AN ABSTRACT OF THE THESIS OF

Futoshi Kobayashi for the Master of Science Degree

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Title: Characterization of oviductal aromatase in the northern leopard frog (*Rana pipiens*)

Abstract Approved: Katherine N. Swadley

Estrogens increase the growth of the frog oviduct. Testosterone also has a growth promoting effect in the northern leopard frog, *Rana pipiens*, while dihydrotestosterone does not. To investigate the possibility that the testosterone effect is due to its conversion to estradiol, the oviduct was examined for the presence of an aromatase, first by looking for metabolites with thin layer chromatography, and then by using an enzymatic technique. The characteristics and kinetic parameters of the amphibian enzyme were then examined. Human placental aromatase was also studied for comparative purposes. Oviducts were obtained from summer frogs in various stage of oogenesis. After the animals were sacrificed, the oviducts were quickly removed, frozen on dry ice, and later homogenized in Tris-EDTA-sucrose buffer, pH 7.4, at 4 °C. Microsomes were isolated by ultracentrifugation, and aromatase activity was assayed by a tritiated water release method using [1β-^{3}H(N)]androstenedione as a substrate. Incubation time totaled 7.5 hours. Under such conditions, oviductal aromatase produced estrogen at a constant rate. The optimum temperature for the enzyme appears to lie between 33 and 37 °C. The pH has a broad optimum between 7.4 and 10.4.

The K_m and the V_{max} for androstenedione were 188.1±30.2 nM and 1.42±0.11 fmol of estrogen produced/min/mg microsomal protein, respectively. Comparison of these parameters with those of human placental aromatase (K_m = 123.0 nM, V_{max} = 113.57 fmol of estrogen produced/min/mg microsomal protein) shows that both enzymes had similar affinity for androstenedione, but frog oviduct had much less aromatase activity than human placenta. To further characterize the frog enzyme, the effects of two mammalian aromatase inhibitors, 10-propargylestr-4-ene-3,17-dione (PED) and 4-hydroxyandrostenedione (4-OH-A) were tested. Since progesterone has been found to inhibit estrogen production in the frog ovary, it was also tested for inhibitory activity. Although PED did not inhibit the aromatase activity, 4-OH-A inhibited it in a dose-dependent fashion. Progesterone also showed inhibitory ability. Since testosterone has been reported to be the primary androgen in the circulation of female frogs, serum testosterone levels in frogs with mature ovaries were measured and compared with the K_m of the oviductal aromatase. The testosterone levels showed a wide range from 17.4 to 176.6 nM with a mean value of 90.6±17.6 nM. These results suggest that the oviductal aromatase could function in converting blood androgens to estrogens to stimulate oviduct growth.

CHARACTERIZATION OF OVIDUCTAL AROMATASE IN THE NORTHERN LEOPARD FROG (*RANA PIPIENS*)

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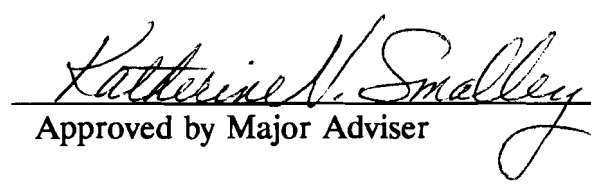

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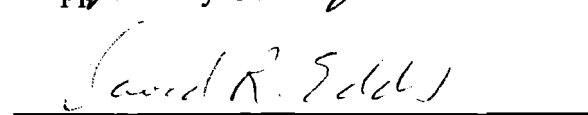

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PREFACE

This thesis is being submitted for publication in General and Comparative Endocrinology; therefore, I followed their publication style.

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INTRODUCTION

The conversion of androgens to estrogens is generally referred to as aromatization and is catalyzed by the enzyme aromatase. Aromatase is a membrane-bound enzyme of the smooth endoplasmic reticulum which is included in microsomes during cell fractionation. It belongs to a cytochrome P-450 complex (Thompson and Siiteri, 1974b). Since Ryan (1959) introduced a microsomal assay for aromatase in the human placenta, this enzyme has been found in various mammalian tissues including the testis (Kelch *et al.*, 1972), central nervous system (Naftolin *et al.*, 1975), ovary (Moon *et al.*, 1978), and prostate (Stone *et al.*, 1986).

Pathways for synthesis of estrogens and other major steroid hormones (Appendix 1) are similar in mammals, amphibians, and other nonmammalian vertebrates (Ozon, 1972a, b). Although aromatase is being studied most actively in mammals, there are some interesting studies in nonmammalian vertebrates. For example, Callard *et al.* (1978b) found extremely high aromatase activity in the testis of the urodele amphibian *Necturus maculosus*. Teleost fishes have brain aromatase activity 100-1,000 times greater than that of mammals (Callard, 1983; Pasmanik and Callard, 1985). Aromatase activity has also been described in the brain of reptiles (Callard *et al.*, 1977) and amphibians (Callard *et al.*, 1978a).

Dubowsky (1991) raised the possibility that an aromatase may also exist in the oviduct of the northern leopard frog, *Rana pipiens*. She investigated effects of androgens on the oviduct in this frog, and found that testosterone causes oviduct hypertrophy but dihydrotestosterone does not. She also demonstrated that

incubation of oviduct tissue with testosterone resulted in estradiol production. It is known that estrogens increase the growth of the frog oviduct (Lofts, 1974; Menghi *et al.*, 1986). Because an aromatase can biochemically link androgens to estrogens, it is possible that testosterone is aromatized to estrogen in the oviduct, and that this conversion accounts for its growth promoting effect. Therefore, the existence of an aromatase is strongly suspected in this organ. Confirming the existence of this enzyme in the oviduct and determining the amount present would help us understand its function in the frog reproductive system. The present study was carried out in order to examine these parameters. After the detection of aromatase activity in the frog oviduct, this study proceeded to a series of examinations for optimum temperature, optimum pH, K_m, and V_{max} of the enzyme.

Inhibition studies of frog oviductal aromatase were also attempted. The development of specific inhibitors is essential to understanding the physiological role of the enzyme and its product. As potential inhibitors, 10-propargylestr-4-ene-3,17-dione (PED) and 4-hydroxyandrostenedione (4-OH-A) were utilized. Both of these are androstenedione derivatives (Appendix 2). Their inhibitory properties were first described by Covey *et al.* (1981) for PED and by Brodie *et al.* (1976) for 4-OH-A. Both inhibit mammalian aromatase and are currently under active study because of their potential for treatment of estrogen-dependent tumors (Wing *et al.*, 1985; Brandt *et al.*, 1988a). For comparison, it is of considerable interest to investigate whether or not mammalian inhibitors are active on

amphibian aromatase.

The effect of progesterone on frog aromatase was also examined. Progesterone is not considered to be an aromatase inhibitor in mammals, but it reduces follicular estrogen production in *R. pipiens* (K. N. Smalley, personal communication). In order to ascertain if this situation is true in the oviduct, and if this reduction might be due to inhibition of aromatase, progesterone was assayed under the same experimental condition as the other aromatase inhibitors.

In general, the oviduct is not regarded as a steroidogenic tissue. Accordingly, its capacity to produce steroids is mainly dependent upon the supply of available substrates. Therefore, the amount of precursor in the circulation should be the same as or higher than the K_m value of the enzyme. Callard *et al.* (1978a) suggested that testosterone was the primary steroid in the circulation of both male and female bullfrogs, *Rana catesbeiana*. In addition, Licht *et al.* (1983) reported that the androgen level in female bullfrogs was significantly higher than that of estrogen and even higher than that in males of the same species. If this situation is true of the leopard frog, testosterone should be a major substrate for the oviductal aromatase. Radioimmunoassay (RIA) was carried out to measure serum testosterone levels in this frog. The amount of this steroid in serum would help us understand its physiological role.

MATERIALS AND METHODS

Supplies. Bovine serum albumin, the Lowry reagents (Folin-Ciocalteu reagent, cupric sulfate, sodium carbonate, sodium hydroxide, and sodium tartrate), NADPH, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase were purchased from Sigma Chemical Company (St. Louis, MO). [4-^{14}C]Testosterone (specific activity: 51.4 mCi/mmol) and [1β-^{3}H(N)]androstenedione (27.5 Ci/mmol) were obtained from New England Nuclear Corp. (Boston, MA). Sucrose was acquired from Mallinckrodt, Inc. (Paris, KY). Dioxane, 2,5-diphenyloxazole, naphthalene, and xylene were purchased from Research Products International Corp. (Elk Grove Village, IL). Human placental microsome, PED, and 4-OH-A were kindly provided by Dr. S. J. Zimniski. Mature female frogs in various stages of oogenesis were obtained from Kons Scientific Corp., Inc. (Germantown, WI). Testosterone antibody was acquired from Wien Laboratories, Inc. (Succasunna, NJ).

Thin layer chromatography (TLC). Frogs were sacrificed by pithing, then oviducts were removed and cut into small pieces. The oviduct (25 mg) was added to 1.5 ml amphibian Ringer's solution (Petrino and Schuetz, 1986), and incubated in each well with 2 μl [4-^{14}C]testosterone for 4 hours at 23 °C. The medium was removed by aspiration and mixed with 4.5 ml ether:chloroform (3:1) to extract steroids. After centrifugation at 2,000*g* for 7 minutes, the upper layer containing the steroids was reserved. This procedure was repeated three times, and the pooled extract was evaporated in a warm water bath (40-50 °C) under an air stream. The medium extract was dissolved in 50 μl of 100% ethanol and spotted

on a TLC plate (aluminum plates, pre-coated with 0.25 mm silica gel F 254 containing a UV indicator). The plate was developed in one of the following two solvents: benzene:ethyl acetate (5:1), or chloroform:ethyl acetate (4:1). The plate was dried overnight, and 5 mm samples of the gel between the solvent front and the baseline were scraped into scintillation vials and mixed with 10 ml of scintillation fluid (Schatz and Morrill, 1975). Each vial was counted using an LKB Rack Beta Liquid Scintillation Counter for 5 minutes. R_f values of standard steroids on the same plates were identified by UV or by spraying with H_2SO_4:CH_3OH (7:3) and heating at 110 °C for 15 minutes.

Preparation of oviductal microsome. The slightly modified method of Brandt *et al.* (1988a) was used for this procedure. Frogs were sacrificed by decapitation. Oviducts were removed and quickly frozen on dry ice. Oviducts from 3-7 frogs were pooled and homogenized in 4 vol of Tris-EDTA-sucrose buffer (20 mM Tris-HCl, 20 mM KCl, 1 mM EDTA, and 250 mM sucrose, pH 7.4 at 4 °C). The homogenate was centrifuged at 9,000*g* for 15 minutes at 4 °C. The supernatant was ultracentrifuged at 105,000*g* for 60 minutes at 4 °C to obtain a microsome pellet. The pellet was resuspended in Tris-EDTA-sucrose-glycerol buffer (Tris-EDTA-sucrose buffer and 10% (v/v) glycerol, pH 7.4 at 4 °C) and stored at -70 °C as the enzyme preparation. The protein content of the microsome preparations was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Aromatase assay. Ryan (1959) discovered that both NADPH and oxygen

are necessary cofactors for aromatization. Thompson and Siiteri (1974a) found a stoichiometry for the reaction (3 moles NADPH and 3 moles O_2/1 mole estrogen produced) and that $NADP^+$ is an inhibitor of aromatase. Therefore, the assay buffer contained not only excessive NADPH but also an NADPH regenerating system. Reaction vials were left open during the assay. Aromatization was stereochemically characterized as specific dehydrogenation from the 1β and 2β positions of androstenedione into water (Townsley and Brodie, 1968; Brodie *et al.*, 1969a, b). Taking advantage of this stereospecificity, a tritiated water release method measures the production of tritiated water corresponding to estrogens. The mechanism of this reaction is summarized in Appendix 3.

Aromatase activity in the microsomal preparations was assayed by a tritiated water release method as modified by Zimniski *et al.* (1985). Aliquots of the microsome preparation (0.7 ml) were incubated with 2.5 ml assay buffer (9.98 mM KH_2PO_4, 20.02 mM K_2HPO_4, and 250 mM sucrose, pH 7.4) containing 1 mM NADPH, an NADPH regenerating system (4.9 mM glucose 6-phosphate and 0.5 U/ml glucose 6-phosphate dehydrogenase), and the tritiated androgen substrate ([1β-^{3}H(N)]androstenedione). At defined intervals (90 minutes for frog oviduct), a 0.1 ml aliquot of each sample was taken and mixed with 10 ml scintillation fluid (1,000 ml dioxane, 495 ml xylene, 120 g naphthalene, and 7.5 g diphenyloxazole). Each scintillation vial was counted using a liquid scintillation counter for 10 minutes to obtain "Total Counts". Then water (2 ml) was mixed into each vial to create a separate water phase containing the tritiated water produced, removing it

from the organic phase containing hydrophobic steroids. Each vial was counted again by the above procedure to obtain "Steroid Counts". Because no scintillant remained in the aqueous phase, the tritiated water was not counted. The amount of tritiated water was calculated by subtracting "Steroid Counts" from "Total Counts". Tritiated water production *versus* time was plotted and analyzed by least squares linear regression to determine enzyme velocity.

Human placental microsomes were assayed under the same conditions as the frog aromatase except that shorter incubations (2-3 minutes intervals) were required. Because of its high concentration of aromatase, human placenta has been a primary enzyme source for studies of aromatization, and it was of interest to compare the properties of the frog oviductal aromatase with that of the well characterized placental enzyme.

A series of examinations for properties of the frog oviductal aromatase were undertaken. In order to examine optimum temperature, the aromatase assay was performed in duplicate (using two aliquots from the same microsome preparation) at each of four different temperatures (15, 24, 37, and 45 °C). To investigate optimum pH, the assay was conducted in duplicate at each of four different pH levels (pH 6.4, 7.4, 8.5, and 10.4). For the enzyme kinetic experiments, five different concentrations (from 10.0 to 105.0 nM) of substrate were used. Results were analyzed by the methods of Lineweaver and Burk (1934), and Eisenthal and Cornish-Bowden (1974). Enzyme parameters were reported as mean ± SEM of four independent studies. In the inhibitor

experiments, inhibitor solutions were added to assay buffer in the above procedure. For percent aromatase activities, the experiment was undertaken in duplicate (using two aliquots from the same microsome preparation) at each of three different concentrations of inhibitors. In the enzyme kinetic experiments using inhibitors, the assay was carried out once for PED, and independently twice for both 4-OH-A and progesterone.

Radioimmunoassay (RIA). The modified method of Chen *et al.* (1971) was used to measure testosterone levels in serum. Steroids were extracted from serum by mixing an aliquot of each sample (from 14.0 to 140.0 μl) and 0.5 ml distilled water with 10 ml methylene chloride. After centrifugation at 2,000*g* for 5 minutes, the lower layer containing the steroids was removed by aspiration and evaporated by the same procedure as for TLC. The testosterone level was measured by RIA and was reported as mean$\pm$SEM of six frogs. This assay is sensitive for testosterone levels as low as 100 pg. Because all frogs used in this study had mature follicles (stage 4 and 5), those testosterone levels should represent maximal values throughout oogenesis.

RESULTS

In an attempt to characterize the metabolites of testosterone by frog oviduct, TLC was carried out using two solvent systems. Benzene:ethyl acetate (5:1) was not able to clearly separate androstenedione, estradiol, and dihydrotestosterone. Chloroform:ethyl acetate (4:1) separated androstenedione and estrone from estradiol and dihydrotestosterone (Fig. 1). However, it did not separate estradiol and dihydrotestosterone, and it was not clear whether the large peak (R_f=0.6) was estradiol, dihydrotestosterone, or a combination of the two.

Aromatase activity in the human placenta was independently studied seven times. A microsomal preparation (about 2.8 mg/ml protein concentration) was used. Estrogen production was proportional to the incubation time (Fig. 2). The velocity of human placental aromatase was found to be 44.2±3.4 fmol of estrogen produced/min/mg microsomal protein at 37 °C (pH 7.4).

The enzyme kinetic experiment for human placental aromatase was performed at 37 °C (pH 7.4). The Lineweaver-Burk plot (Lineweaver and Burk, 1934) of the reciprocal of the substrate concentration *versus* the reciprocal of the enzyme velocity yielded a straight line (Fig. 3). The line intersected on the abscissa and the ordinate indicating the negative reciprocal of K_m and the reciprocal of V_{max}, respectively. The K_m was 123.0 nM; the V_{max} was 113.57 fmol of estrogen produced/min/mg microsomal protein.

In the study of placental aromatase carried out using PED, this inhibitor effectively reduced estrogen production (Fig. 4). The velocity was 38.65 fmol of estrogen produced/min/mg microsomal protein for the control, and 4.76 in the

Fig. 1. Metabolism of [4-^{14}C]testosterone by frog oviduct. Results of TLC using chloroform:ethyl acetate (4:1) as solvent. Abbreviations show R_f values of the standard steroids (T = testosterone; E_2 = estradiol; D = dihydrotestosterone; A = androstenedione; E_1 = estrone; TP = testosterone propionate).

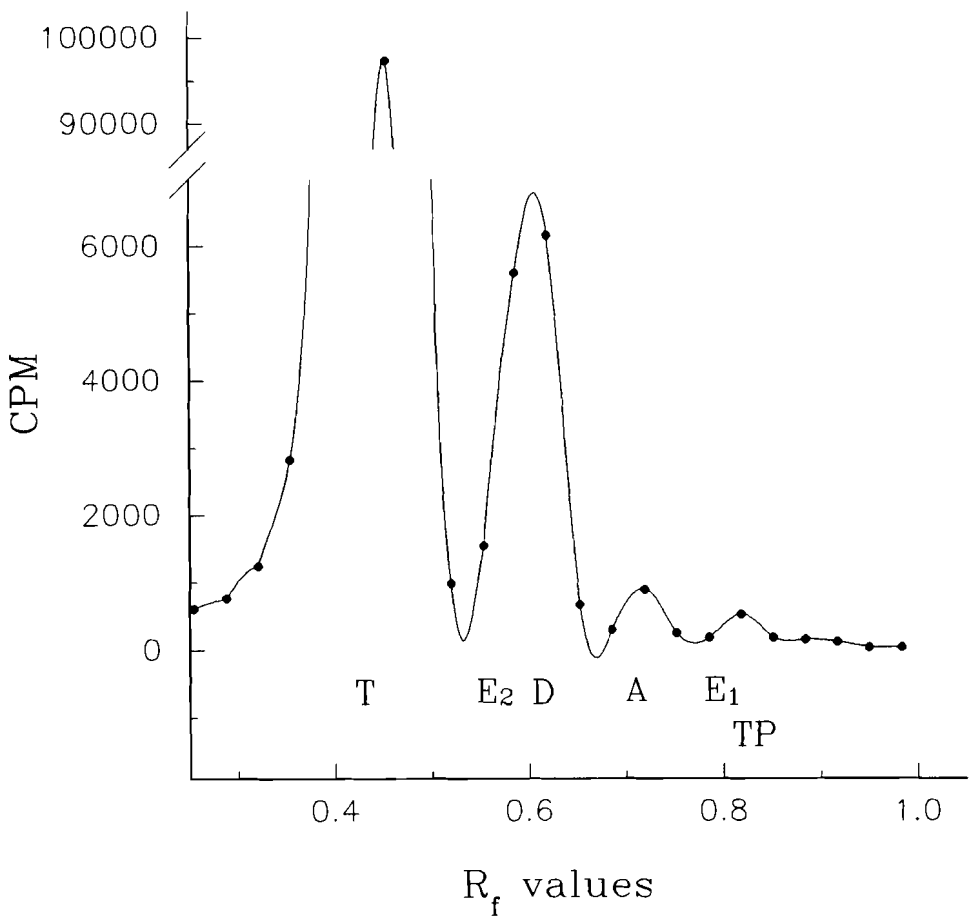

100000
90000
6000
4000
2000
0
CPM
T
E2
D
A
E1
TP
0.4
0.6
0.8
1.0
Rf values

Fig. 2. Estrogen production in a typical experiment with human placental microsomes. The same experiment was independently performed seven times. Androstenedione (70 nM) was used as substrate. An aliquot of the enzyme preparation was incubated with assay buffer (pH 7.4) at 37 °C. The velocity from this plot is 37.48 fmol of estrogen produced/min/mg microsomal protein.

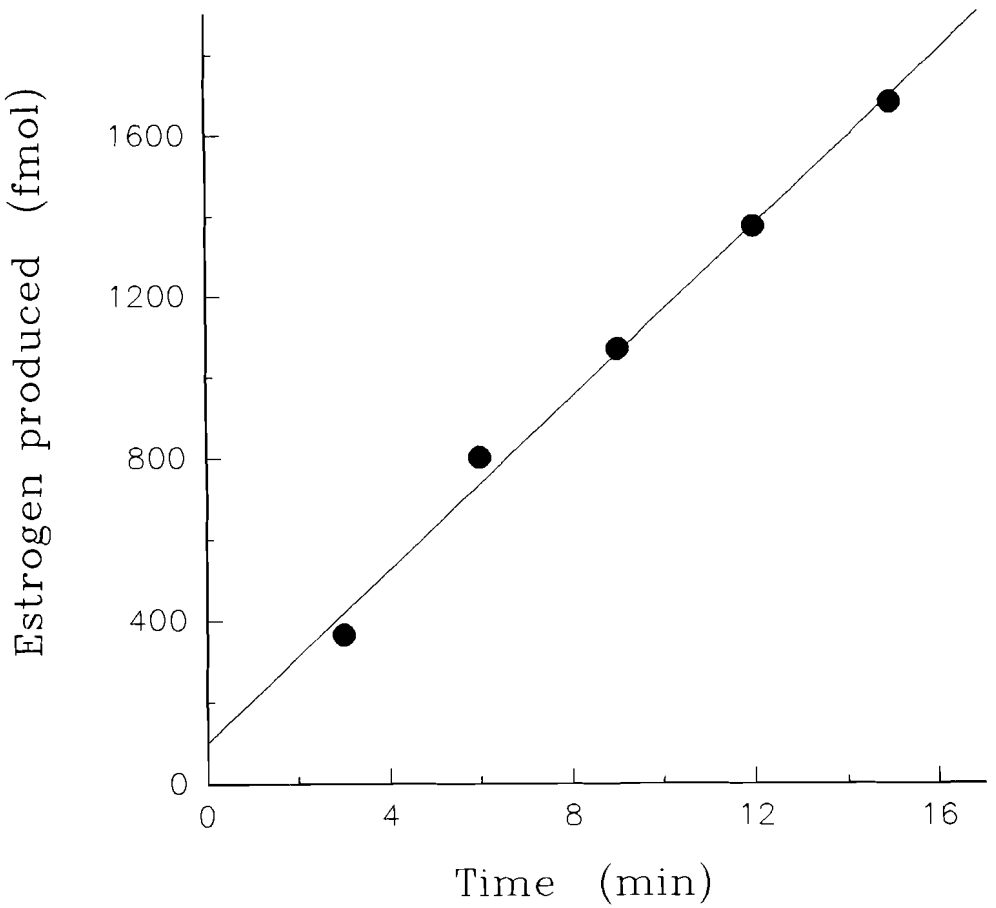

Estrogen produced (fmol)
1600
1200
800
400
0
0
4
8
12
16
Time (min)

Fig. 3. Lineweaver-Burk plot for human placental aromatase. Aliquots of the enzyme preparation were incubated with assay buffer (pH 7.4) at 37 °C.

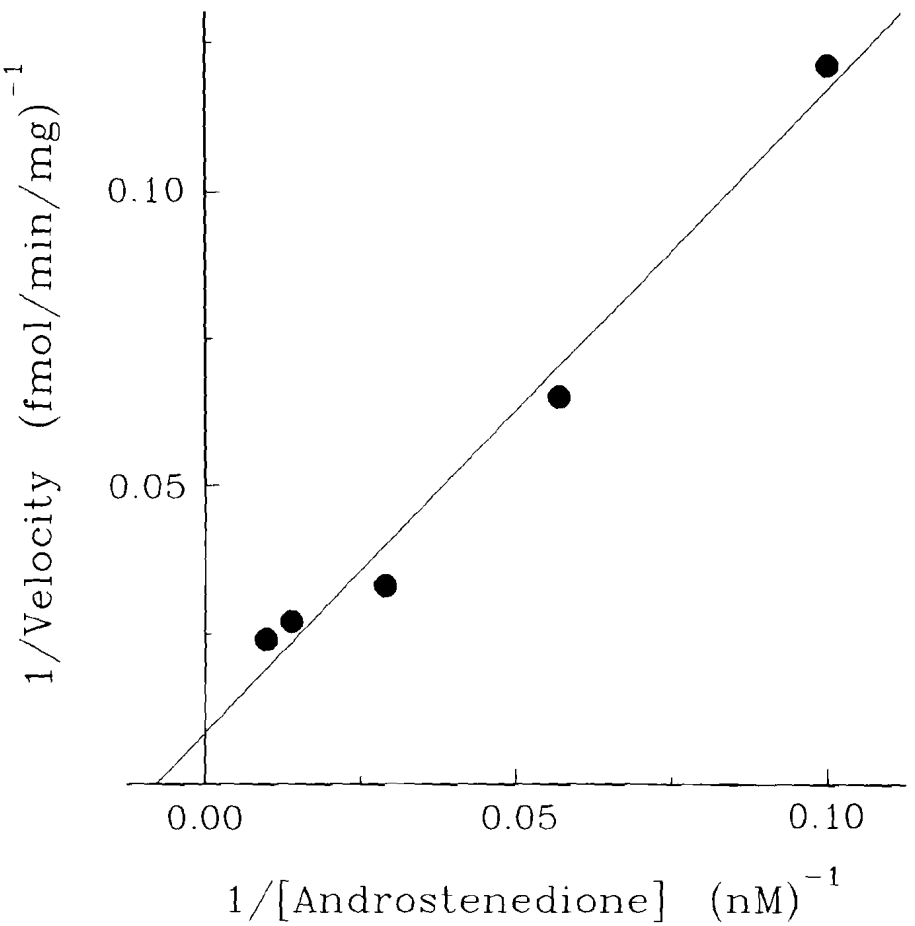
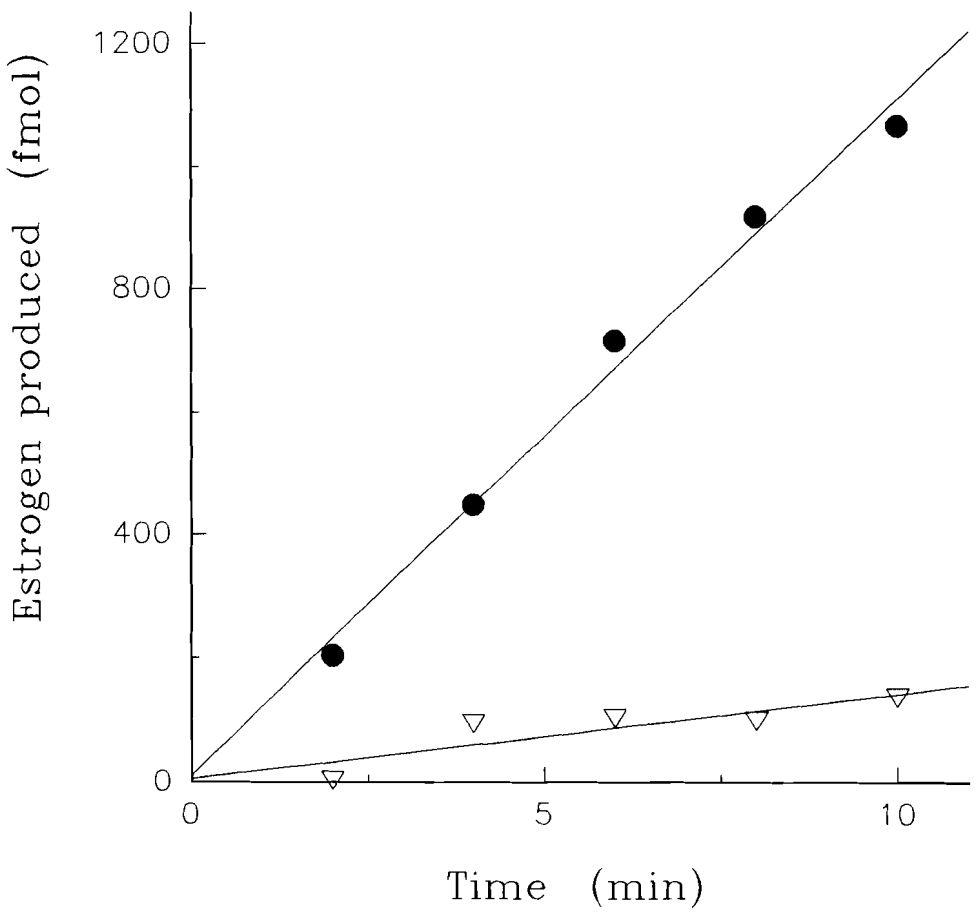

1/Velocity (fmol/min/mg)$^{-1}$
0.10
0.05
0.00
0.05
0.10
1/[Androstenedione] (nM)$^{-1}$

Fig. 4. Effect of PED on human placental aromatase activity. Solid circles represent the absence of PED and open triangles represent the presence of the inhibitor. Androstenedione (70 nM) and PED (0.05 μM) were used as substrate and inhibitor, respectively. Aliquots of the enzyme preparation were incubated with assay buffer (pH 7.4) at 37 °C.


Estrogen produced (fmol)
1200
800
400
0
0
5
10
Time (min)

presence of PED. Thus, PED reduced enzyme activity to 12.3% of the control level.

Studies of aromatase activity in the frog oviduct were begun using microsome preparations with protein concentration similar to those of placental samples, and by prolonging incubation to a total of 100 minutes at 37 °C (pH 7.4). However, there was no detectable aromatase activity in those assays. Both the protein concentration of the oviductal microsome preparation and the incubation time were then increased in a stepwise fashion until aromatase activity was detected. In the final assay procedure, the microsome preparation contained about 7.9 mg protein/ml. This corresponds to approximately one frog's oviducts per one microsome sample, or to one run of the aromatase assay. Incubation time was prolonged up to a total of 450 minutes and aliquots were taken at 90 minute intervals. Under such conditions, estrogen production was proportional to incubation time (Fig. 5). It is apparent that the frog oviduct has extremely low aromatase activity compared to the human placenta.

In the optimum temperature investigation, aromatase activity gradually increased between 15 and 24 °C (Fig. 6). At 37 °C, the enzyme showed more than twice as much activity as at 24 °C, and then rapidly decreased at 45 °C. Obviously, the enzyme was denatured by heat at 45 °C. The optimum temperature of frog aromatase appears to lie between 33 and 37 °C.

In the optimum pH investigation, aromatase activity dramatically increased between pH 6.4 and 7.4 (Fig. 7). In contrast, it was very stable at high pH levels.

Fig. 5. Estrogen production in a typical experiment with frog oviductal microsomes. The same experiment was performed ten times. Androstenedione (70 nM) was used as substrate. An aliquot of the enzyme preparation was incubated with assay buffer (pH 7.4) at 37 °C. Velocity from this plot is 0.43 fmol of estrogen produced/min/mg microsomal protein.

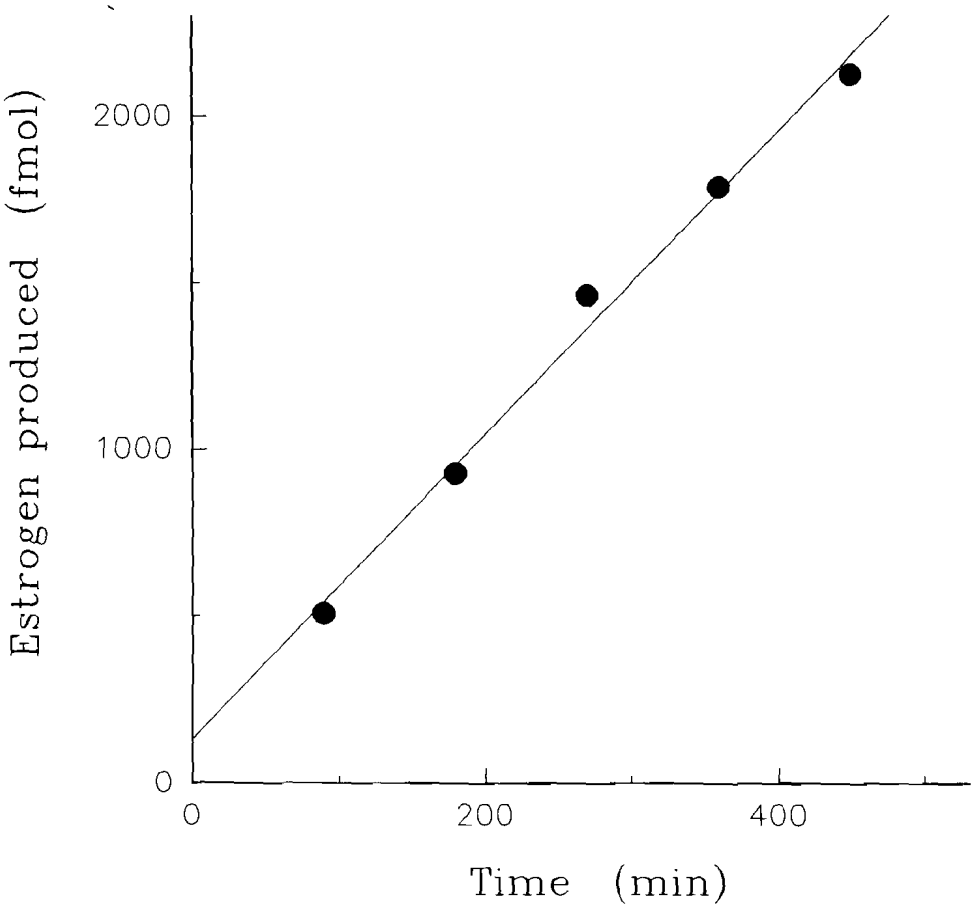

Estrogen produced (fmol)
2000
1000
0
0
200
400
Time (min)

Fig. 6. Effect of temperature on frog oviductal aromatase activity. Mean values of the activities in a duplicated experiment are expressed as fmol of estrogen produced/min/mg microsomal protein. Androstenedione (70 nM) was used as substrate. Aliquots of the enzyme preparation were incubated with assay buffer (pH 7.4).

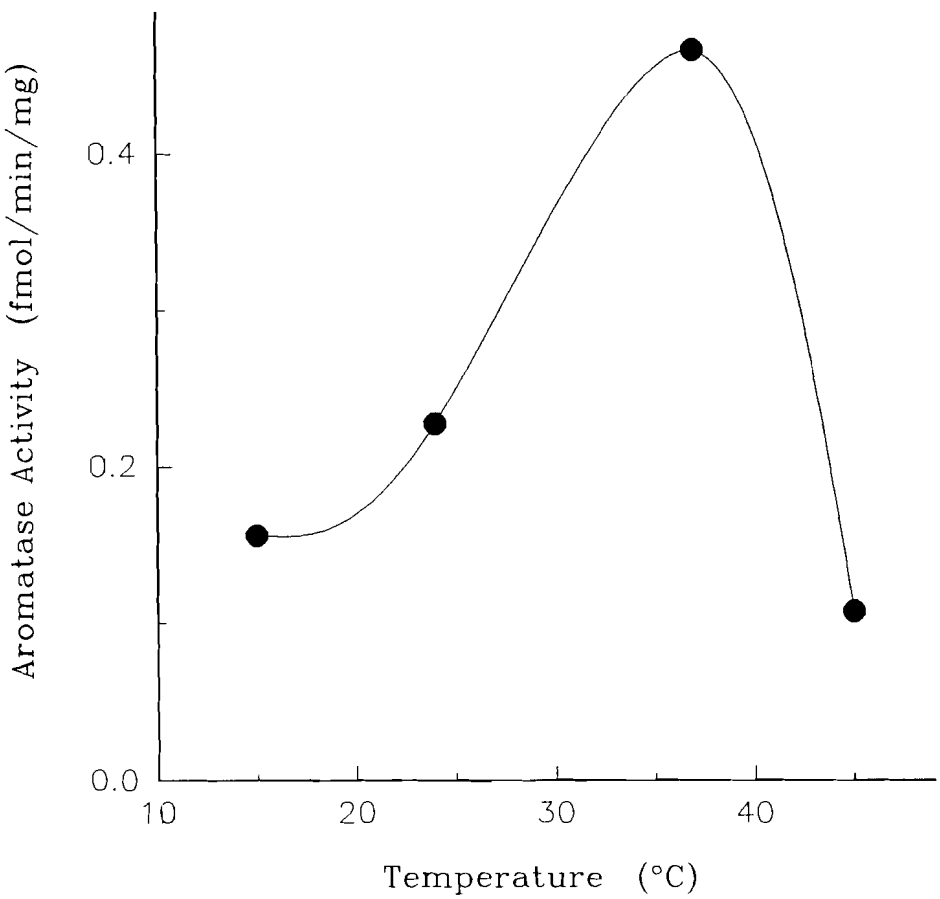
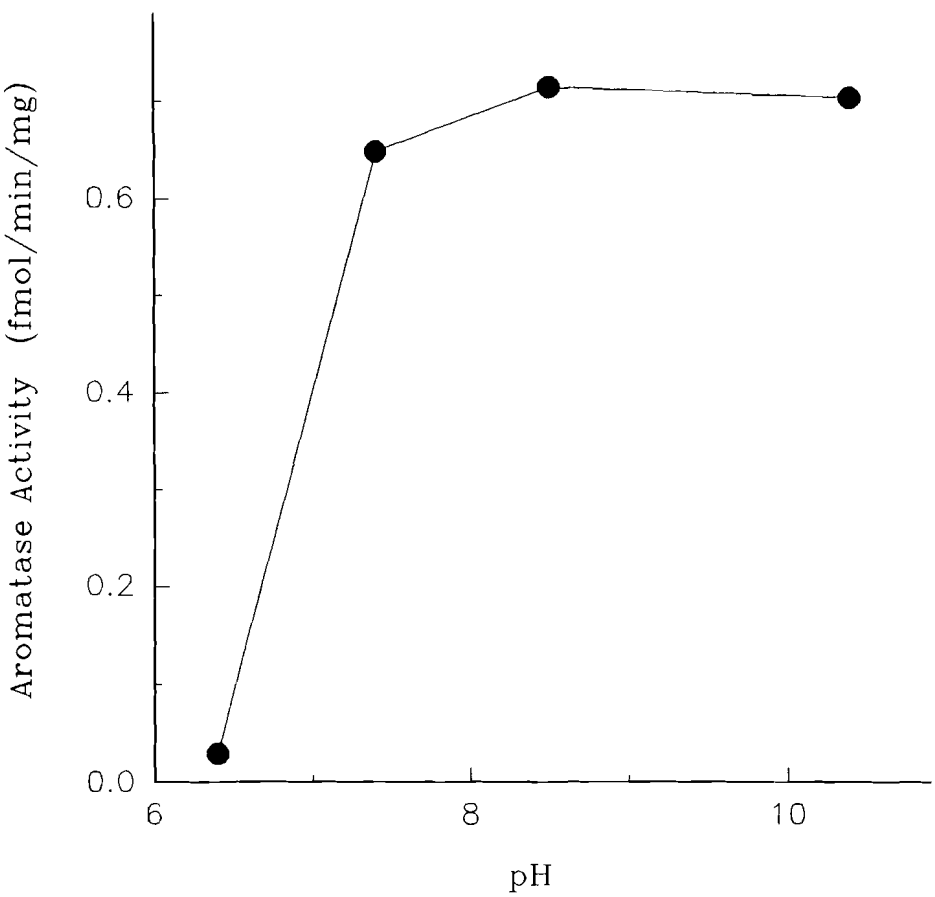

Aromatase Activity (fmol/min/mg)
0.0
0.2
0.4
10
20
30
40
Temperature (°C)

Fig. 7. Effect of pH on frog oviductal aromatase activity. Mean values of the activities in a duplicated experiment are expressed as fmol of estrogen produced/min/mg microsomal protein. Androstenedione (70 nM) was used as substrate. Enzyme preparations were incubated at 37 °C.


Aromatase Activity (fmol/min/mg)
0.0
0.2
0.4
0.6
6
8
10
pH

Even at pH 10.4, there was no difference in activity. Frog aromatase appears to have a broad pH optimum between 7.4 and 10.4.

Because of these results, enzyme kinetic experiments were performed at 37 °C using assay buffer at pH 7.4. The Lineweaver-Burk plot (Lineweaver and Burk, 1934) yielded a straight line with two intersections on the abscissa and the ordinate (Fig. 8). Using this plotting method, the average K_m of the frog oviductal aromatase for androstenedione was 188.1±30.2 nM, and the average V_{max} was 1.42±0.11 fmol of estrogen produced/min/mg microsomal protein. The direct linear plot (Eisenthal and Cornish-Bowden, 1974) of the negative of the substrate concentration *versus* the enzyme velocity for each data point yielded five lines which intersected in the +x,+y quadrant (Fig. 9). Using this plotting method, the average K_m for the same experimental results was 177.1±47.6 nM, and the average V_{max} was 1.33±0.24 fmol of estrogen produced/min/mg microsomal protein. Thus, there was no difference in the enzyme parameters using these different plotting methods.

In the inhibitor experiments, two inhibitors of mammalian aromatase, PED and 4-OH-A, and progesterone were utilized at three different concentrations (Fig. 10). None of them reduced the frog aromatase activity at 0.05 μM. PED also did not reduce the aromatase activity at 0.3 or 0.5 μM, but both 4-OH-A and progesterone did. Interestingly, 4-OH-A inhibited the activity in a dose-dependent fashion, whereas there was no difference in the inhibition by progesterone between 0.3 and 0.5 μM.

Fig. 8. Lineweaver-Burk plot for frog oviductal aromatase. For clarity, only the results of one experiment are shown, but the same experiment was independently performed four times. The K_m from this plot is 176.8 nM; the V_{max} is 1.31 fmol of estrogen produced/min/mg microsomal protein.

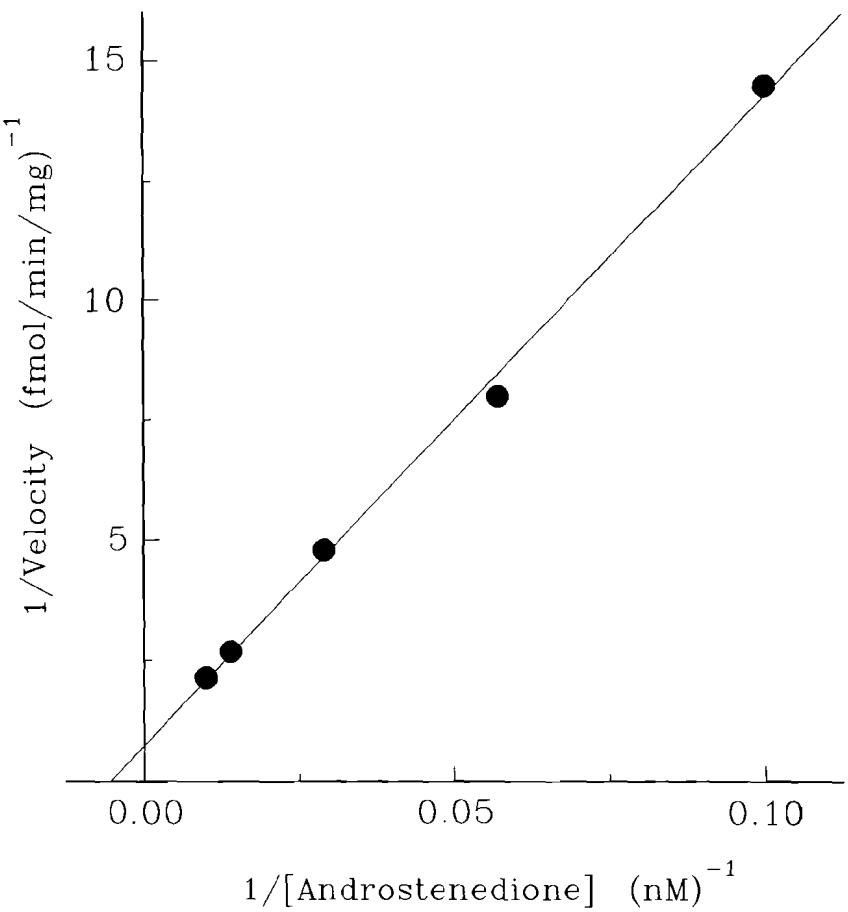

15
10
5
0.00
0.05
0.10
1/Velocity (fmol/min/mg)$^{-1}$
1/[Androstenedione] (nM)$^{-1}$

Fig. 9. Direct linear plot for the data from Fig. 8. The K_m from this plot is 140.7 nM; the V_{max} is 1.09 fmol of estrogen produced/min/mg microsomal protein.

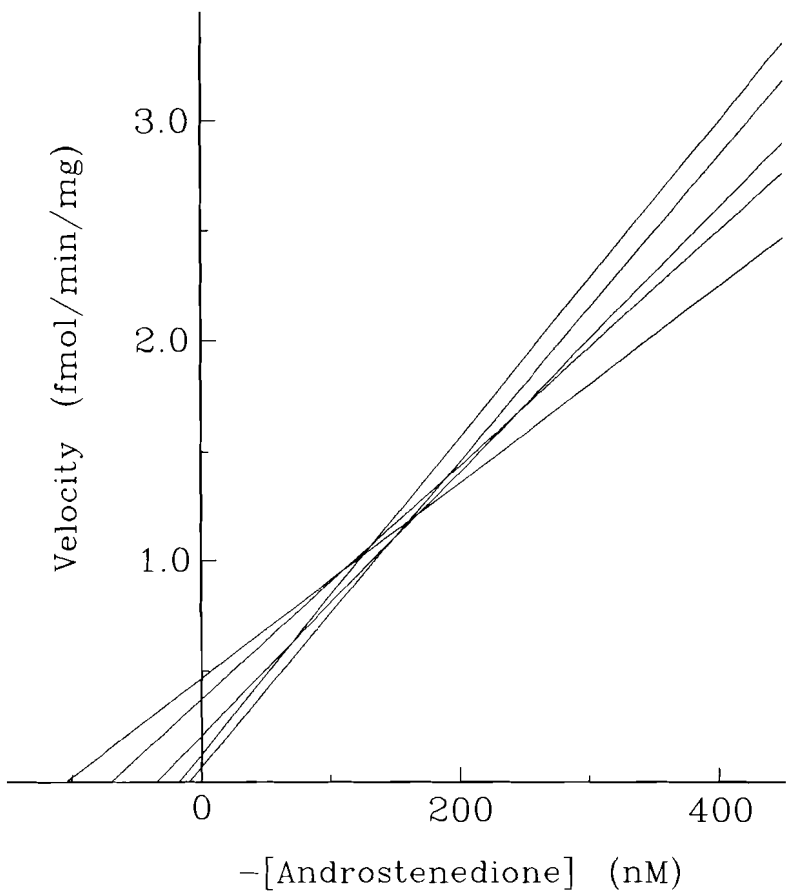
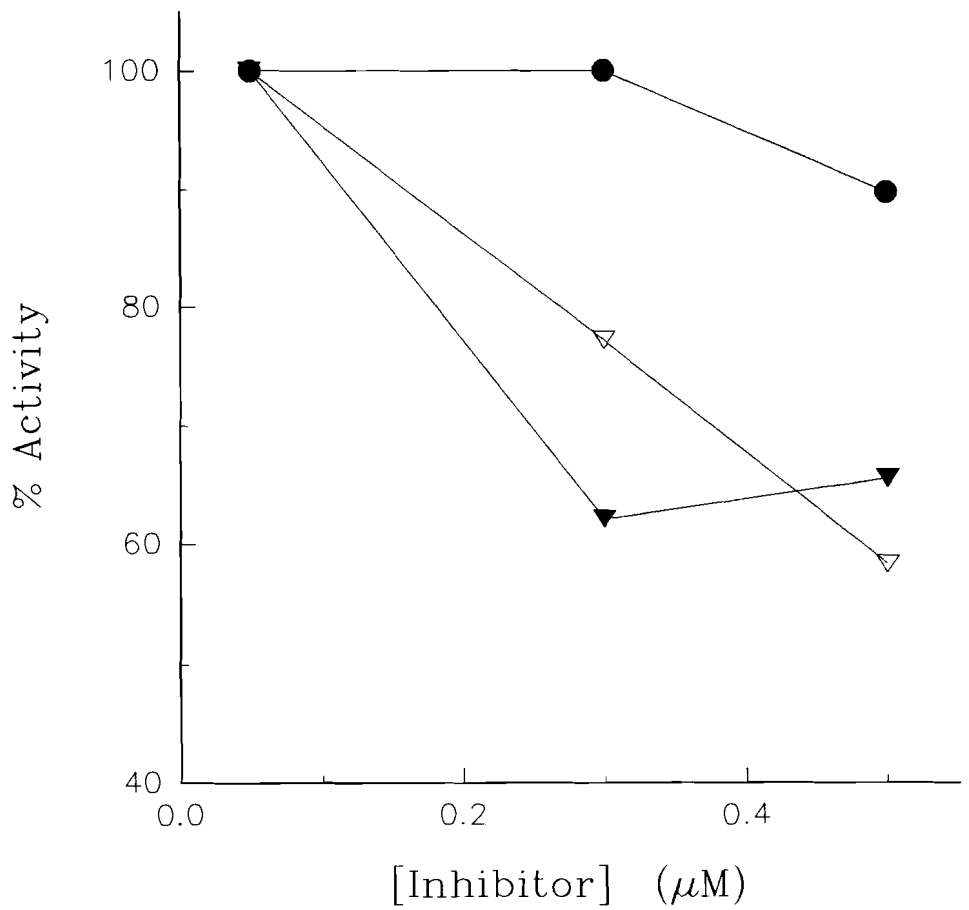

3.0
2.0
1.0
Velocity (fmol/min/mg)
0
200
400
-[Androstenedione] (nM)

Fig. 10. Percent aromatase activities for each concentration of two inhibitors and progesterone: solid circles = PED, open triangles = 4-OH-A, and solid triangles = progesterone. Mean values in a duplicated experiment are expressed. Androstenedione (70 nM) was used as substrate.


100
80
60
40
% Activity
0.0
0.2
0.4
[Inhibitor] (μM)

In order to investigate types of inhibition, identical enzyme kinetic experiments for K_m and V_{max} were carried out with the inhibitor solutions (0.5 μM). As seen in Fig. 11, PED did not inhibit frog aromatase. In contrast, both 4-OH-A and progesterone increased the slope of the Lineweaver-Burk plot (Figs. 12 and 13, respectively). For 4-OH-A, the two lines intersected in the +x,+y quadrant, being slightly apart from the ordinate (Fig. 12). Therefore, 4-OH-A mathematically yielded higher K_m (10974.05 nM) and higher V_{max} (29.67 fmol of estrogen produced/min/mg microsomal protein) than the control (230.31 nM, 1.57 fmol of estrogen produced/min/mg microsomal protein, respectively). For progesterone, the two lines intersected in the -x,-y quadrant, being slightly apart from the abscissa (Fig. 13). Therefore, progesterone mathematically yielded lower K_m (142.83 nM) and lower V_{max} (0.55 fmol of estrogen produced/min/mg microsomal protein) than the control (203.14 nM, 1.52 fmol of estrogen produced/min/mg microsomal protein, respectively).

Serum testosterone levels of females with mature follicles were measured by RIA. Testosterone concentrations showed a wide range from 17.4 to 176.6 nM (from 5.0 to 50.9 ng/ml) with a mean value of 90.6$\pm$17.6 nM (26.1$\pm$5.1 ng/ml).

Fig. 11. Lineweaver-Burk plot for frog oviductal aromatase using PED. Solid circles represent the absence of PED and open triangles represent the presence of the inhibitor.

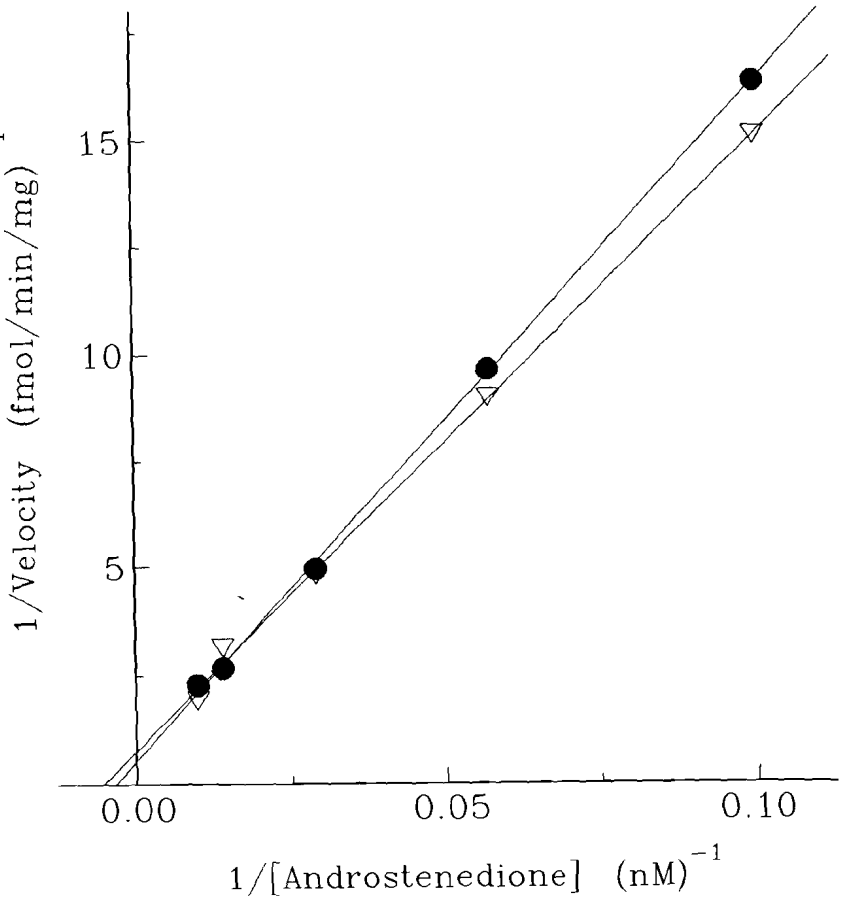
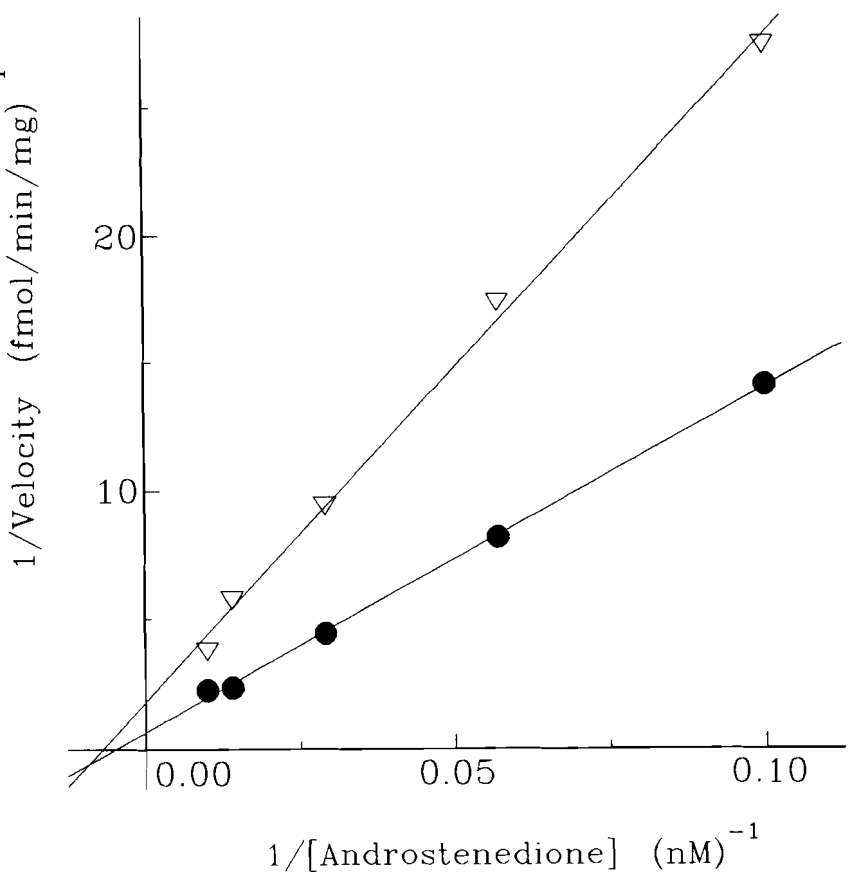

15
10
5
0.00
0.05
0.10
1/Velocity (fmol/min/mg)$^{-1}$
1/[Androstenedione] (nM)$^{-1}$

Fig. 13. Lineweaver-Burk plot for frog oviductal aromatase using progesterone. Mean values in two independent experiments are expressed. Solid circles represent the absence of progesterone and open triangles represent the presence of the steroid.


1/Velocity (fmol/min/mg)$^{-1}$
20
10
0.00
0.05
0.10
1/[Androstenedione] (nM)$^{-1}$

DISCUSSION

The present study examined the frog oviduct for the presence of an aromatase. A tritiated water release method detected aromatase activity in this organ, suggesting local conversion of testosterone to estradiol. The activity was very low and showed different characteristics and kinetic parameters from human placental aromatase. The present study also examined the effects of two mammalian aromatase inhibitors, PED and 4-OH-A, and progesterone on frog oviductal aromatase. Of these, both 4-OH-A and progesterone proved to be potential inhibitors. Characterized properties of this enzyme are summarized in Table 1.

TLC results confirmed that testosterone is metabolized by the frog oviduct, but the system was not able to clearly characterize the metabolites. There was a large peak which migrated with standard testosterone on the chromatogram (Fig. 1). However, a second peak could have been either estradiol or dihydrotestosterone. Because estrogen production was of more interest than the identity of other testosterone metabolites, additional studies were concentrated on possible oviductal aromatase activity. For future studies of testosterone metabolism using TLC, different solvent systems should be tested, and the concentration of testosterone should be adjusted to maximize results.

Aromatase activity was first studied in human placenta, and K_m was found to be 123.0 nM. K_m indicates the affinity between an enzyme and its substrate, and this low value of K_m implies that the enzymatic reaction can proceed even at low concentrations of substrate. Since K_m has an intrinsic value for each enzyme,

Table 1. Characterized parameters of oviductal aromatase in the northern leopard frog, *Rana pipiens*.

Parameter	Characterized value
Optimum temperature	33-37 °C
Optimum pH	7.4-10.4
K_m for androstenedione	188.1±30.2 nM
V_{max} for androstenedione	1.42±0.11 fmol/min/mg
K_i for 4-OH-A	10.7 nM
K_i for progesterone	283.5 nM

it is an important kinetic parameter to characterize enzymes.

Several kinetic studies of aromatase have been performed on human placenta (Bellino and Osawa, 1974; Brodie *et al.*, 1977; Barbieri *et al.*, 1981; Covey and Hood, 1982a; Johnston *et al.*, 1984; Kellis and Vickery, 1984). However, K_m values for androstenedione ranged from 10 to 20,000 nM in these studies, probably because different experimental procedures make comparisons difficult. The result of the present study is close to the value of 100 nM reported by Reed and Ohno (1976). They also used a tritiated water release method although [1β, 2β-^{3}H]androstenedione was utilized as a substrate.

V_{max} for human placental aromatase in this study was found to be 113.57 fmol of estrogen produced/min/mg microsomal protein. V_{max} indicates the maximal velocity of the enzymatic reaction, and is related to enzyme activity. The determination of an accurate V_{max} requires purification of the enzyme. Therefore, V_{max} is less important than K_m as a kinetic parameter. Literature values for human placental aromatase are 76.5 (Bellino and Osawa, 1974) and 28 pmol of estrogen produced/min/mg microsomal protein (Kellis and Vickery, 1984). These are quite different from the V_{max} found in the present study; however, it should be emphasized that the present study dealt with crude enzyme preparations.

A comparable aromatase assay was then carried out on frog oviduct. Aromatase activity was very low and could only be detected by using a higher concentration of the oviductal microsome preparation and a longer incubation time.

The optimum temperature of frog aromatase appears to lie between 33 and 37 °C (Fig. 6). It is interesting that the enzyme prepared from a cold-blooded animal had an optimum temperature similar to that of warm-blooded animals. According to Brattstrom (1963), mean body temperature of *R. pipiens* was 24.4 °C. However, it varied from 17.8 to 34.7 °C depending upon sampling locations, seasons and weather. It should be noted that frogs used for this assay were captured in late May and had been maintained in the laboratory at room temperature for about two months. Aromatase from summer frogs might be more adaptable to high temperature.

Callard *et al.* (1977) found that brain aromatase activity was greater at 27 °C than at 17 or 37 °C in the turtle, *Chrysemys picta.* Ovarian estradiol production was greatest at 24-29 °C in common carp, *Cyprinus carpio* maintained at 24 °C for one month (Manning and Kime, 1984). Because enzymatic activity can be modified by ambient temperature in adapted animals (Licht, 1967; Eckert, 1988), differences in optimum temperature may be caused by this modification.

Frog aromatase had a broad pH optimum between 7.4 and 10.4 (Fig. 7). Reed and Ohno (1976) reported an optimum pH between 7.5 and 8.0 for human placental aromatase. Like frog oviductal aromatase, the placental enzyme was more stable at higher than at lower pH. An optimal pH of 7.4 was clearly characterized for brain aromatase in rats (Roselli *et al.*, 1984). Silberzahn *et al.* (1988) found an optimum between 8.0 and 9.0 in equine testicular microsomes. These differences suggest that the optimum pH of aromatase is a species- or

organ-specific characteristic.

There are several graphic methods to linearize the Michaelis-Menten equation. The Lineweaver-Burk plot is undoubtedly the most famous. The biggest advantage of this plot is its clear presentation of the relationship between substrate concentration and enzyme velocity. However, because it is a double reciprocal plot, small differences are exaggerated and large ones are minimized. As a result, the accuracy of the plot for the determination of kinetic parameters is questionable (Dowd and Riggs, 1965). In contrast, the direct linear plot is less widely used; however, it has been statistically evaluated as one of the most reliable methods to determine kinetic parameters (Atkins and Nimmo, 1975). The only disadvantage of this plot is its complexity on graphic presentation. While the Lineweaver-Burk plot uses only a single line (Fig. 8), the direct linear plot requires five lines (Fig. 9). When several lines need to be plotted at the same time (for example, in inhibitor experiments), the direct linear plot is too complicated to display their relationship.

Thus, these plotting methods have both advantages and disadvantages. The best way to determine kinetic parameters might be to use both methods together. On the Lineweaver-Burk plot, the K_m for frog aromatase was found to be 188.1 ± 30.2 nM and the V_{max} was 1.42 ± 0.11 fmol of estrogen produced/min/mg microsomal protein. The direct linear plot of the same data yielded 177.1 ± 47.6 nM and 1.33 ± 0.24 fmol of estrogen produced/min/mg microsomal protein, respectively. Thus there was no difference in these enzyme parameters between

these different methods; therefore, the Lineweaver-Burk plot was used in the present study because of its advantages.

The K_m value of frog aromatase (188.1±30.2 nM) was slightly greater than that of human placental aromatase (123.0 nM) indicating that the frog aromatase has a lower affinity for androstenedione. The V_{max} for frog aromatase was much smaller (1.42±0.11 fmol of estrogen produced/min/mg microsomal protein) than that of placental aromatase (113.57 fmol of estrogen produced/min/mg microsomal protein) indicating that the frog oviduct has much less aromatase activity than human placenta. The testicular aromatase of another amphibian, the urodele *Necturus maculosus* also had slightly greater affinity for androstenedione (K_m=120 nM) than frog oviductal aromatase, but showed much higher activity (V_{max}=62-87 pmol of estrogen produced/min/mg microsomal protein) (Canick *et al.*, 1984). These data show that all these aromatases have similar K_m, but the activities vary widely between tissues.

Although PED effectively reduced estrogen production by human placental aromatase (Fig. 4), it did not inhibit aromatase activity in frog oviduct (Figs. 10 and 11). In contrast, 4-OH-A inactivated the enzyme in a dose-dependent fashion (Fig. 10). The difference in the effectiveness of these inhibitors is probably due to different mechanisms of inhibition. Both PED and 4-OH-A are classified as suicide inhibitors of mammalian aromatase (Brodie *et al.*, 1977; Zimniski *et al.*, 1985; Brandt *et al.*, 1988b). The term "suicide" intimates that these inhibitors irreversibly (covalently) bind to the enzyme after activation by the enzyme itself.

Despite this similarity, inactivation by these inhibitors does not use the same mechanism. PED requires the C-19 position (Appendix 2) to irreversibly bind to aromatase (Metcalf *et al.*, 1981; Covey *et al.*, 1981). On the other hand, the inactivation mediated by 4-OH-A is based on a covalent bond formed at the C-4 position (Covey and Hood, 1982a) (Appendix 2).

There are also differences in the physiological characteristic of PED and 4-OH-A. MacIndoe *et al.* (1982) proposed that 4-OH-A possessed estrogenic activity in cultured human breast cancer cells, while PED did not. In a human ovarian carcinoma, 4-OH-A bound slightly to androgen receptors, whereas PED did not (Brandt *et al.*, 1988b). Thus, it is possible that fundamental differences between these inhibitors were responsible for their different effects on the frog oviductal aromatase.

The ability of 4-OH-A to inhibit frog oviductal aromatase was indicated by the increased slope of the Lineweaver-Burk plot when 4-OH-A was present (Fig. 12). Of the various plots showing different types of inhibition, this plot most resembles that for competitive inhibition. Furthermore, a number of studies have suggested competitive inhibition of aromatase by 4-OH-A (Schwarzel *et al.*, 1973; Brodie *et al.*, 1977; Hsiang *et al.*, 1987). Therefore, competitive inhibition seems to be a reasonable interpretation of the data, although other interpretations are possible since the lines do not intersect on the ordinate.

Assuming competitive inhibition, the K_i (inhibitor dissociation constant) of the frog oviductal aromatase for 4-OH-A was calculated to be 10.7 nM (Rawn,

1989). The K_i for 4-OH-A was 170 nM in human placental aromatase (Covey and Hood, 1982b) and 2.7 nM in aromatase of cultured foreskin fibroblasts (Hsiang *et al.*, 1987). Thus, frog aromatase showed a higher affinity for 4-OH-A than the placental aromatase and a lower affinity than that in fibroblasts. Because the K_m of the frog oviductal aromatase for androstenedione was 188.1 ± 30.2 nM, 4-OH-A can bind more avidly to the aromatase than can the substrate. This higher affinity of aromatase for 4-OH-A than for its substrate agrees with the previous studies in human placenta (Covey and Hood, 1982a, b) and human fibroblasts (Hsiang *et al.*, 1987).

Like 4-OH-A, progesterone inhibited frog oviductal aromatase. The Lineweaver-Burk plot for progesterone revealed noncompetitive inhibition (Fig. 13). This is different from competitive inhibition in that the inhibitor can associate with either a free enzyme or an enzyme-substrate complex (Stryer, 1988; Rawn, 1989). Because the two lines intersect below the abscissa, the inhibitor shows a higher affinity for the free enzyme than for the enzyme-substrate complex. However, it is doubtful if the deviation of the intersection from the abscissa is important. To conclude that the affinities are different would require more studies.

If the intersection falls on the abscissa and the affinities of the inhibitor for a free enzyme and for an enzyme-substrate complex are equal, the K_i of the frog oviductal aromatase for progesterone would be 283.5 nM (Rawn, 1989). Compared with the K_i for 4-OH-A (10.7 nM) and the K_m for androstenedione

(188.1±30.2 nM), this high K_i value for progesterone implies that frog oviductal aromatase has very low affinity for progesterone.

Progesterone is considered to be neither a substrate nor an inhibitor of human placental aromatase (Thompson and Siiteri, 1974b). However, this steroid inhibited frog oviductal aromatase in the present study. A probable interpretation is that progesterone is metabolized by another enzyme in the oviduct, producing a metabolite which acts as the aromatase inhibitor. There are two lines of evidence that support this idea. First, a progesterone metabolite, 16α-hydroxytestosterone, has been shown to inhibit aromatization of androstenedione by human placenta in a noncompetitive fashion (Canick and Ryan, 1976). Second, there is no difference in inhibition between 0.3 and 0.5 μM of progesterone (Fig. 10). The existence of a progesterone metabolite would explain this result quite well. Assuming that there is an oviductal enzyme which converts progesterone to an aromatase inhibitor during incubation, this enzyme might be saturated at a substrate concentration of 0.3 μM. In that case, the same amount of aromatase inhibitor would be produced at both 0.3 and 0.5 μM of progesterone. Therefore, no difference in inhibition would be expected at these two concentrations. If this is the case, the high value of K_i calculated for progesterone is of questionable accuracy because the calculation requires knowing the precise concentration of an inhibitor. In fact, progesterone was a more potent inhibitor than 4-OH-A at 0.3 μM (Fig. 10).

Serum testosterone levels in mature female frogs were 26.1±5.1 ng/ml;

however, these levels varied widely from 5.0 to 50.9 ng/ml. Part of the variability is probably due to the fact that these animals had been maintained in the laboratory at room temperature for about two months. Licht *et al.* (1983) found that stress and captivity caused a dramatic decrease in blood levels of steroids in some bullfrogs. Because all frogs used in this experiment had mature follicles (stage 4 and 5), they should show high serum testosterone levels. The results in the present study were approximately in the same range as the maximum testosterone levels of about 15 ng/ml reported in female *R. esculenta* (D'Istria *et al.*, 1974), and 74.3±16.5 ng/ml reported in *R. catesbeiana* (Licht *et al.*, 1983).

It is impossible to directly compare the K_m value of oviductal aromatase for androstenedione to the serum testosterone level. In human placenta (Bellino and Osawa, 1974; Reed and Ohno, 1976) and *Necturus* testis (Canick *et al.*, 1984), aromatase has a higher affinity for androstenedione than for testosterone. However, it is unknown whether this is true of frog aromatase or not. The properties characterized in the present study suggest that frog oviductal aromatase may be quite different from that of human placenta.

If one assumes that there is no significant difference between K_m values for androstenedione and for testosterone, then one can compare the K_m value to the serum testosterone level. The K_m of oviductal aromatase for androstenedione was 188.1±30.2 nM. This value is quite similar to the highest testosterone level (176.6 nM) suggesting that the oviductal aromatase could function under physiological conditions. In other words, it is possible that estrogens are produced in the

oviduct by local conversion of circulating testosterone.

If the frog oviductal aromatase has a higher affinity for androstenedione than for testosterone, even the highest testosterone level (176.6 nM) is too low for local conversion to occur. Nevertheless, the frog aromatase could function under certain physiological conditions. In mammals, follicle stimulating hormone induced aromatase activity in the ovary (Dorrington *et al.*, 1975; Erickson and Hsueh, 1978; Hsueh *et al.*, 1984; Steinkampf *et al.*, 1987). Pituitary gonadotropin of bullfrog also increased estrogen production in a rat granulosa cell (Dahl *et al.*, 1989). Petrino *et al.* (1990) suggested that pituitary extract activated ovarian aromatase in the killifish, *Fundulus heteroclitus*, and tilapia prolactin enhanced estradiol production in the oocytes of the guppy, *Poecilia reticulata* (Tan *et al.*, 1988). These results suggest that there might be some mechanisms to increase oviductal aromatase activity in frogs. However, it seems unlikely that these mechanisms involve steroid hormones, since Dubowsky (1991) found that the pretreatment of frogs with testosterone or estradiol has no influence on estradiol production by the oviducts during incubation.

In conclusion, the results of the present study provide evidence for the existence of aromatase in the frog oviduct. Final determination of the physiological role of this aromatase requires determination of its K_m for testosterone and more data regarding serum testosterone and androstenedione levels. As discussed before, however, frog oviductal aromatase could function in converting blood androgens to estrogens to stimulate oviduct growth under certain

conditions.

The present study also suggests further studies. 4-OH-A is an effective aromatase inhibitor in frog oviduct *in vitro*. This should also be studied *in vivo*. If growth of the oviduct caused by testosterone is reduced by 4-OH-A, that would confirm that the growth is facilitated by estrogens locally produced from testosterone. In order to clarify the physiological role of progesterone in regulating oviductal aromatase activity, it will be necessary to characterize the metabolites produced from progesterone in the oviduct.

The oviduct plays an important role in the reproductive system of amphibians because it secretes jelly to coat the eggs. This jelly deposit ensures fertilization by capacitating sperm in *R. pipiens* (Shaver, 1966; Shivers and James, 1970; Elinson, 1971; Elinson, 1973). The present study suggests that the oviduct may play another important role for reproduction in this species by playing a role in steroid metabolism.

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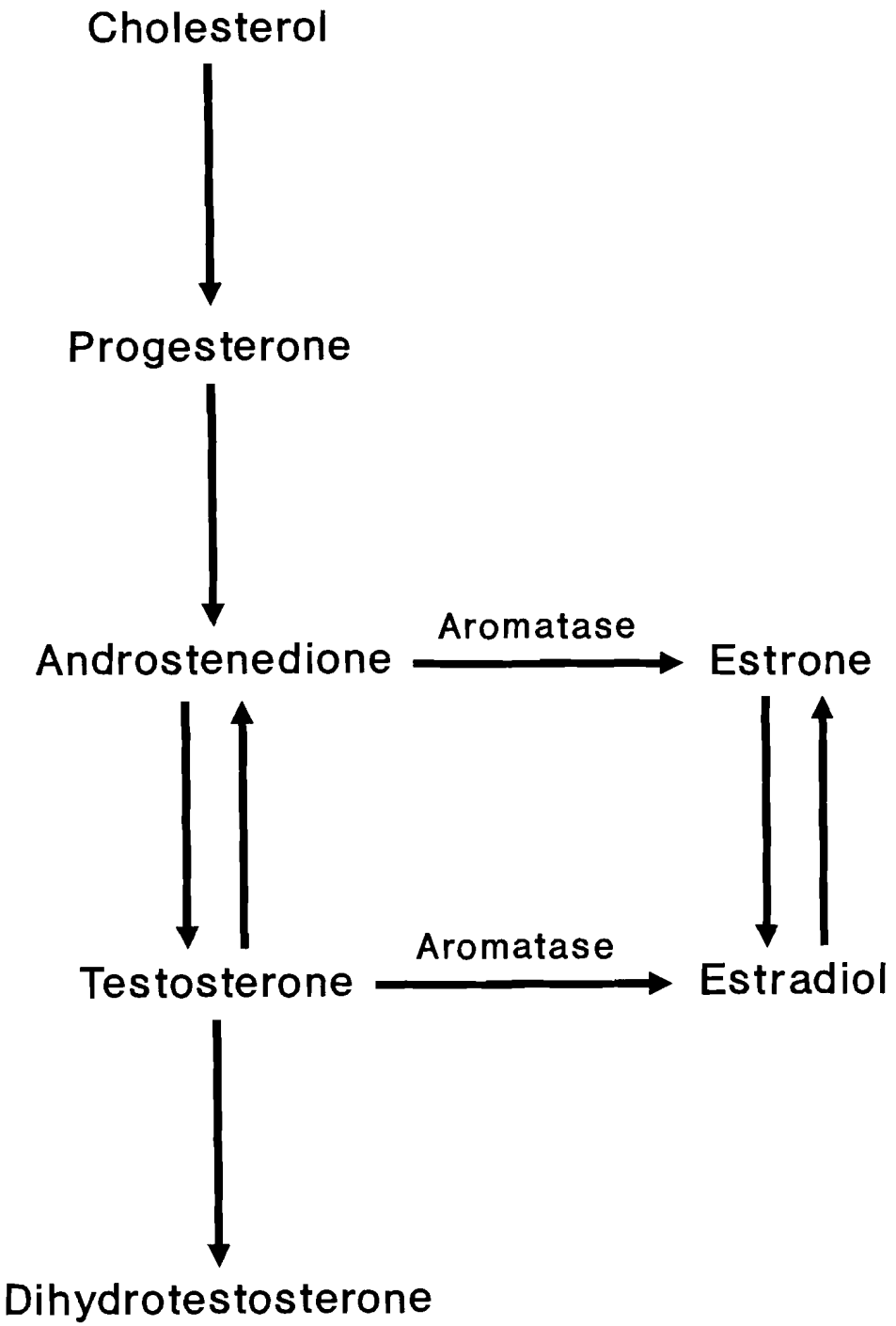
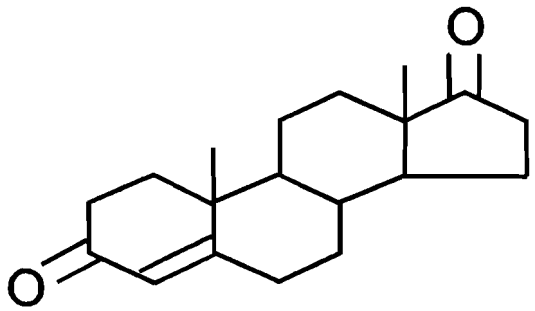
APPENDIXES

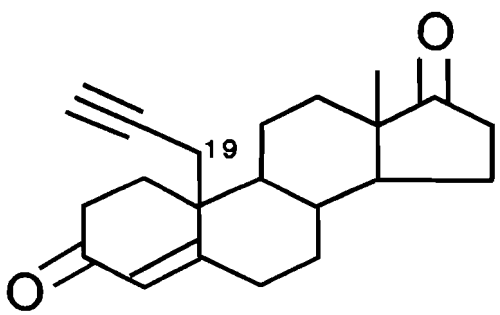
Appendix 1. General biosynthetic pathway from cholesterol to sex steroids. Androstenedione, testosterone, and dihydrotestosterone represent androgens, whereas estrone and estradiol are estrogens. Androstenedione is a precursor for estrone and testosterone is a precursor for estradiol.


Cholesterol
Progesterone
Androstenedione
Aromatase
Estrone
Testosterone
Aromatase
Estradiol
Dihydrotestosterone

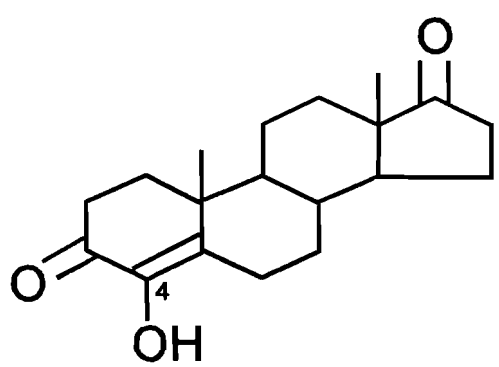
Appendix 2. Chemical structures of androstenedione and aromatase inhibitors. Both PED and 4-OH-A are inhibitors derived from androstenedione.



Androstenedione

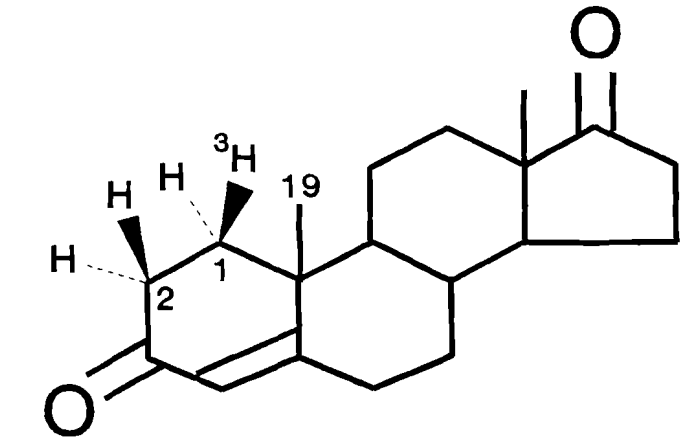


10-Propargylestr-4-ene-3,17-dione
(PED)



4-Hydroxyandrostenedione
(4-OH-A)

Appendix 3. Summarized mechanism of a tritiated water release method for measuring aromatization. [1β-^{3}H(N)]Androstenedione releases its tritium into water through this reaction. The C-19 methyl group is removed as formic acid (Skinner and Akhtar, 1969).

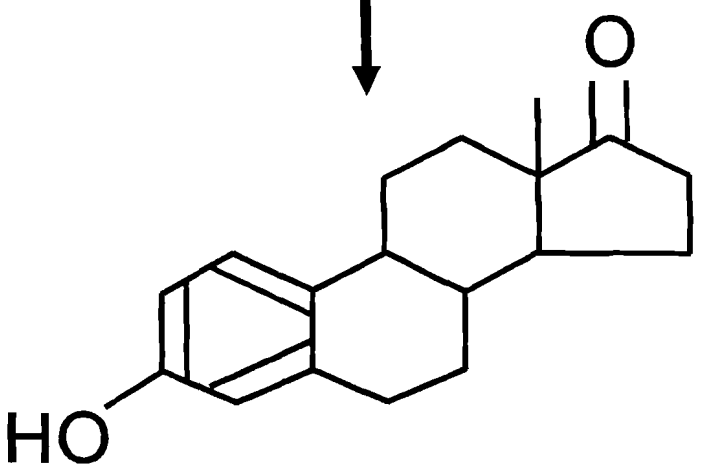


Androstenedione

3 NADPH
+ 3 O_2

Aromatase

3 $NADP^+$
+ HCOOH
+ $[^3H]H_2O$



Estrone

Futoshi Kobayashi
Signature of Graduate Student

Katherine N. Smalley
Signature of Major Advisor

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