ovaries assayed simultaneously, was 15.4 ± 1.6 for homogenates of ovaries removed between 1500 hr of Day 3 and 1400 hr of Day 4 but it increased to 48.9 ± 6 in homogenates of ovaries removed on Day 2. In contrast to the changes seen with hydroxylase and lyase, aromatase remained essentially unchanged throughout the cycle (Fig. 2).

Steroid assays. 17-Hydroxyprogesterone

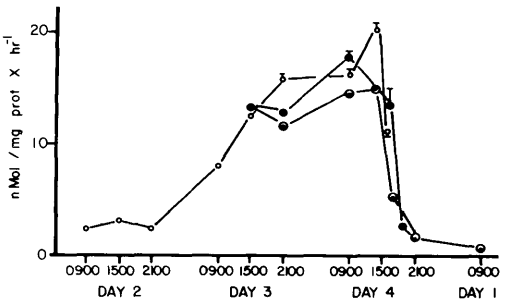

FIG. 1. The 17α-hydroxylase activity of the microsomal fraction of hamster ovaries that were removed at various times during the estrous cycle (Day 4 = proestrus). The different symbols indicate results obtained with three series of animals. SEM of four replicates of homogenates made up of a pool of ovaries from five to seven animals is indicated by a vertical line if it exceeds the area covered by the symbol.

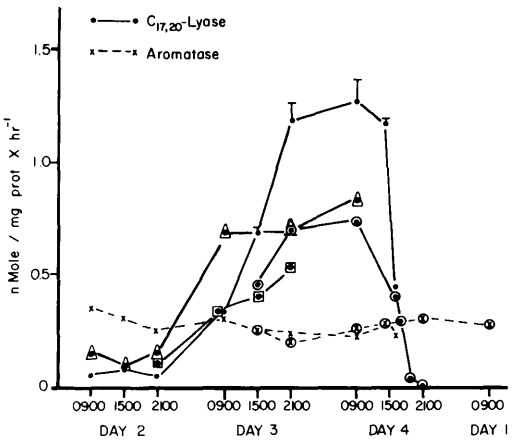

FIG. 2. The C17,20-lyase and aromatase activities of ovaries removed at various times during the estrous cycle. Four kinds of symbols indicate four series of animals used for the assay of lyase; results of two series are shown for aromatase. SEM is indicated by vertical line if it exceeds the area of the symbol.

was detectable in the sera of only 4 of 10 animals killed on Day 3 (Fig. 3). The level of this steroid increased significantly during Day 4, reached a peak of about 2 ng/ml at 1600 hr, and then rapidly declined by 2100 hr. However, the concentration of 17-hydroxyprogesterone on the morning of Day 1 was not significantly different from that found at 2100 hr of Day 4. Although not shown in Fig. 3, the steroid was present in the sera of all animals killed on Day 2 (N = 7/group; 108 ± 18 pg/ml at 0900 hr; 220 ± 49 pg/ml at 1400 hr and 189 ± 15 pg/ml at 2100 hr) but was less than 50 pg/ml in animals killed on the morning of Day 3.

Serum androstenedione concentrations also increased during Day 4, reaching a peak value of about 600 pg/ml at 1600 hr. The concentration then quickly declined to undetectable levels (<50 pg/ml) by 0900 hr of Day 1, and the steroid was undetectable in 100 μl of serum from animals killed at any time on Day 2 or at 0900 hr of Day 3.

Estradiol was assayed in only five groups of animals (Fig. 3). This steroid was readily detectable in the serum obtained at 0900 hr of Day 4, increased in concentration at 1400 hr, and then declined to undetectable levels by 0900 hr of Day 1.

Discussion. The present study has clearly confirmed results, obtained by indirect anal-

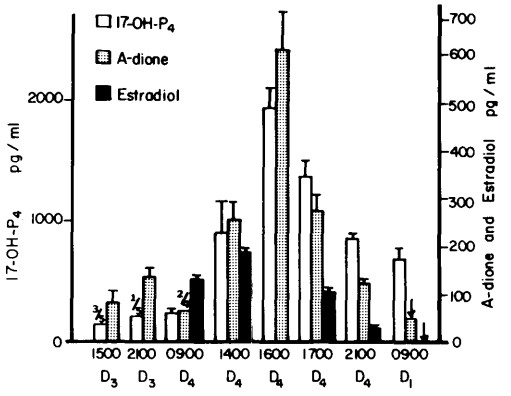

FIG. 3. The concentration, determined by radioimmunoassay, of steroids (17-hydroxyprogesterone (17-OH-P4), androstenedione (A-dione), and estradiol) in the sera of hamsters during the estrous cycle when the most drastic changes in 17α-hydroxylase and C17,20-lyase activities occur. Vertical line indicates SEM for groups of at least four animals. Numbers over columns indicate how many animals of the group had detectable levels of steroid; arrow indicates undetectable level.

yses (reviewed in (3)), that changes in ovarian aromatase activity are not responsible for the fall in serum estradiol levels associated with the ovulatory surge of LH in the proestrous hamster. When measured in homogenates of the whole ovary, this enzyme appears to be particularly stable. On the other hand, the methods employed in the present study would not detect changes that occur in various ovarian compartments. That is, aromatase is limited to the granulosa cells of the hamster ovary (16) and changes that occur in maturing follicles or follicles undergoing atresia would not be distinguished. Previous studies (1, 3) have suggested that inhibition of C17,20-lyase activity, thus denying aromatase an androgenic substrate, was the principal mechanism for the well-known decreased estrogen synthesis on the evening of proestrus. This is supported by the pattern of change in androstenedione concentrations which was similar to that reported (1) for testosterone. However, the finding that the level of serum androstenedione remained elevated ($\cong$1 ng/ml) and relatively stable throughout the cycle (1) was not confirmed in the present study. The reason for the discrepancy is not known but could involve use of a different batch of antiserum and/or different methodologies. Other studies (17) have shown that the ability of follicles removed from proestrous hamsters to synthesize androstenedione *in vitro* in response to LH is drastically curtailed by the *in vivo* ovulatory surge in LH. Thus there seems no doubt about the importance of the loss of C17,20-lyase activity for reduction in androgen and estrogen synthesis on proestrus.

A large body of evidence has accumulated to indicate that C17,20-lyase is a function of a specific cytochrome *P*-450 that also hydroxylates progesterone, or pregnenolone, at the 17α position (10). A confusing factor for interpretation of the data for the hamster is that the concentration of 17-hydroxyprogesterone, the product of the hydroxylase, increases with the LH surge and remains elevated when androgen levels disappear (6–8). Shaikh (6) demonstrated that the concentration of 17-hydroxyprogesterone in the ovarian venous effluent was high after the LH surge and remained elevated through 0100 hr of Day 1. The levels decreased significantly by 1200 hr of Day 1 and reached their lowest levels on the morning of Day 4. The present results, using peripheral blood, essentially follow this same pattern but the steroid became undetectable in the serum on the morning of Day 3. For interpretation of these results we must acknowledge either two separate enzymes or a control mechanism specific for the lyase activity of the cytochrome *P*-450. The latter alternative, which has gained general acceptance, has been discussed but mechanisms remain obscure (17).

Several studies suggest that the changes in production of progesterone are important for controlling hydroxylase and lyase activities. First, Ekholm and Hillensjo (19) found that the inhibition of androgen production by rat ovarian follicles *in vitro* induced by LH was dependent upon active steroidogenesis. Second, Saidapur and Greenwald (3) reported that a small dose of progesterone (50 μg) administered to hamsters at 1000 hr of proestrus produced a fall in serum estradiol levels within 3 hr without altering serum LH concentrations. Third, administration of aminoglutethimide to inhibit synthesis of pregnenolone from cholesterol increased the already elevated levels of ovarian microsomal 17α-hydroxylase and C17,20-lyase activities in immature hypophysectomized rats treated chronically with hCG; i.e., loss of substrate resulted in increased enzyme activity when gonadotropin was pres-

ent (12). Finally, the concentration of progesterone in the serum, measured at 2-hr intervals during the estrous cycle of the hamster, declines significantly between midnight of Day 2 and 0600 hr of Day 3 (20) just prior to the rise in enzyme activities (Figs. 1, 2). The latter study also showed that progesterone levels rose rapidly after 1200 hr and reached at peak at 2000 hr of Day 4, the latter coinciding with the large reduction in hydroxylase and lyase activities. Taken together these data suggest that progesterone indirectly controls cytochrome *P*-450 (17α-hydroxylase/C17,20-lyase) activities. The preponderance of hydroxylase over lyase activity has been suggested as a mechanism for disposal of excess progesterone analogous to the function of 20α-dihydroprogesterone in the rat (7). The cellular mechanisms involved in the control process, which may be tissue and/or gland specific, remain to be determined.

Supported in part by grant from NICHD (HD-15307). Special thanks are due Dr. P. Kremers for the synthesis of 17α^3-progesterone and to Dr. J. Resko and Dr. D. C. Collins for the gifts of antisteroid antisera.

1. Saidapur SK, Greenwald GS. Peripheral blood and ovarian levels of sex steroids in the cyclic hamster. Biol Reprod **18**:401–408, 1978.
2. Butcher RL, Collins WE, Fugo NW. Plasma concentrations of LH, FSH, prolactin, progesterone and estradiol 17β throughout 4-day estrous cycle of the rat. Endocrinology (Baltimore) **94**:1704–1708, 1974.
3. Saidapur SK, Greenwald GS. Regulation of 17β estradiol synthesis in the proestrus hamster: Role of progesterone and luteinizing hormone. Endocrinology (Baltimore) **105**:1432–1439, 1979.
4. Katz Y, Armstrong DT. Inhibition of ovarian estradiol 17β secretion by luteinizing hormone in prepubertal pregnant mare serum-treated rats. Endocrinology (Baltimore) **99**:1442–1447, 1976.
5. Erickson GR, Magoffin DA, Dyer CA, Hofeditz C. The ovarian androgen producing cells: A review of structure/function relationships. Endocrine Rev **6**: 317–399, 1985.
6. Shaikh AA. Estrone, estradiol, progesterone and 17α hydroxyprogesterone in the ovarian venous plasma during the estrous cycle of the hamster. Endocrinology (Baltimore) **91**:1136–1140, 1972.
7. Hubbard CJ, Greenwald GS. Cyclic nucleotide, DNA, and steroid levels in ovarian follicles and corpora lutea of the cyclic hamster. Biol Reprod **26**:230–240, 1982.
8. Greenwald GS, Limback D. Effects of treatment with cycloheximide at proestrus on subsequent in vitro follicular steroidogenesis in the hamster. Biol Reprod **30**:1105–1116, 1984.
9. Bast JD, Greenwald GS. Serum profiles of follicle stimulating hormone, luteinizing hormone and prolactin during the estrous cycle of the hamster. Endocrinology (Baltimore) **94**:1295–1299, 1974.
10. Suhara K, Fujimura Y, Shiroo M, Katagiri M. Multiple catalytic properties of the purified reconstituted cyctochrome *P*450 (*P*450 SccII) system of pig testis microsomes. J Biol Chem **259**:8729–8736, 1984.
11. Johnson DC, Griswold T. Ovarian C17,20-lyase: Changes in intact and hypophysectomized immature rats treated with pregnant mare's serum gonadotropin. J Steroid Biochem **20**:733–739, 1984.
12. Johnson DC, Griswold T. Relationship between in vivo and in vitro 17α-hydroxylase and C17,20-lyase activity in ovaries of immature hypophysectomized rats treated chronically with human chorionic gonadotropin. J Steroid Biochem **24**:637–643, 1986.
13. Osawa Y, Spaeth DG. Estrogen biosynthesis stereospecific distribution of tritium in testosterone 1,2-t. Biochemistry **10**:66–71, 1971.
14. Bradford M. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem **72**:248–254, 1976.
15. Sashida T, Johnson DC. The response of the immature rat ovary to gonadotropins: Acute changes in cyclic AMP, progesterone, testosterone, androstenedione and oestradiol after treatment with PMS or FSH + LH. Acta Endocrinol (Copenhagen) **82**:413–425, 1976.
16. Makris A, Ryan KJ. Aromatase activity of isolated and recombined hamster granulosa cells and theca. Steroids **29**:65–72, 1977.
17. Terranova PF, Garza F. Relationship between the preovulatory luteinizing hormone (LH) surge and androstenedione synthesis of preantral follicles in the cyclic hamsters: Detection by in vitro responses to LH. Biol Reprod **29**:630–636, 1983.
18. Nakajin S, Shinoda M, Hall PF. Purification and properties of 17α-hydroxylase from microsomes of pig adrenal: A second C21 side-chain cleavage system. Biochem Biophys Res Commun **111**:512–517, 1983.
19. Ekholm C, Hillensjo T. LH-induced inhibition of follicular androgen formation requires intact steroidogenesis. Mol Cell Endocrinol **27**:67–75, 1982.
20. Bast JD. A comprehensive profile of peripheral progesterone levels in cyclic hamsters sequentially bled at 2-hr intervals. Proc Soc Exp Biol Med **162**:199–201, 1979.

Received April 10, 1986. P.S.E.B.M. 1987, Vol. 184.
Accepted September 15, 1986.