AN ABSTRACT OF THE THESIS OF

Atsuko Fukuike for the Master of Science Degree in Biology presented on 7 December 1993

Title: Pregnenolone metabolism in developing follicles of the frog Rana pipiens

Abstract Approved: Katherine N. Sinsabaugh

To investigate ovarian steroid metabolism in the frog Rana pipiens, follicles at different stages of development were incubated with ^{3}H-pregnenolone, and the metabolites were analyzed by thin layer chromatography. A decrease in the percentage of unmetabolized ^{3}H-pregnenolone in the medium and in the follicles suggested that total steroidogenesis increased in later stage follicles. The main metabolites formed were estradiol by early stage follicles and testosterone by later stage follicles. Besides these two main metabolites, androstenedione, estrone, and progesterone were also identified, suggesting the presence of the $\Delta 4$ pathway of steroid metabolism in Rana follicles. The change in the main metabolite from estradiol to testosterone might be due to inhibition of aromatase activity. The possibility that a steroidal aromatase inhibitor might be synthesized during follicular development was investigated but none was identified. Stage 3 follicle incubation in the presence of a known aromatase inhibitor, 4-hydroxy-androstenedione (4-OH-A), resulted in a large increase in androstenedione as well as a smaller increase in testosterone. Such an increase in androstenedione did not occur in untreated late stage

follicles. Therefore, synthesis of a steroidal aromatase inhibitor seems unlikely. Follicles also metabolized ^{3}H-pregnenolone to several polar metabolites. However, the polar metabolites synthesized by stage 3 follicles pre-incubated with or without 4-OH-A were similar. Therefore, it is suggested that the polar metabolites are not derived from either estradiol or testosterone, but are probably progesterone metabolites.

PREGNENOLONE METABOLISM IN DEVELOPING FOLLICLES OF THE FROG *RANA PIPIENS*

A Thesis

Submitted to

the Division of Biological Sciences

Emporia State University

In Partial Fulfillment

of the Requirement for the Degree

Master of Science

by

Atsuko Fukuike

December, 1993

Katherine N. Smalley
Approved by Major Adviser

Gaylen Youngblood
Approved by Committee Member

Richard P. Keeven
Approved by Committee Member

Rodney J. Schuetz
Approved for Major Department

Faye N. Vowell
Approved for Graduate Council

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to the faculty members of the Division of Biological Sciences. I greatly appreciate my committee members, Dr. Gaylen Neufeld and Dr. Richard Keeling, who gave me helpful advice and comments. I appreciate Sondra Dubowsky for her kindness and advice. I also appreciate Dr. Larry Scott for helping me with data analysis.

Very special thanks go to Dr. Katherine Smalley. She always showed me what science is and how a scientist should be. I could not complete my degree without her support, guidance, advice, and encouragement.

I would like to extend my thanks to my friends and ex-coworkers, who provided me with references and information along with encouragement.

Finally, I would like to express my deepest gratitude to my parents, Tatsumasa and Mitsue Fukuike, and my sisters, Toshiko Kaneshiro and Kyoko Fukuike, whose support took me to the goal of this degree.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Many studies indicate that frog ovarian follicles synthesize and secrete various steroid hormones, including estrogens, androgens, and progestins. The growth and final maturation of oocytes are regulated by these hormones.

Estrogens stimulate the synthesis of vitellogenin, the precursor of yolk protein, in the liver (Wallace and Bergink, 1974; Ho, 1987). Estrogens are ineffective in inducing oocyte maturation (Schuetz, 1972; Spiegel et al., 1978), and progesterone is the primary steroid hormone responsible for this process. The role of androgens in follicular development is not well understood.

The secretion of steroid hormones changes during follicular development. The early vitellogenic follicles of Rana pipiens (stages 1-3) secrete large amounts of estradiol, whereas follicles in later stages of development (stages 4-5) secrete large amounts of testosterone (Smalley, 1989). A similar change has been reported in Xenopus laevis (Fortune, 1983) and Rana nigromaculata (Kwon et al., 1991). The secretion of the predominant hormone is greatly stimulated by gonadotropin (Smalley, 1989; Fortune, 1983; Kwon et al., 1991). Therefore, this secretion pattern seems to be follicular stage specific. However, other possible products have not been well investigated, and it is not clear whether estradiol and testosterone are the main metabolites in early stage and late stage follicles,

respectively. Moreover, the mechanism which causes the change in steroid secretion is not understood.

Estradiol is synthesized from testosterone by aromatase. Therefore, the change in secretion from estradiol to testosterone might be due to the inhibition of aromatase activity. Some androgens are known aromatase inhibitors (Brodie et al., 1977; Brueggmeier et al., 1982), so it is possible that transitional follicles may be able to synthesize an aromatase inhibitor which causes the shift from estradiol to testosterone.

Reynhout and Smith (1973) reported that the metabolites formed from progesterone by mature oocytes of Rana pipiens were 5α-pregnanedione, 5α-pregnan-20α-ol-3-one and 5α-pregnan-3β-20α-diol. The main metabolites from progesterone synthesized by mature follicles of Xenopus laevis were androstenedione and 17α,20α-dihydroxy-pregn-4-ene-3-one (Thibier-Fouchet et al., 1976). However, neither of these studies looked at steroidogenesis during early development.

Ozon (1972a, b) indicated that the main steroidogenic pathway in ovarian follicles appears to be similar in mammals, amphibians, and other vertebrates. However, his studies of amphibian follicles were not complete.

This study was undertaken to investigate the changes in steroid metabolism by ovarian follicles of Rana pipiens during follicular development. The objectives of this study were:

1) to investigate steroidogenesis in follicles at different stages of development.

2) to investigate whether estradiol and testosterone are the main metabolites in early and late stage follicles, respectively.

3) to examine whether follicles synthesize an aromatase inhibitor.

4) to investigate changes in other metabolites and clarify their origin.

To accomplish these objectives, follicles of different stages were incubated with ^{3}H-pregnenolone and tritiated metabolites were separated by thin layer chromatography. Some stage 3 follicles were incubated with or without 4-hydroxy-androstenedione (4-OH-A), a competitive aromatase inhibitor.

The results indicate that ovarian steroid metabolism changes during follicular development. However, this study did not reveal the mechanism which causes the change in metabolism.

MATERIALS AND METHODS

Animals. Freshly-caught female adult frogs, Rana pipiens, were obtained from Kons Scientific Co., Inc. (Germantown, WI) in June and September of 1992 and June of 1993. Frogs were maintained at room temperature in large tanks with a small amount of water for 3 to 45 days. They were fed live crickets ad lib. twice a week until they were sacrificed.

Chemicals. Tritium-labeled pregnenolone (7-^{3}H, specific activity: 23.5 Ci/mmol), ^{3}H-labeled estradiol (2,4,6,7-^{3}H, specific activity: 87.5 Ci/mmol), and ^{14}C-labeled testosterone (4-^{14}C, specific activity: 57.3 mCi/mmol) were obtained from Du Pont Research Products (Boston, MA). Unlabeled steroids were obtained from Sigma Chemical Company (St. Louis, MO).

Follicle preparation and Incubation. Frogs were anesthetized with MS-222 (3-aminobenzoic acid ethyl ester) or pithed, and the ovaries were removed from the body cavity. Individual follicles were dissected out in amphibian Ringer's (AR) (Lin and Schuetz, 1985) with watchmaker's forceps under a microscope and sorted into developmental stages (Smalley and Nace, 1983). The follicles used for incubation were stage 2-3 (a mixture of stages 2 and 3), stage 3, early stage 4 (4E), late stage 4 (4L), and stage 5. Stage 4L follicles have lighter vegetal hemispheres and darker animal hemispheres than stage 4E follicles. The difference between animal and vegetal hemispheres is

very clear. In preliminary studies, 20 follicles of stage 2-3 were put into each well of a 24-well tissue culture dish and incubated with ^{3}H-pregnenolone (100,000 cpm) in 1 ml of AR for 2, 4, 6, or 8 hours at 24-25°C. Maximum uptake of radioactivity by follicles (about 30%) was observed after 6 hours. Therefore, all subsequent incubations were carried out for 6 hours at 24-25°C. Final incubations were carried out with 20 follicles for stages 2-3, 3, 4E, and 4L and 10 follicles for stage 5. Incubations were carried out using follicles from at least 3 animals per stage except for stage 4L where 2 animals were used. At least 4 wells were used per animal. One well per dish was used as a control and incubated with AR and ^{3}H-pregnenolone but without follicles.

To examine the effects of an aromatase inhibitor, 20 stage 3 follicles were put into each of 4 wells and incubated with 4-hydroxy-androstenedione (4-OH-A, 10^{-7}M) in 1 ml of AR for 1 hour at 24-25°C. Then ^{3}H-pregnenolone (100,000 cpm) was added to the incubation medium and follicles were incubated for an additional 6 hours. Control wells were incubated under the same conditions but without prior incubation in the presence of 4-OH-A.

After incubation, the medium was removed by aspiration and the follicles were washed twice with 0.5 ml of AR each time. The first washing was added to the incubation medium. Then follicles were extracted by adding 1 ml of methanol and shaking for 15 minutes.

The methanol extract was separated from the follicles by aspiration. Unlabeled steroids, androstenedione, estradiol, progesterone, and testosterone, 50 μg each, were added to the extract as carriers to help in identification of radioactive metabolites. The extract was dried under an air stream at room temperature and stored at 4°C for later analysis. The recovery of radioactivity was about 93% of that from the control well.

Incubation medium was passed through a C-18 Sep Pak cartridge (Waters Associates; Milford, MA) to remove the steroid metabolites. The cartridge was washed with 4 ml of water. About 7% of the radioactivity appeared in this water phase. The metabolites retained on the cartridge were eluted with 7 ml of methanol. Unlabeled steroids, androstenedione, estradiol, progesterone, and testosterone, 50 μg each, were added to the methanol fraction. The methanol fraction was also dried under an air stream at room temperature and stored at 4°C.

Thin layer chromatography (TLC). Pregnenolone metabolites in the medium or in the follicle extract were dissolved in 100 μl of methanol and 40 to 80 μl of the solution were spotted onto a TLC plate (Whatman 20 cm silica gel aluminum backed plate with fluorescent indicator). Spots for two different samples and one spot for marker steroids were put on each plate. Unlabeled steroids, androstenedione, estradiol, estrone, pregnenolone, progesterone,

testosterone, and dihydrotestosterone, 20 μg each, were used as marker steroids. The TLC plate was developed until the solvent had run 15 cm.

The separation of marker steroids was examined using 3 solvent systems.

Solvent system 1: chloroform:ethylacetate:water=80:20:1

Solvent system 2: benzene:ethylacetate=5:1

Solvent system 3: benzene:acetone=5:1

The Rf values of marker steroids are shown in Table 1. Solvent system 1 gave the best overall separations, but it did not separate progesterone and estrone. Solvent system 2 could separate progesterone and estrone, but it could not separate estradiol from progesterone or androstenedione from dihydrotestosterone. Solvent system 3 could not separate pregnenolone from dihydrotestosterone. Therefore, TLC was carried out using solvent system 1 first, then solvent system 3. The actual numbers of samples chromatographed in solvent system 1 were: 4 for stage 2-3 medium, 2 each for stage 4L medium and follicles, and 3 for all other stages. One sample per stage was chromatographed in solvent system 3.

The plate was dried overnight and the sample lanes were cut into 5 mm strips. The silica gel was scraped off each strip and put into a scintillation vial with 5 ml of scintillation fluid. Vials were counted using an LKB Rack

Table 1. Rf values of standard steroids chromatographed in 3 solvent systems.

Steroids	S1[a]	S2[b]	S3[c]
Testosterone	0.43	0.16	0.49
Estradiol	0.52	0.35	0.60
Dihydrotestosterone	0.60	0.32	0.65
Pregnenolone	0.66	0.35	0.65
Androstenedione	0.72	0.32	0.73
Progesterone	0.80	0.43	0.79
Estrone	0.78	0.65	0.83

[a] solvent system 1: chloroform:ethylacetate:water=80:20:1
[b] solvent system 2: benzene:ethylacetate=5:1
[c] solvent system 3: benzene:acetone=5:1

Beta Liquid Scintillation Counter. Recovery of radioactivity from the TLC plates was 91%.

Identification of standard steroids in the extract was performed by visualizing the spots with UV light before cutting the plate. Identification of standards in the separate lane was performed, after cutting the plate, by spraying with H_2SO_4:CH_3OH (7:3) and heating at 100°C for 15 minutes.

Recrystallization. After estradiol and testosterone were identified as the main metabolites, the positive identification of these steroids was carried out by recrystallization to a constant $^3H/^{14}C$ ratio as described below.

For testosterone recrystallization, the medium extract from stage 4L follicles was dissolved in 100 µl of methanol and 40 µl of solution were spotted on a TLC plate. ^{14}C-testosterone (10,000 dpm) was then added to the same spot. The TLC plate was developed in solvent system 1. The plate was dried overnight and then scraped in the area where testosterone migrated. The scraped silica gel was mixed with 1 ml of methanol in a test tube to extract the steroids. Then the test tube was centrifuged at 10,000 rpm for 10 minutes to eliminate most of the silica gel. The supernatant was decanted into a sintered glass funnel and vacuum filtered. The extraction was repeated two times, and a total of 3 ml of methanol was filtered. The funnel and the flask

were washed 3 times with 3 aliquots of 0.5 ml methanol. All methanol extracts and washings, a total of 6 ml, were put into a test tube and evaporated under an air stream at room temperature. The residue was dissolved in 1 ml of methanol and 100 μl of this solution were used for counting radioactivity.

Twenty mg of non-radioactive testosterone were added to the remaining 900 μl as a carrier and the volume was reduced by evaporation to less than 0.5 ml. Water was then added slowly to the sample until a cloud appeared. The average volume of water added was about 500 μl. The test tube was stored at 4°C for several hours to ensure complete crystallization. The test tube was centrifuged for 10 minutes at 5000 rpm, and the supernatant, mother liquor, was decanted into a second test tube. The remaining crystals were washed twice with 2 aliquots of 1 ml methanol-water (7:3). The crystals were then dried and dissolved in 1 ml of methanol. One hundred μl of this solution were used for counting radioactivity. The washings from the crystals were added to the mother liquor. The volume of mother liquor was reduced to 2 ml by evaporation, and 200 μl of mother liquor were used for counting radioactivity. This procedure was repeated 3 times. The $^{3}H/^{14}C$ ratios of the crystals and mother liquor for each crystallization were then calculated. The ratios from the extract and first crystallization differed by only 5.5% and no change was observed in

subsequent recrystallization.

To identify ^{3}H-estradiol, ^{14}C-estradiol was first prepared by incubating 20 stage 3 follicles with ^{14}C-testosterone (80,000 cpm) in 1 ml of AR for 6 hours at 24-25°C. The incubation medium was extracted using a Sep-pak as described above. About 4% of the ^{14}C-testosterone was converted to ^{14}C-estradiol. The medium extract was dissolved in 100 μl of methanol and 80 μl of this solution were spotted on a TLC plate. To get enough radioactivity (about 10,000 dpm) the extract from 4 different wells were spotted on the same plate at 0.5 cm intervals. Tritiated-estradiol (10,000 dpm) was then added to the same spots. The TLC plate was developed in solvent system 1, dried over night, and then scraped in the region of estradiol migration. The silica gel was removed from the steroids as described above. Identification of ^{14}C-estradiol was carried out by using the same recrystallization procedure as described for testosterone.

Identification of ^{3}H-estradiol derived from ^{3}H-pregnenolone was then carried out using the ^{14}C-labeled estradiol prepared above. The extract from 20 stage 4E follicles was dissolved in 100 μl of methanol, and 60 μl of this solution were spotted on a TLC plate. ^{14}C-estradiol (about 10,000 dpm) was added to the same spot. Identification of ^{3}H-estradiol was then carried out by following the same procedure used for ^{14}C-estradiol

recrystallization. The difference between $^{3}H/^{14}C$ ratios from the extract and first crystallization was 5.8% and did not change during subsequent recrystallization.

Data treatment. Results are based on total dpm recovered. This was greater than 30,000 dpm per incubation and greater than 13,000 dpm per TLC plate. Total dpm recovered varied between animals, follicles, medium, and stages. Therefore, all data are expressed as a percentage of the total dpm recovered on each plate.

Statistical analysis of data was carried out, after arcsine transformation, using a t-test or one way analysis of variance (ANOVA) followed by Duncan's multiple range test. Differences were considered significant at $p<0.05$.

RESULTS

Total steroidogenesis

When ^{3}H-pregnenolone was added to follicles of different stages, the percentage of total radioactivity remaining as unchanged pregnenolone decreased in later stages (Fig. 1).

Since pregnenolone is the precursor of all other steroid hormones, this decrease is evidence that total steroid metabolism by follicles increased in later stages.

The total uptake of radioactivity by follicles also increased in later stages (stage 2-3: 2140±229 dpm /follicle (Mean±SEM, n=6); stage 5: 5181±1285 dpm /follicle (n=3)). This is additional evidence which indicates that there was an increase in total steroidogenesis in later stages.

Major metabolites in the medium and follicles

The major metabolites identified in the medium and in the follicles were estradiol, estrone, testosterone, androstenedione, and progesterone. The percentage of major metabolites varied at different stages.

The main metabolite in the medium was estradiol in stage 2-3, 3 and 4E. The percentage of estradiol was greater than 20% of the total radioactivity in stages 2-4E, but decreased in stages 4L and 5. Testosterone became the main metabolite in stages 4L and 5 (Fig.2).

In the follicles, estradiol remained the main metabolite somewhat longer, through stage 4L, and

Fig.1 The percentage of ^{3}H-pregnenolone remaining in the medium and in the follicles after 6 hour incubation. ^{3}H-pregnenolone was separated using solvent system 1. Each bar represents Mean±SEM. N(number of animals)=2 for stage 2-3 medium, 4 for stage 4L medium and follicles, and 3 for all other stages. Means with different letter are significantly different by at least $p<0.05$ based on Duncan's multiple range test. Capital letters (medium) and small letters (follicles) cannot be compared.

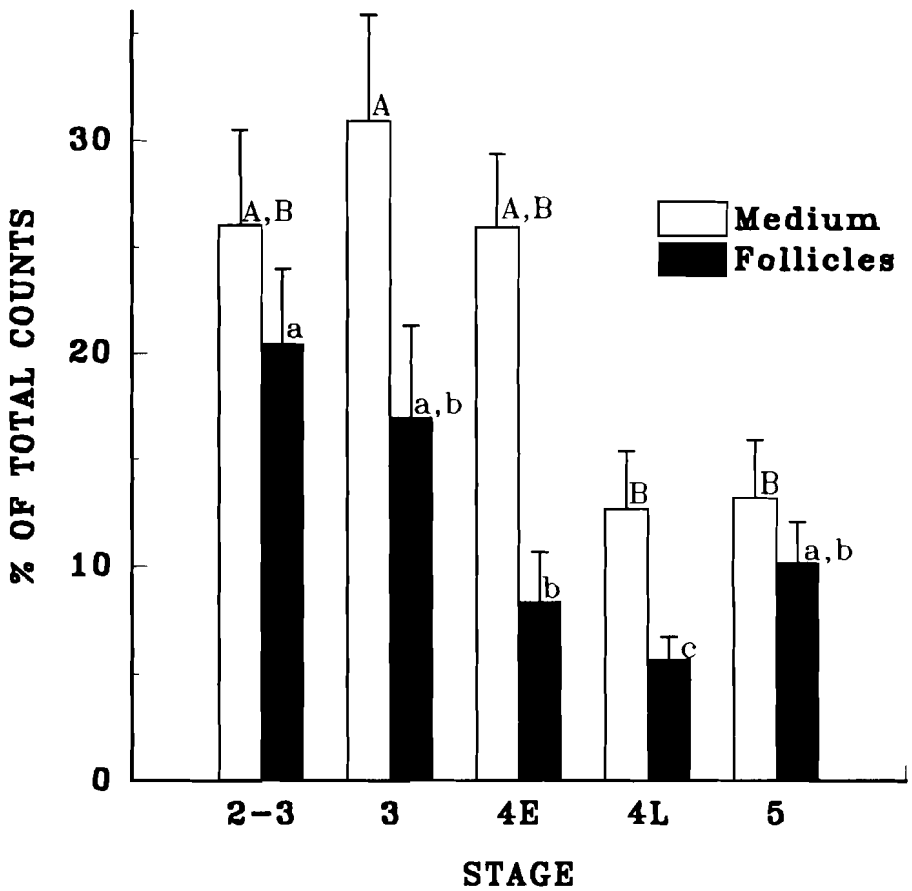
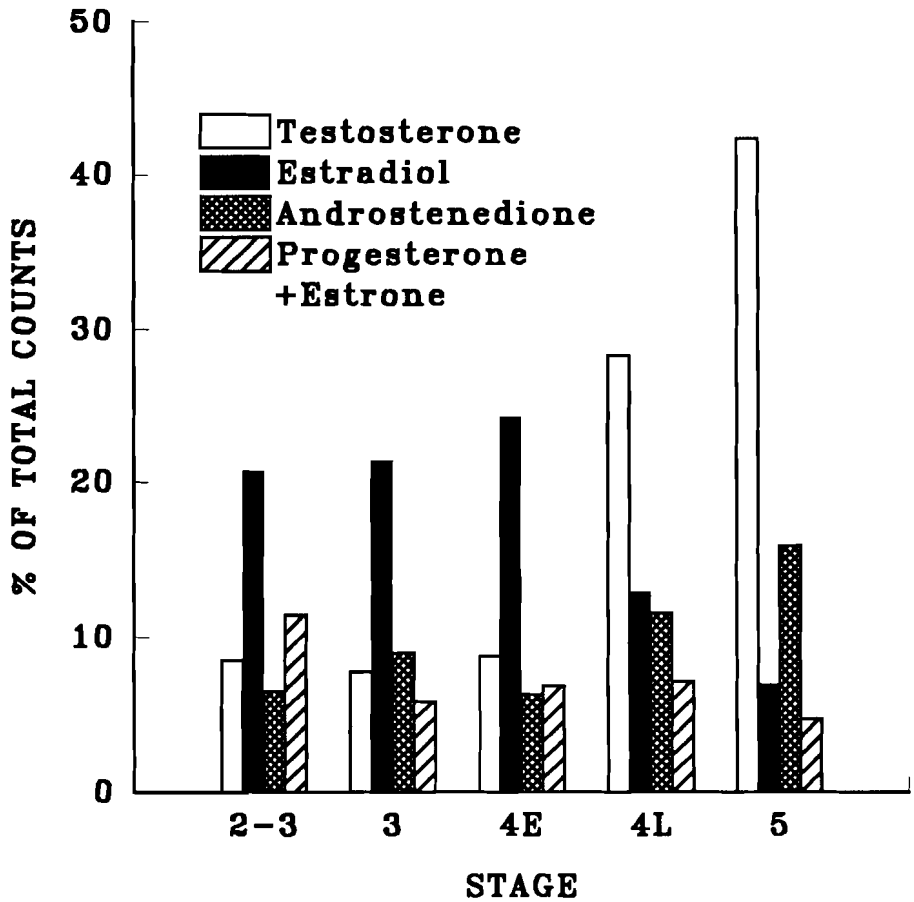

% OF TOTAL COUNTS
30
20
10
0
A,B
a
A
a,b
A,B
b
B
c
B
a,b
Medium
Follicles
2-3
3
4E
4L
5
STAGE

Fig.2 Tritiated-pregnenolone metabolites in the medium after 6 hour incubation. Each bar represents the mean of percentages. N: same as Fig.1.


50
40
30
20
10
0
% OF TOTAL COUNTS
Testosterone
Estradiol
Androstenedione
Progesterone
+Estrone
2-3
3
4E
4L
5
STAGE

testosterone did not become the main metabolite until stage 5 (Fig.3). Stage 4L seemed to be a transitional stage with the percentage of estradiol falling and the percentage of testosterone rising.

Interestingly, estradiol always formed a higher percentage of total radioactivity in the follicles than in the medium (Fig.4). Estrone showed a similar pattern to that of estradiol with a higher percentage in the follicles (Fig.5). Since estradiol and estrone are both estrogens, these results suggest that estrogen receptors might be present and binding these hormones in the follicle.

The percentage of testosterone in the medium and in the follicles was very low in early stages and increased in later stages. Unlike estradiol, the percentages of testosterone in the medium and in the follicles were relatively close (Fig.6). The percentage of androstenedione in the medium and in the follicles also increased in later stages. However, the change was smaller than for testosterone. The percentage of androstenedione in the medium was higher than in the follicles (Fig.7).

The percentages of progesterone in the medium and in the follicles were low during early follicle development. It increased slightly in stage 5 in the follicles (Fig.8).

No peak for dihydrotestosterone was identified in any of samples.

Fig.3 Tritiated-pregnenolone metabolites in the follicles after 6 hour incubation. N: same as Fig.1.

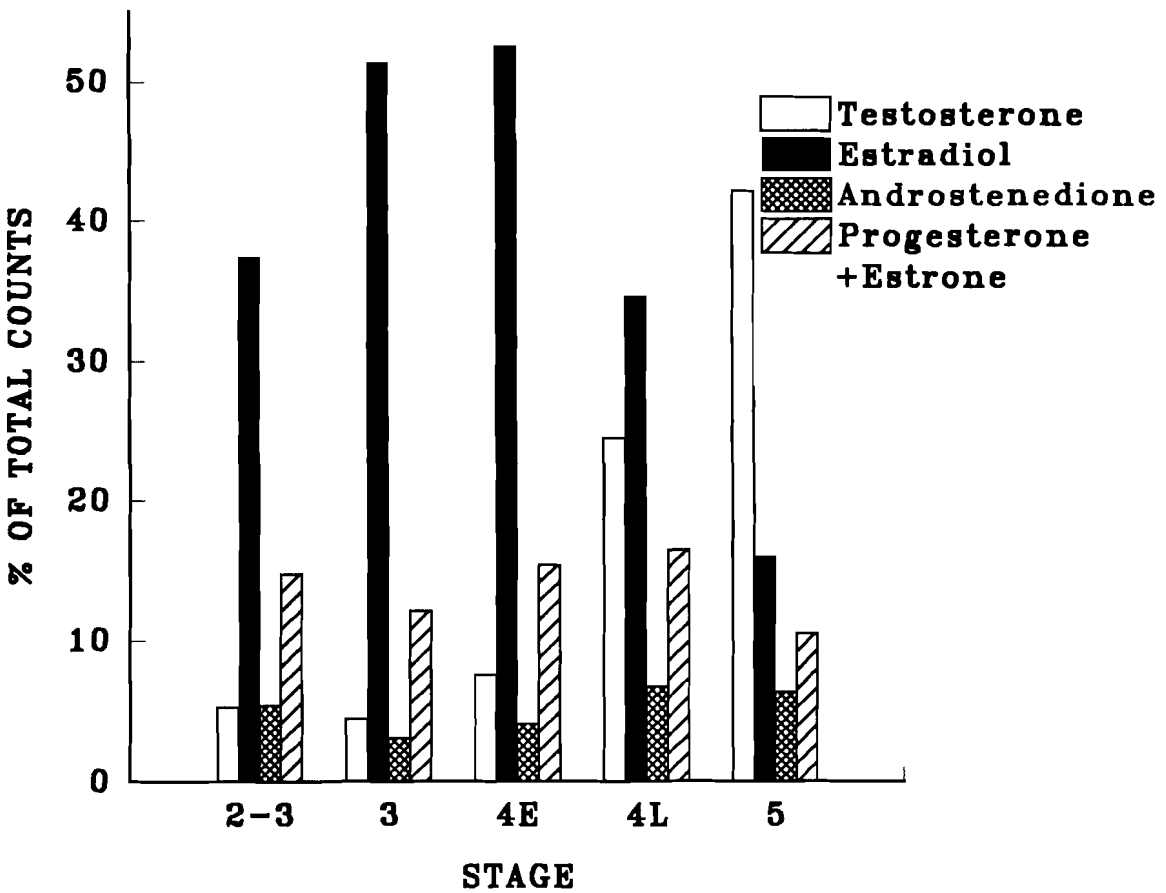

% OF TOTAL COUNTS
50
40
30
20
10
0
2-3
3
4E
4L
5
STAGE
Testosterone
Estradiol
Androstenedione
Progesterone
+Estrone

Fig.4 The percentage of ^{3}H-estradiol in the medium and in the follicles after 6 hour incubation with ^{3}H-pregnenolone. Other conventions are the same as Fig.1.

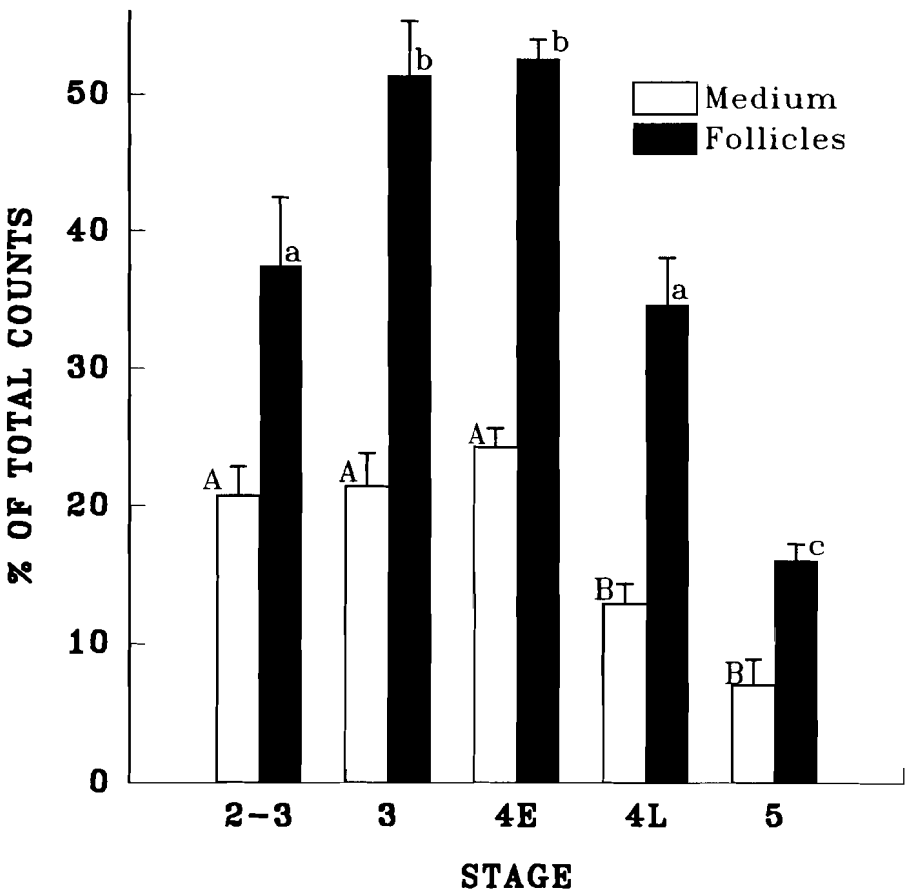

% OF TOTAL COUNTS
0
10
20
30
40
50
Medium
Follicles
A
a
A
b
A
b
B
a
B
c
2-3
3
4E
4L
5
STAGE

Fig.5 The percentage of ^{3}H-estrone in the medium and in the follicles after 6 hour incubation with ^{3}H-pregnenolone. Separation was performed using solvent system 3. N=1.

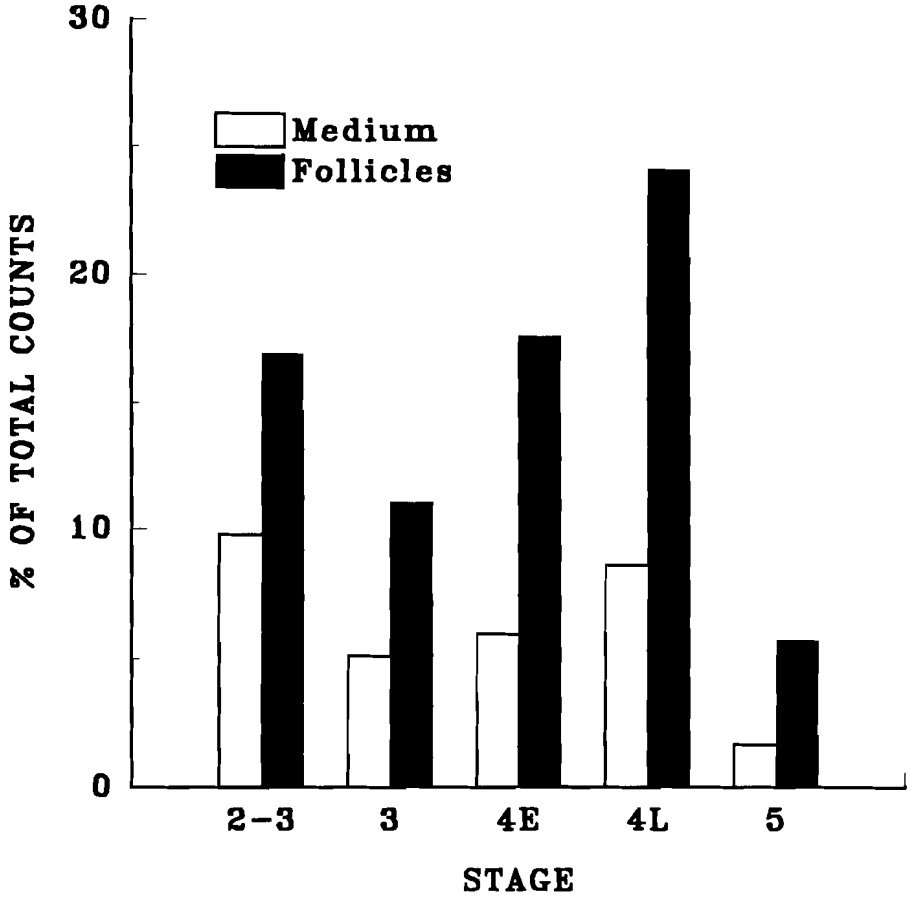

30
20
10
0
% OF TOTAL COUNTS
Medium
Follicles
2-3
3
4E
4L
5
STAGE

Fig.6 The percentage of ^{3}H-testosterone in the medium and in the follicles after 6 hour incubation with ^{3}H-pregnenolone. Other conventions are the same as Fig.1.

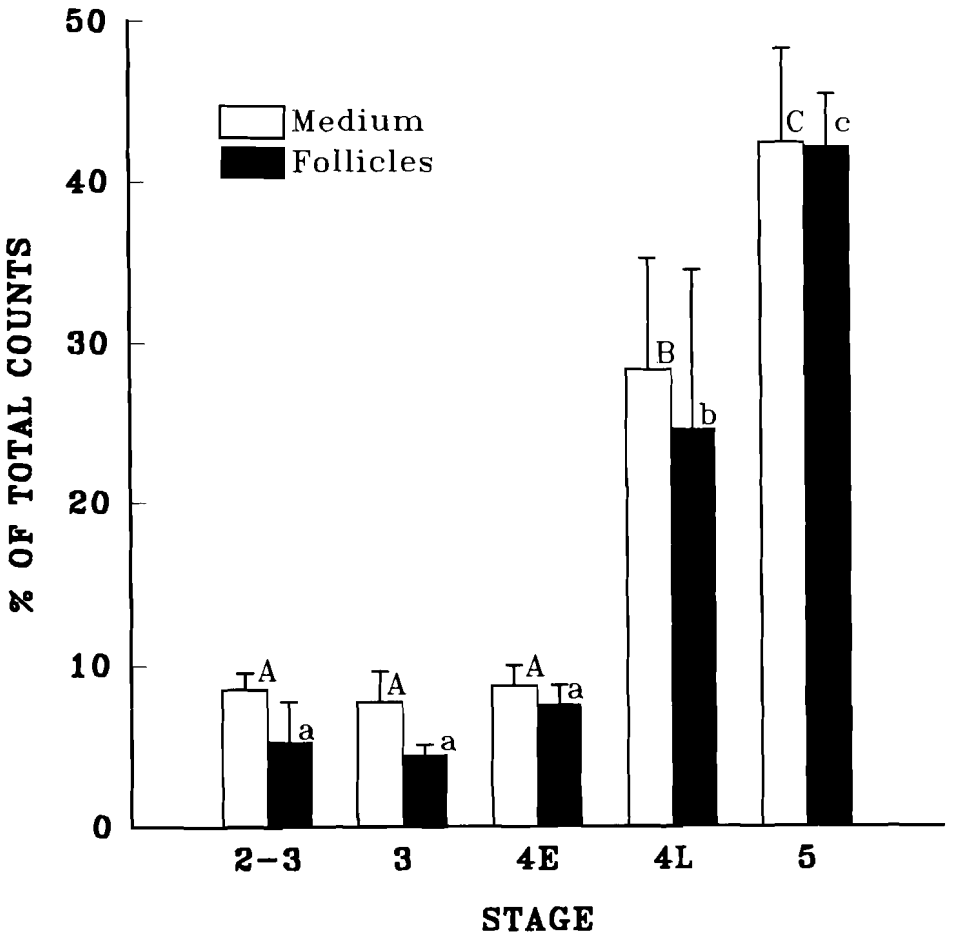

50
40
30
20
10
0
% OF TOTAL COUNTS
Medium
Follicles
A
a
A
a
A
a
B
b
C
c
2-3
3
4E
4L
5
STAGE

Fig.7 The percentage of ^{3}H-androstenedione in the medium and in the follicles after 6 hour incubation with ^{3}H-pregnenolone. Other conventions are the same as Fig.1.

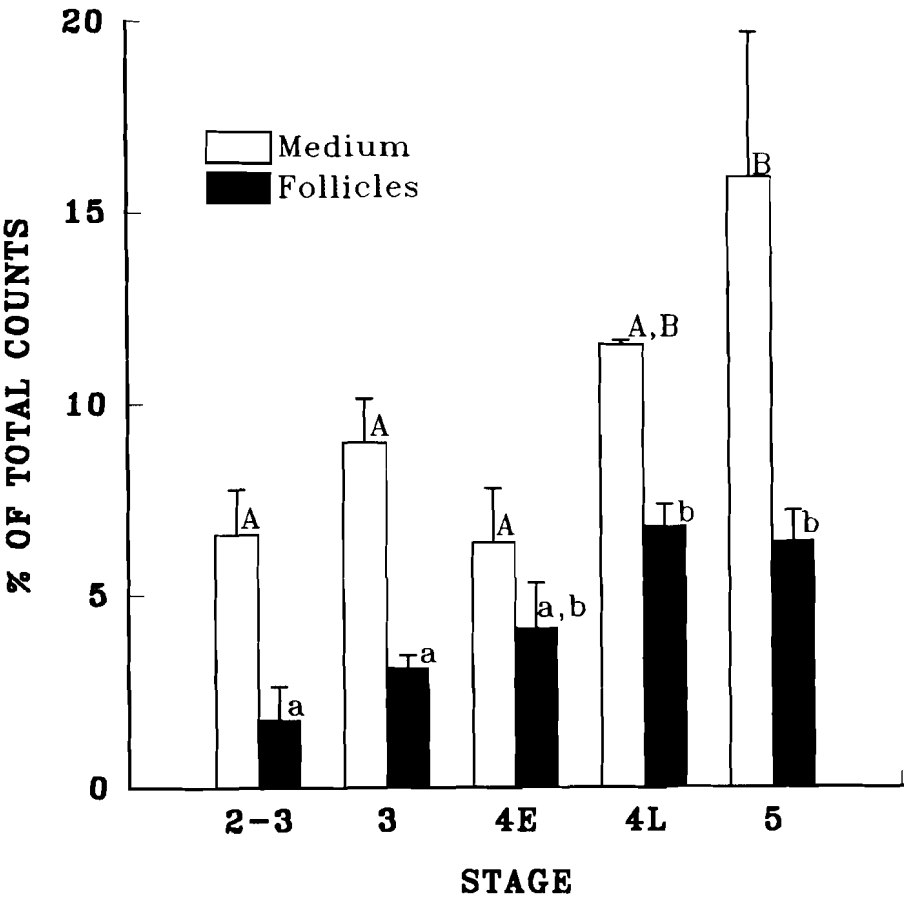
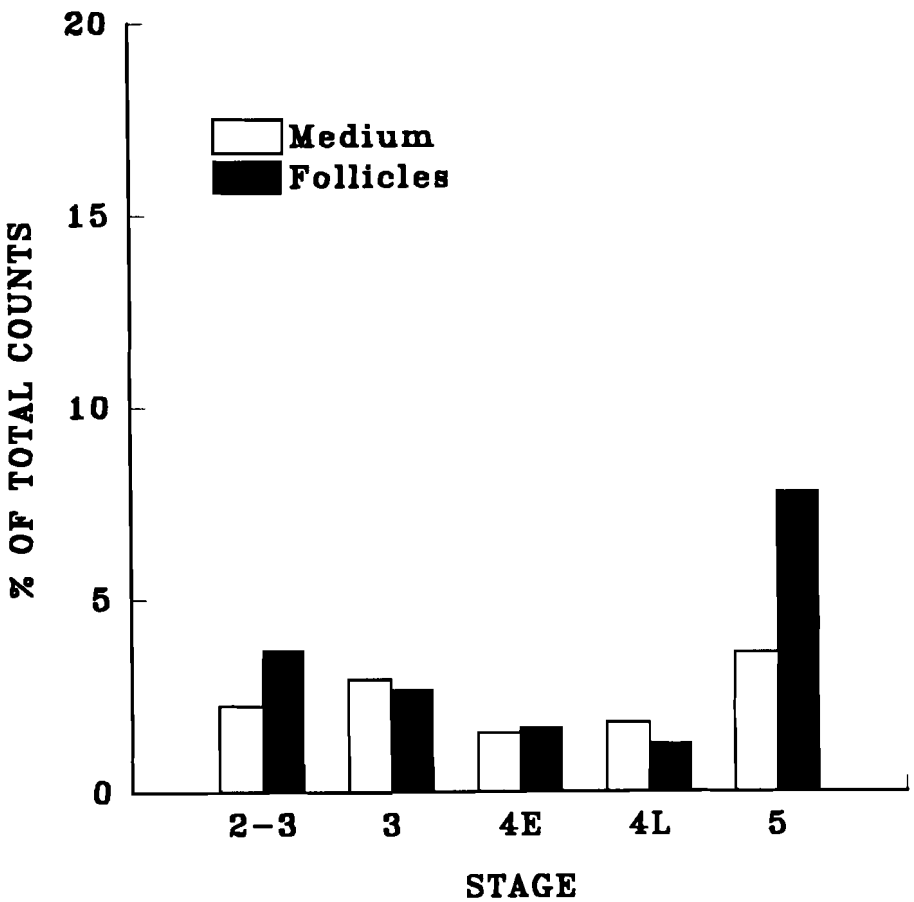

20
15
10
5
0
% OF TOTAL COUNTS
Medium
Follicles
A
a
A
a
A
a,b
A,B
b
B
b
2-3
3
4E
4L
5
STAGE

Fig.8 The percentage of ^{3}H-progesterone in the medium and in the follicles after 6 hour incubation with ^{3}H-pregnenolone. Separation was performed using solvent system 3. N=1.


20
15
10
5
0
% OF TOTAL COUNTS
Medium
Follicles
2-3
3
4E
4L
5
STAGE

Other metabolites

An attempt was made to identify several additional steroids. The Rf values of these steroids are shown in Table 2. Dehydroepiandrosterone could not be detected in any sample. Five-androstene-3,17β-diol and 5α-androstane-3,17β-diol could not be separated from testosterone in any solvent systems, and 17α-hydroxyprogesterone could not separated from estradiol in solvent system 1' and 3. Therefore, it was not possible to identify these metabolites. However, recrystallization of estradiol and testosterone showed that other steroids could not account for more than 6% of the radioactivity associated with these compounds on the TLC plates.

Polar metabolites

In addition to the major metabolites, several metabolites with low Rf values were detected in the medium and in the follicles (Fig.12). The percentages of these metabolites were higher in the medium than in the follicles, suggesting that they were hydrophilic polar compounds. The percentages of these polar metabolites decreased in stage 5 (Fig.9).

These metabolites were separated into 4 groups (PM1, PM2, PM3, and PM4), according to their Rf values (0.1, 0.2, 0.3, 0.4, respectively). The percentages of these polar metabolites varied among the stages (Fig.10, 11). The percentages also varied between animals at the same stage.

Table 2. Rf values of steroids chromatographed in 4 solvent systems

Steroids	S1	S1'[a]	S2	S3
5-androstene-3,17β-diol	0.42		0.18	0.46
5α-androstane-3,17β-diol	0.42		0.18	0.43
4-hydroxy-androstenedione	0.79		0.50	0.85
11-keto-testosterone	0.11		0.04	0.26
dehydroepiandrosterone	0.60			
17α-hydroxyprogesterone		0.57		0.62
4-pregnen-20α-ol-3-one		0.59		0.55
5α-pregnan-20α-ol-3-one		0.76		0.73

[a] solvent system 1': chloroform:ethylacetate=4:1,
Rf: estradiol=0.61, pregnenolone=0.68,
androstenedione=0.74.

S1, S2, S3: same as Table 1.

Fig 9 The percentage of total tritiated polar metabolites in the medium and in the follicles after 6 hour incubation with ^{3}H-pregnenolone. Other conventions are the same as Fig.1.

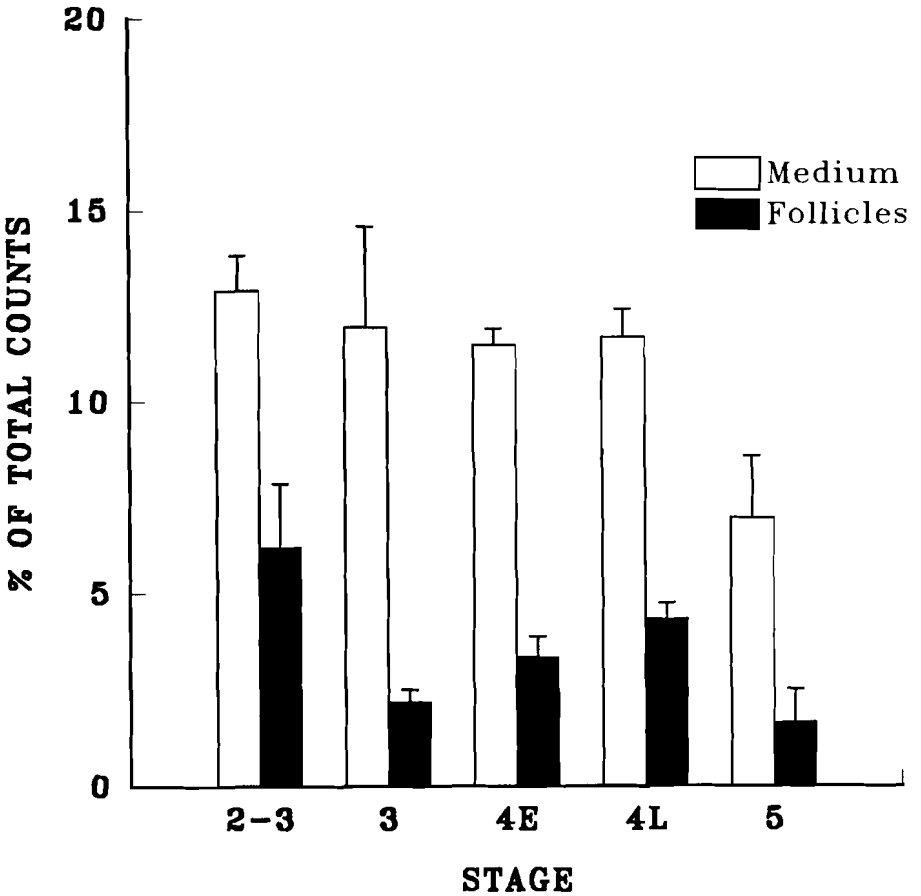

20
15
10
5
0
% OF TOTAL COUNTS
Medium
Follicles
2-3
3
4E
4L
5
STAGE

Fig.10 The percentages of 4 different tritiated polar metabolites in the medium after 6 hour incubation with ^{3}H-pregnenolone. PM=polar metabolite, PM1:Rf=0.1, PM2:Rf=0.2, PM3:Rf=0.3, PM4:Rf=0.4. PM2 and PM3 were not separated in stage 3. Other conventions are the same as Fig.1.

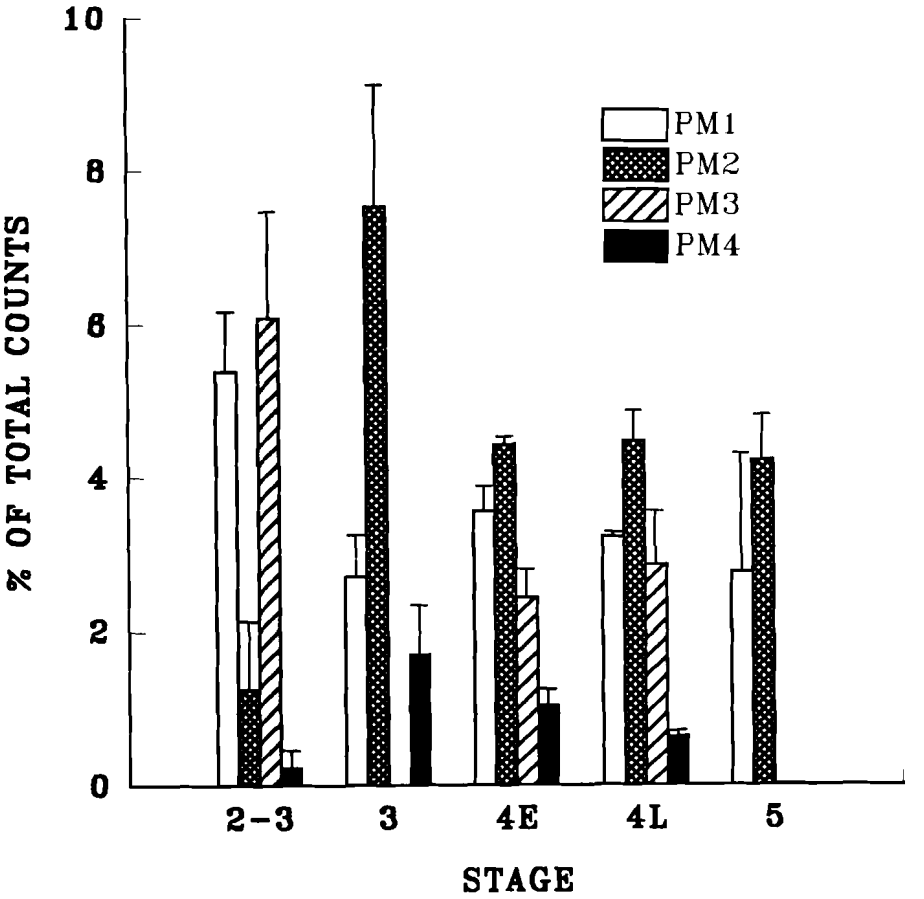

10
8
6
4
2
0
% OF TOTAL COUNTS
PM1
PM2
PM3
PM4
2-3
3
4E
4L
5
STAGE

Fig.11 The percentages of 4 different tritiated polar metabolites in the follicles after 6 hour incubation with ^{3}H-pregnenolone. Other conventions are the same as Fig.1.

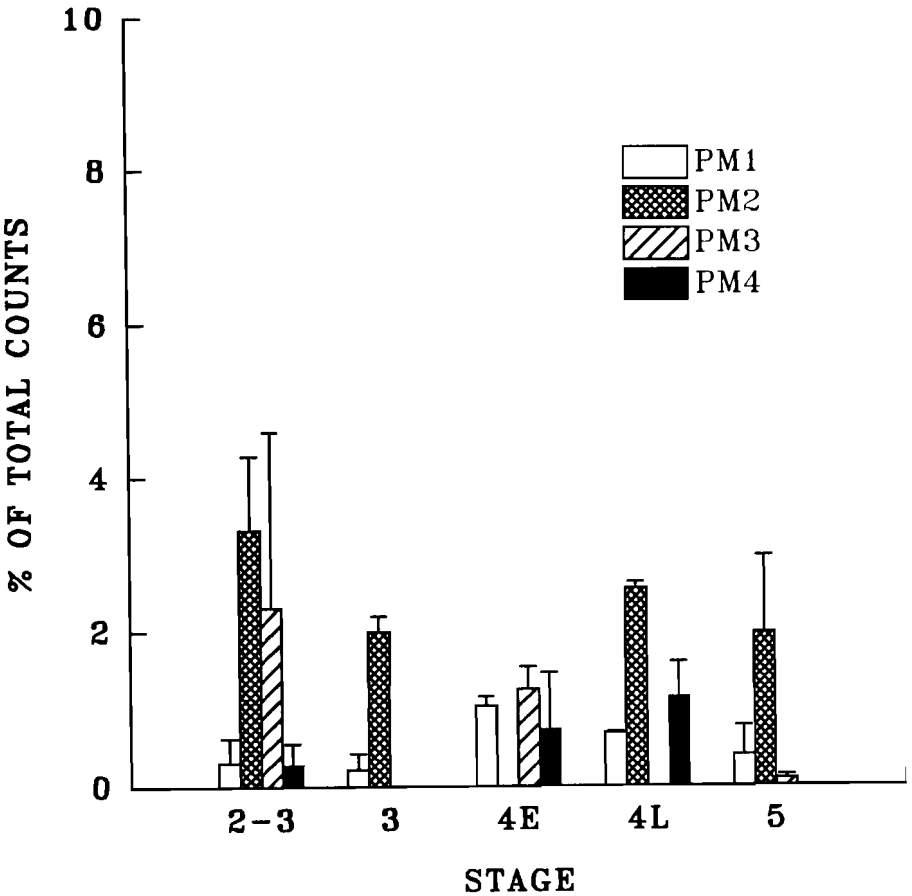
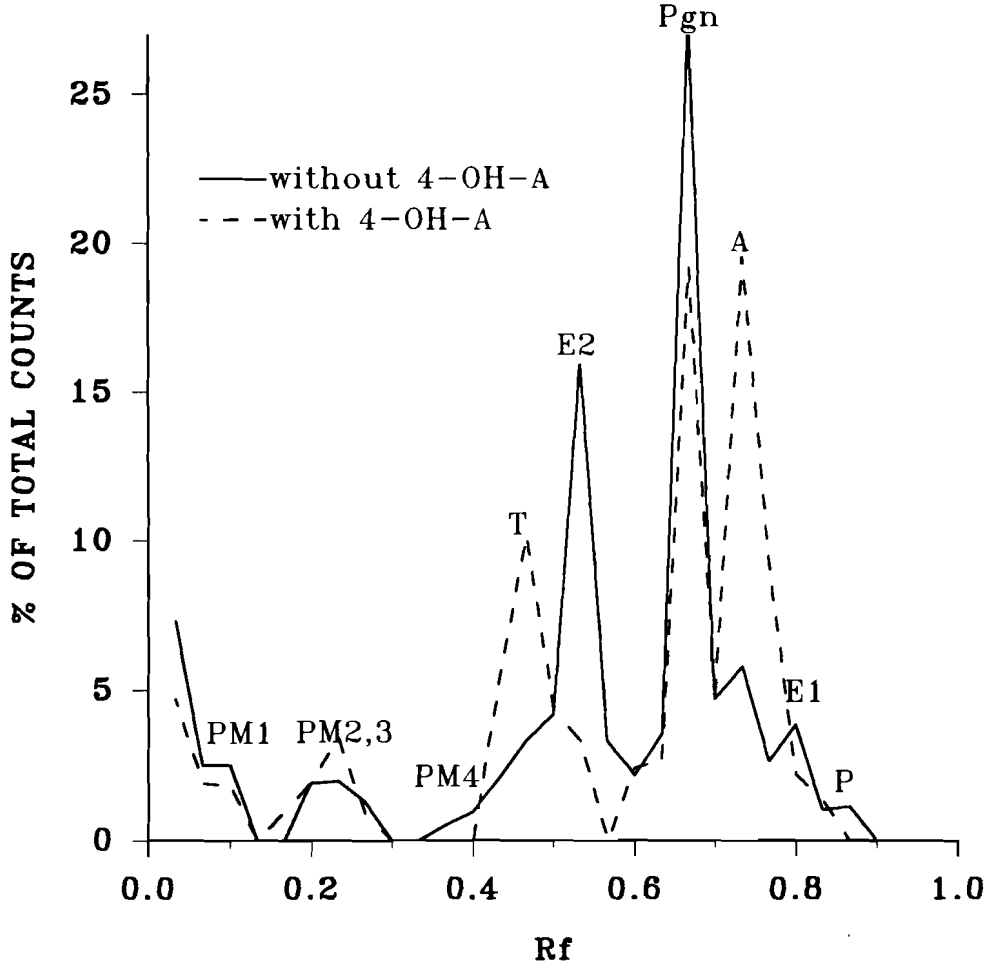

10
8
6
4
2
0
% OF TOTAL COUNTS
PM1
PM2
PM3
PM4
2-3
3
4E
4L
5
STAGE

Fig.12 The TLC profile of ^{3}H-pregnenolone metabolites in the medium from follicles preincubated with or without 4-hydroxy-androstenedione (4-OH-A). Abbreviations above peaks represent steroids (T=testosterone, E2=estradiol, Pgn=pregnenolone, A=androstenedione, E1=estrone, P=progesterone, PM1, PM2, PM3, PM4= polar metabolites)


Pgn
25
20
15
10
5
0
——without 4-OH-A
- - -with 4-OH-A
A
E2
T
E1
P
PM1
PM2,3
PM4
% OF TOTAL COUNTS
0.0
0.2
0.4
0.6
0.8
1.0
Rf

Since the percentage of polar metabolites decreased in stage 5, it was thought that they might be metabolites of estradiol. It was also hypothesized that they might be metabolites of testosterone. In order to test these hypotheses, stage 3 follicles were incubated with 4-OH-A. The data are shown in Table 3 and Fig.12. The aromatase inhibitor caused a significant decrease in the percentage of estradiol and a significant increase in the percentage of testosterone and androstenedione. The percentage of pregnenolone, which is the index of total steroidogenesis, showed a significant decrease. However, the percentages of polar metabolites were the same in the presence or absence of 4-OH-A (Fig.12). No significant changes were observed (Table 3). These results suggest that the polar metabolites were not formed from either estradiol or testosterone. These results also suggest that PM1 is not identical to 11-keto-testosterone, although the Rf value of PM1 is similar to that of 11-keto-testosterone.

Aromatase inhibitor

Since some androgens are known to be aromatase inhibitors, it was thought that follicles might synthesize a steroidal aromatase inhibitor. If such an aromatase inhibitor were present, it should appear in stage 4, when estradiol synthesis begins to fall, but not be present in earlier stages. However, a particular metabolite which appeared only in late stages could not be identified.

Table 3. The effect of an aromatase inhibitor on the percentages of pregnenolone and various metabolites in medium. Stage 3 follicles were incubated with ^{3}H-pregnenolone with or without preincubation with 4-hydroxy-androstenedione (4-OH-A).

	Percentage (Mean±SEM)		
Steroids	without 4-OH-A	with 4-OH-A	T-test
Pregnenolone	30.88±4.99	26.51±4.74	P<0.05
Estradiol	21.41±2.48	4.78±0.68	P<0.05
Testosterone	7.74±1.90	17.13±1.16	P<0.01
Androstenedione	8.99±1.15	29.14±5.07	P<0.05
Polar metabolites	11.94±2.64	8.77±0.16	N.S.
PM1(Rf=0.1)	2.70±0.54	2.05±0.12	N.S.
PM2(Rf=0.2) and PM3(Rf=0.3)	7.52±1.60	6.17±0.59	N.S.
PM4(Rf=0.4)	1.72±0.62	0.55±0.32	N.S.

DISCUSSION

The present study demonstrates that steroidogenesis by follicles of Rana pipiens changes during follicular development. Estradiol, estrone, progesterone, testosterone, androstenedione, and several polar metabolites were identified in the incubation medium and in follicles incubated with tritiated pregnenolone. The presence of these compounds indicates that pregnenolone was metabolized by Rana follicles through the $\Delta 4$ pathway as previously reported by Ozon (1972a, b). The absence of dehydroepiandrosterone and possibly 5-androstene-3,17β-diol suggests that the $\Delta 5$ pathway is not important in these follicles.

Change in total steroidogenesis.

The percentage of unmetabolized pregnenolone in the medium and in the follicles decreased in later stages, while total counts in the follicles increased. These results suggest that total steroidogenesis increased as follicles matured. Such an increase is also supported by earlier studies. Smalley (1989), Fortune (1983), and Kwon et al.(1991) all showed that the amount of testosterone secreted by later stage follicles was much larger than the amount of estradiol secreted by early stage follicles. Since the present study shows that testosterone and estradiol are the principal steroids secreted in these stages, these results again suggest that total steroidogenesis increases at later stages.

Main metabolites

The main metabolite formed by early stage follicles was estradiol (Fig.2, 3). The percentage of estrone was also high in early stages, similar to that of estradiol (Fig.5). Estrogens stimulate the synthesis of vitellogenin in the liver. Vitellogenin is then transported to the ovary, where it is converted to yolk protein (Wallace and Bergink, 1974; Ho, 1987). Therefore, it is significant that the highest percentage of conversion from pregnenolone to estradiol occurred in early stage follicles. By stimulating vitellogenin production, these follicles are responsible for their own growth.

In every developmental stage, the percentages of both estradiol and estrone were higher in the follicles than in the medium. Similar observations for estradiol have been reported in Xenopus laevis (Fortune, 1983) and Rana nigromaculata (Kwon et al., 1991). Since such differences were not observed with testosterone or androstenedione, it is suggested that receptors for estradiol might be present in the follicles. In other words, the ovary may be not only an organ which secretes estrogens, but also a target organ for them. Estrogen receptors have been identified in several mammalian tissues including the ovary (Kudolo et al., 1987; Billiar et al., 1992). In amphibia, they have been identified in the liver (Westley and Knowland, 1978; Paolucci and Botte, 1988) and testis (Mak et al., 1983), but

not yet in the ovary. Nevertheless they do affect the ovary. Estrogens inhibit oocyte maturation in Rana pipiens by blocking progesterone synthesis or raising the intrafollicular threshold for progesterone to induce maturation (Spiegel et al., 1978; Lin and Schuetz, 1983). This latter observation also suggests that a decline in estradiol synthesis might be important for oocyte maturation.

Testosterone was the main metabolite in later stage follicles (stages 4 and 5, Fig.2, 3). The percentage of androstenedione also increased in later stages (Fig.7). The function of these androgens is not clear. Le Goascogne et al. (1985) reported that testosterone induced maturation in Xenopus oocytes. In rat ovarian follicles, testosterone has been reported to stimulate progesterone synthesis (Hillier et al., 1977). Therefore, one possibility is that testosterone may facilitate oocyte maturation. Another possibility is that testosterone may be secreted as a precursor of estradiol. The presence of an aromatase has been reported in the oviduct (Dubowsky and Smalley, 1993) and brain (Callard et al., 1978) of amphibia. This enzyme could convert testosterone to estradiol in peripheral tissues and allow it to have estrogen effects.

Progesterone is widely accepted as the maturation inducing substance in amphibian oocytes, and previous studies indicated that progesterone synthesis increases

before the onset of final oocyte maturation (reviewed by Nagahama, 1987). However, the percentage of progesterone showed only a slight increase in stage 5 follicles in the present study (Fig.8). This may be because the stage 5 follicles incubated in this study were obtained in October, and so may not have been ready for final oocyte maturation. Another, more likely, explanation of low progesterone formation is that the follicles were incubated without any added gonadotropins, and gonadotropins greatly stimulate the synthesis of progesterone (Fortune, 1983; Kwon et al., 1991).

The only increase in progesterone observed in stage 5 incubations occurred in the follicles and not in the medium. This is consistent with the result of Petrino and Schuetz (1986) who reported that most of the progesterone formed from exogenous pregnenolone was retained in the follicle. This provides an interesting parallel with the follicular retention of estradiol discussed above, because progesterone is known to act through a progesterone receptor in the oocyte.

Aromatase inhibitor

The change of the main metabolite from estradiol to testosterone occurred during stage 4. This is consistent with earlier reports of a change from estradiol secretion by early stage follicles to testosterone secretion by late stage follicles (Smalley, 1989; Fortune, 1983; Kwon et al.,

1991). Since estradiol is synthesized from testosterone by aromatase, it is possible that the change from estradiol to testosterone secretion could be due to a decrease in aromatase activity. Such a decline in aromatase activity is associated with oocyte maturation in several species of fish (Young et al., 1983; Kagawa et al., 1984; Sakai et al., 1988). Recently, a loss of aromatase activity in amphibian follicles was reported by Kwon et al.(1993). They showed that late stage follicles collected from Rana nigromaculata in fall and early winter (late stage) were unable to convert testosterone to estradiol.

In addition to the decrease in estradiol synthesis, a decrease in estrone and an increase in androstenedione was observed in later stages. Because estrone is synthesized from androstenedione by aromatase, this is additional evidence for a decrease in aromatase activity.

The mechanism responsible for the decrease in aromatase activity is unknown. One hypothesis is that an aromatase inhibitor is synthesized during stage 4. This seemed likely because androgen synthesis increased at this time and some androgens are known to be aromatase inhibitors (Brodie et al., 1977; Brueggmeier et al., 1982). For example, naturally occurring non-aromatizable androgens, such as 5α-dihydrotestosterone and 5α-androstanedione can be aromatase inhibitors in rat granulosa cells (Hillier et al., 1980) and porcine granulosa cells (Chan and Tan, 1986). Unfortunately,

no particular metabolite could be identified as a possible aromatase inhibitor.

Failure to find an aromatase inhibitor can be interpreted in two ways. One interpretation is that an aromatase inhibitor was actually synthesized but could not be separated by TLC because it co-migrated with some other major metabolite. Some androgens, such as 5-androstenediol and 5α-androstanediol, co-migrated with testosterone in solvent system 1. However, the result from recrystallization suggests that no particular compound co-migrated with testosterone.

The results of incubating stage 3 follicles with a known aromatase inhibitor, 4-OH-A, argue against the possibility of a natural aromatase inhibitor. The change in metabolism caused by 4-OH-A was not similar to that of late stage follicles. 4-OH-A caused a significant increase in androstenedione in addition to a smaller increase in testosterone (Fig.12). Normal stage 4 follicles produce much more testosterone than androstenedione (Fig.2). Schroeder et al., (1988) reported that androstenedione-type aromatase inhibitors activate 17β-hydroxysteroid-dehydrogenase. The high conversion to androstenedione in the presence of 4-OH-A is probably due to this enzyme activation. These results all indicate that the synthesis of an androgen-type aromatase inhibitor in Rana follicles is questionable.

Another interpretation is that an aromatase inhibitor

is not synthesized in the follicles, but that other hormones can modify the enzyme activity. In mammals, it is widely established that two gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH) play an important role in ovarian steroidogenesis. The secretion of FSH and LH is regulated by gonadal negative feedback. Several studies indicate that gonadal feedback on gonadotropin secretion also occurs in amphibia. Estradiol suppressed the increase in plasma gonadotropin levels in gonadectomized male and female bullfrogs, Rana catesbeiana (McCreery and Licht, 1984), and male Rana pipiens (Pavgi and Licht, 1989) in vivo. Estradiol also inhibited pituitary FSH and LH secretion in Rana pipiens in vitro (Pavgi and Licht, 1993). Therefore, an increase in estradiol secretion may cause inhibition of gonadotropin secretion which, in turn, might modify ovarian steroidogenesis. However, the plasma levels of FSH and estradiol in Rana catesbeiana did not show this relationship clearly (Licht et al., 1983). Moreover, feedback inhibition cannot explain the increase in total steroidogenesis observed in later stage follicles. Therefore, it is difficult to explain the change in ovarian steroidogenesis by a simple feedback mechanism, and more studies are needed to determine the exact mechanism responsible for this change.

Polar metabolites

Several metabolites with low Rf values were observed

(Fig. 12). The low Rf values suggest that these compounds were more polar than known steroids, but they could not be identified. The percentage of these polar metabolites decreased in stage 5. This suggests two hypotheses about their origin. One hypothesis is that they come from estradiol and therefore decrease in stage 5 when estradiol decreases. Another hypothesis is that they come from testosterone. In this case, the hypothesis is that early stage follicles actively metabolize testosterone to several metabolites, including estradiol. In later stages, the enzymes which metabolize testosterone become inactive, and as a result, secretion of large amounts of testosterone is observed, and there is a decrease in its metabolites.

Preincubation with the aromatase inhibitor, 4-OH-A, was carried out to examine these possibilities. However, the results from these incubations were not consistent with either of these possibilities. If these metabolites came from estradiol, 4-OH-A should cause a decrease in these metabolites. If they came from testosterone, an increase in these metabolites should be observed. However, the percentages of these metabolites was the same in the presence or absence of 4-OH-A. Therefore, it is suggested that the polar metabolites are not derived from either estradiol or testosterone.

Another possibility is that the polar metabolites are derived from progesterone and are C_{21} steroid compounds.

Some polar metabolites of progesterone have been identified in previous studies. 5α-Pregnane-3β,20α diol and possibly tri-hydroxy-pregnane were identified as Rana oocyte metabolites from progesterone by Reynhout and Smith (1973). 17α,20α-dihydroxy-progesterone and possibly 17α,20α-dihydroxy-5β/α-pregnane-3-one were identified as Xenopus follicle metabolites from progesterone by Thiber-Fouchet et al, (1976). In addition, several dihydroxy, trihydroxy, and tetrahydroxy C_{21} steroid metabolites were identified from follicles incubated with pregnenolone, progesterone or 17-hydroxyprogesterone in several species of fish (Suzuki et al., 1981; Petrino et al., 1993; Canario and Scott, 1989; Yeung and Chan, 1985; Schoonen et al., 1988; Kime, 1990; Venkatesh et al., 1992). The polar metabolites detected from Rana follicles in this study may be similar or identical to these compounds.

It is not clear that these metabolites have a physiological function. Some multi-hydroxylated steroids are effective in inducing oocyte maturation in certain species of fish. However, these metabolites decreased in stage 5 in the present study. Therefore, it seems likely they are not maturation inducing substances. Reynhout and Smith (1973) and Thibier-Fouchet et al.,(1976) reported that the metabolites formed by frog follicles were less effective than progesterone in inducing oocyte maturation.

One possibility is that the polar metabolites are

conjugated steroids, such as glucuronides or sulfates to be excreted into intercellular fluid. Such compounds have been reported in the fish ovary (Canario and Scott, 1989; Schoonen et al., 1988).

In conclusion, this study demonstrates that ovarian steroid metabolism in Rana pipiens changes during follicular development. Follicles secrete large amounts of estradiol or testosterone as their main metabolite in early or late stages, respectively. However, this study could not determine the mechanism which causes the metabolic change.

LITERATURE CITED

LITERATURE CITED

Billiar, R. B., Loukides, J. A., and Miller, M. M. (1992). Evidence for the presence of the estrogen receptor in the ovary of the baboon (Papio anubis). J. Clin. Endocrinol. Metab. 75, 1159-1165.

Brodie, A. M. H., Schwarzel, W.C., Shaikh, A. A., and Brodie, H. J. (1977). The effect of an aromatase inhibitor, 4-hydroxy-4-androstene-3,17-dione, on estrogen-dependent processes in reproduction and breast cancer. Endocrinol. 100, 1684-1695.

Brueggemeier, R. W., Snider, C. E., and Counsell, R. E. (1982). Substituted C_{19} steroid analogs as inhibitors of aromatase. Cancer Res. 42(Suppl), 3334s-3337s.

Callard, G. V., Petro, Z., and Ryan, K. J. (1978). Phylogenetic distribution of aromatase and other androgen-converting enzymes in the central nervous system. Endocrinol. 103, 2283-2290.

Canario, A. V. M. and Scott, A. P. (1989). Synthesis of 20α-hydroxylated steroids by ovaries of the dab (Limanda limanda). Gen. Comp. Endocrinol. 76, 147-158.

Chan, W. K., and Tan, C. H. (1986). FSH-induced aromatase activity in porcine granulosa cells: non-competitive inhibition by non-aromatizable androgens. J. Endocrinol. 108, 335-341.

Dubowsky, S., and Smalley, K. N. (1993). Testosterone

induced growth of the oviduct in the frog, Rana pipiens: evidence for local aromatization. Gen. Comp. Endocrinol. 86, 276-282.

Fortune, J. E. (1983). Steroid production by Xenopus ovarian follicles at different developmental stages. Dev. Biol. 99, 502-509.

Hillier, S. G., Knazek, R. A., and Ross, G. T. (1977). Androgenic stimulation of progesterone production by granulosa cells from preantral ovarian follicles: further in vitro studies using replicate cell cultures. Endocrinol. 100, 1539-1549.

Hillier, S.G., van den Boogaard, A. M. J., Reichert, L. E., and van Hall, E. V. (1980). Alterations in granulosa cell aromatase activity accompanying preovulatory follicular development in the rat ovary with evidence that 5α-reduced C_{19} steroids inhibit the aromatase reaction in vitro. J. Endocrinol, 84, 409-419.

Ho, S-M. (1987). Endocrinology of vitellogenesis. In "Hormones and Reproduction in Fishes, Amphibians, and Reptiles" (D. O. Norris and R. E. Jones, eds.) pp.145-169. Plenum Press, New York.

Kagawa, H., Young, G., and Nagahama, Y. (1984). In vitro estradiol-17β and testosterone production by ovarian follicles of the goldfish, Carassius auratus. Gen. Comp. Endocrinol. 54, 139-143.

Kime, D. E. (1990). In vitro metabolism of progesterone, 17-

hydroxyprogesterone and 17,20β-dihydroxy-4-pregnen-3-one by ovaries of the common carp Cyprinus carpio: production rates of polar metabolites. Gen. Comp. Endocrinol. 79, 406-414.

Kudolo, G. B., Elder, M. G., and Myatt, L. (1987). Estrogen receptor in the granulosa cell during postnatal development of the rat ovary. J. Endocrinol. 112, 333-338.

Kwon, H. B., Ahn, R. S., Lee, W. K., Im, W.-B., Lee, C. C., and Kim, K. (1993). Changes in the activities of steroidogenic enzymes during the development of ovarian follicles in Rana nigromaculata. Gen. Comp. Endocrinol. 92, 225-232.

Kwon, H. B., Choi, H. H., Ahn, R. S., and Yoon, Y. D. (1991). Steroid production by amphibian (Rana nigromaculata) ovarian follicles at different developmental stages. J. Exp. Zool. 260, 66-73.

Le Goascogne, C., Sananes, N., Gouezou, M., and Baulieu, E.-E. (1985). Testosterone-induced meiotic maturation of Xenopus laevis oocytes: evidence for an early effect in the synergistic action of insulin. Dev. Biol. 109, 9-14.

Licht, p., McCreery, B. R., Barnes, R., and Pang, R. (1983). Seasonal and stress related changes in plasma gonadotropins, sex steroids and corticosterone in the bullfrog, Rana catesbeiana. Gen. Comp. Endocrinol. 50,

124-145.

Lin, Y.-W. P. and Schuetz, A. W. (1983). In vitro estrogen modulation of pituitary and progesterone-induced oocyte maturation in Rana pipiens. J. Exp. Zool. 226, 281-291.

Lin, Y.-W. P. and Schuetz, A. W. (1985). Intrafollicular action of estrogen in regulating pituitary-induced ovarian progesterone synthesis and oocyte maturation in Rana pipiens: temporal relationship and locus of action. Gen. Comp. Endocrinol. 58, 421-435.

Mak, P., Callard, I. P., and Callard, G. V. (1983). Characterization of an estrogen receptor in the testis of the urodele amphibian Necturus maculosus. Biol. Reprod. 28, 261-270.

McCreery, B. R. and Licht, P. (1984). Effects of gonadectomy and sex steroids on pituitary-gonadotropin release and response to gonadotropin releasing hormone (GnRH) agonist in the bullfrog, Rana catesbeiana. Gen. Comp. Endocrinol. 54, 283-296.

Nagahama, Y. (1987). Endocrine control of oocyte maturation. In "Hormones and Reproduction in Fishes, Amphibians, and Reptiles" (D. O. Norris and R. E. Jones, eds.) pp.171-202. Plenum Press, New York.

Ozon, R. (1972a). Androgens in fishes, amphibians, reptiles, and birds. In"Steroids in Nonmammalian Vertebrates" (D. R. Idler eds.) pp.328-389. Academic Press, New York.

Ozon, R. (1972b). Estrogens in fishes, amphibians, reptiles,

and birds. In"Steroids in Nonmammalian Vertebrates" (D. R. Idler eds.) pp.390-413. Academic Press, New York.

Paolucci, M., and Botte, V. (1988). Estradiol-binding molecules in the hepatocytes of the female water frog, Rana esculenta, and plasma estradiol and vitellogenin levels during the reproductive cycle. Gen. Comp. Endocrinol. 70, 466-476.

Pavgi, S., and Licht, P. (1989). Effects of gonadectomy and steroids on pituitary gonadotropin secretion in a frog, Rana pipiens. Biol. Reprod. 40, 40-48.

Pavgi, S., and Licht, P. (1993). Inhibition of in vitro pituitary gonadotropin secretion by 17β-estradiol in the frog Rana pipiens. Gen. Comp. Endocrinol. 89, 132-137.

Petrino, T. R., and Schuetz, A. W. (1986). Protein synthesis and steroidogenesis in amphibian (Rana pipiens) ovarian follicles: studies on the conversion of pregnenolone to progesterone. Gen. Comp. Endocrinol. 63, 441-450.

Petrino, T. R., Lin, Y.-W. P., Netherton, J. C., Powell, D. H., and Wallace, R. A. (1993). Steroidogenesis in Fundulus heteroclitus V.: purification, characterization, and metabolism of 17α,20β-dihydroxy-4-pregnen-3-one by intact follicles and its role in oocyte maturation. Gen. Comp. Endocrinol. 92, 1-15.

Reynhout, J. K. and Smith, L. D. (1973). Evidence for steroid metabolism during the in vitro induction of

maturation in oocytes of Rana pipiens. Dev. Biol. 30, 392-402.

Sakai, N., Iwamatsu, T., Yamauchi, K., Suzuki, N., and Nagahama, Y. (1988). Influence of follicular development on steroid production in the medaka (Oryzias latipes) ovarian follicle in response to exogenous substrates. Gen. Comp. Endocrinol. 71, 516-523.

Schoonen, W. G. E. J., Penders, M. T., Van Dam, G. H., and Lambert, J. G. D. (1988). 5β-Pregnane-3α,6α,17α,20β-tetrol and 5β-pregnane-3α,6α,17α-triol-20-one: steroids of ovarian origin in the african catfish, Clarias gariepinus, during oocyte maturation. Gen. Comp. Endocrinol. 69, 181-187.

Schröder, H., Ziegler, M., and El Etreby, M. F. (1988). Aromatase-inhibitors of the androstenedione-type activate the 17β-hydroxysteroid-dehydrogenase in the rat testis tissue suspension model. J. Steroid Biochem. 31, 685-690.

Schuetz, A. W. (1972). Estrogens and ovarian follicular functions in Rana pipiens. Gen. Comp. Endocrinol. 18, 32-36.

Smalley, K. N. (1989). Hormone production by developing follicles of the northern leopard frog. Kans. Acad. Sci. Abst. 8, 30. [Abstract]

Smalley, K. N. and Nace, G. W. (1983). Vitellogenic cycles

in laboratory-maintained females of the leopard frog Rana pipiens. J. Exp. Zool. 226, 211-219.

Spiegel, J., Jones, E., and Snyder, B. W. (1978). Estradiol-17β interference with meiotic maturation in Rana pipiens ovarian follicles; evidence for inhibition of 3β-hydroxysteroid dehydrogenase. J. Exp. Zool. 204, 187-192.

Suzuki, K., Tamaoki, B., and Hirose, K. (1981). In vitro metabolism of 4-pregnenes in ovaries of a fresh water teleost, the ayu (Plecoglossus altivelis); production of 17α,20β-dihydroxy-4-pregnen-3-one and its 5β-reduced metabolites, and activation of 3β- and 20β-hydroxysteroid dehydrogenase by treatment with a fish gonadotropin. Gen. Comp. Endocrinol. 45, 473-481.

Thibier-Fouchet, C., Mulner, O. and Ozon, R. (1976). Progesterone biosynthesis and metabolism by ovarian follicles and isolated oocytes of Xenopus laevis. Biol. Reprod. 14, 317-326.

Venkatesh, B., Tan, C. H., Kime, D. E., Loy, G. L., and Lam, T. J. (1992). Steroid metabolism by ovarian follicles and extrafollicular tissue of the Guppy (Poecillia reticulata) during oocyte growth and gestation. Gen. Comp. Endocrinol. 86, 378-394.

Wallace, R. A. and Bergink, (1974). Amphibian vitellogenin: properties, hormonal regulation of hepatic synthesis and ovarian uptake, and conversion to yolk proteins.

Amer. Zool. 14, 1159-1175.

Westley, B. and Knowland, J. (1978). An estrogen receptor from Xenopus laevis possibly connected with vitellogenin synthesis. Cell. 15, 367-374.

Yeung, W. S. B., and Chan, S. T. H. (1985). The in vitro metabolism of radioactive progesterone and testosterone by the gonads of the protandrous Rhabdosargus sarba at various sexual phases. Gen. Comp. Endocrinol. 59, 171-183.

Young, G., Kagawa, H., and Nagahama, Y. (1983). Evidence for a decrease in aromatase activity in the ovarian granulosa cells of amago salmon (Oncorhynchus rhodurus) associated with final oocyte maturation. Biol. Reprod. 29, 310-315.

Atsuko Fukuike
Signature of Graduate Student

Katherine N. Smalley
Signature of Major Advisor

I, Atsuko Fukuike, Hereby submit this thesis to Emporia State University as partial fulfillment of the requirements of an advanced degree. I agree that the Library of the University may make it available for use in accordance with its regulations governing materials of this type. I further agree that quoting, photocopying, or other reproduction of this document is allowed for private study, scholarship (including teaching), and research purposes of a nonprofit nature. No copying which involves potential financial gain will be allowed without written permission of the author.

Atsuko Fukuike
Signature of Author

8/12/93
Date

Pregnenolone metabolism in developing follicles of the frog Rana pipiens
Title of Thesis

Doug Cooper
Signature of Graduate Office Staff Member

12-21-93
Date Received