STEROIDOGENESIS IN OVARIAN TISSUE OF A VIVIPAROUS TELEOST, THE GUPPY *POECILIA RETICULATA*

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Abstract—1. Homogenates of ovaries of 3- and 12-month-old guppies were incubated with pregnenolone-7α-^{3}H and progesterone-4-^{14}C, and with androstenedione-$1,2$-^{3}H, respectively.

2. From the double-labeled experiment, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, dehydroepiandrosterone, androstenedione and testosterone were isolated and identified by paper- and thin-layer chromatography, derivative formation and by recrystallization to constant specific activity.

3. In the ovary of the guppy the delta-5 pathway in steroidogenesis predominates, as may be concluded fròm the ^{3}H/^{14}C ratios of the identified metabolites.

4. From the androstenedione-$1,2$-^{3}H incubation, ovaries of 12-month-old guppies appeared to be able to synthesize steroids with functional groups in the 11-position (11-ketotestosterone and 11β-hydroxytestosterone).

5. The androgen synthesis in ovaries of older guppies may be considered as a possible explanation of the sex conversion occasionally observed.

INTRODUCTION

IN PREVIOUS studies (Lambert *et al.*, 1971), it was shown that the ovary of the viviparous teleost *Poecilia reticulata* contains aromatizing enzymes, which are able to catalyse the conversions of androstenedione* and testosterone to oestradiol. However, direct evidence for complete steroid synthesis in the ovary of this teleost was not obtained. An incubation experiment with the radioactive precursors ^{3}H-pregnenolone and ^{14}C-progesterone was considered necessary. Such a double-labeled experiment could also be used to discriminate between a delta-4 and a delta-5 pathway in steroid synthesis.

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* The following trivial names are used throughout this paper: progesterone, pregn-4-ene-3,20-dione; pregnenolone, 3β-hydroxypregn-5-ene-20-one; 17α-hydroxyprogesterone, 17α-hydroxypregn-4-ene-3,20-dione; 17α-hydroxypregnenolone, $3\beta,17\alpha$-dihydroxypregn-5-ene-20-one; dehydroepiandrosterone, 3β-hydroxyandrost-5-ene-17-one; androstenedione, androst-4-ene-3,17-dione; testosterone, 17β-hydroxyandrost-4-ene-3-one; adrenosterone, androst-4-ene-3,11,17-trione; 11β-hydroxyandrostenedione, 11β-hydroxyandrost-4-ene-3,17-dione; 11-ketotestosterone, 17β-hydroxyandrost-4-ene-3,11-dione; 11β-hydroxytestosterone, $11\beta,17\beta$-dihydroxyandrost-4-ene-3-one; cortisol, $11\beta,17\alpha,21$-trihydroxypregn-4-ene-3,20-dione; cortisone, $17\alpha,21$-dihydroxypregn-4-ene-3,11,20-trione; oestradiol, $3,17\beta$-dihydroxyoestra-1,3,5(10)-triene; oestrone, 3-hydroxyoestra-1,3,5(10)-triene-17-one.

Another problem in physiology of the fish ovary is its capacity for synthesizing 11-keto- and 11β-hydroxysteroids. 11-Ketotestosterone has a strong androgenic capacity (Idler *et al.*, 1961), and therefore might be considered as a male sex hormone in fish. However, it has also been isolated from ovarian tissue (Eylath & Eckstein, 1969); incubation experiments proved ovaries to be capable of synthesizing 11-ketotestosterone (Eckstein, 1970; Eckstein & Eylath, 1970; Eckstein & Katz, 1971; Reinboth, 1972), and in some cases 11β-hydroxytestosterone (Eckstein and Katz, 1971; Reinboth, 1972). The synthesis of the mentioned steroids could not be demonstrated in ovaries of young guppies (Lambert *et al.*, 1971). It is possible that in older animals the 11-hydroxylation is functioning. For this reason it has been attempted to demonstrate the formation of such steroids in ovaries of virgin guppies which are at least 12 months old.

MATERIALS AND METHODS

Animals

Virgin female guppies (*Poecilia reticulata*) were reared under laboratory conditions at 24°C and artificial light during 12 consecutive hours per day.

Radioactive steroids

The following radioactive steroids, all purchased from New England Nuclear Corp., Boston, Mas., U.S.A.,

were used following purification by thinlayer chromato-
graphy: pregnenolone-7α-³H (sp. act.: 17 Ci/mMol),
androstenedione-1,2-³H (sp. act.: 48 Ci/mMol), pro-
gesterone-4-¹⁴C (sp. act.: 57·3 mCi/mMol), oestradiol-
17β-4-¹⁴C (sp. act.: 52 mCi/mMol), oestrone-4-¹⁴C (sp.
act.: 45·2 mCi/mMol), cortisone-4-¹⁴C (sp. act.: 55
mCi/mMol), cortisol-4-¹⁴C (sp. act.: 55·1 mCi/mMol).

11β-Hydroxyandrostenedione-4-¹⁴C and adreno-
sterone-4-¹⁴C were prepared from cortisol-4-¹⁴C and
cortisone-4-¹⁴C respectively by oxidation with sodium
bismuthate. 11β-Hydroxytestosterone-4-¹⁴C and 11-
ketotestosterone-4-¹⁴C were then prepared from 11β-
hydroxyandrostenedione-4-¹⁴C and adrenosterone-4-¹⁴C
respectively by reduction with sodium borohydride.

Reagents

All chemicals used were of analytical grade; organic
solvents were redistilled once just before use. Cofactors
used in the incubation mixture were obtained from
Boehringer, Mannheim, Germany.

Chromatography

Thin-layer chromatography (TLC) was carried out on
precoated plates (10 × 20 cm) with silica gel, F 254
(Merck, A.G.) or cellulose MN 300, F 254 (Antec, A.G.)
in saturated tanks using the following systems: system 1:
dichloromethane–methanol (97 : 3); system 2: benzene–
acetone (8 : 2); system 3: benzene–methanol (9 : 1);
system 4: benzene–cyclohexane (1 : 1); system 5:
benzene–ethylacetate (7 : 3); system 6: hexane–
ethylacetate (1 : 1); system 7: cyclohexane–ethylacetate
(1 : 3); system 8: ethylacetate–cyclohexane–ethanol (45 :
45 : 10); system 9: toluene–acetone (7 : 3); system 10:
petroleum ether (40–60°C)–tert. butanol (1 : 1); system
11: petroleum ether (40–60°C)–tert. butanol (3 : 1);
system 12: toluene saturated with propylene glycol.

Only cellulose plates were developed in system 12.
The spotted cellulose plates were dipped up to the origin
in a mixture of methanol–propylene glycol (5 : 1), and
allowed to dry for 10 min before development. After
chromatography, the 3-keto-Δ⁴-steroids were located by
u.v. absorption, whereas the other steroids or derivates
were detected by spraying with primuline according to
Wright (1971).

Paper chromatography (PC) was carried out on
Machery and Nagel M.N. 263 ff. paper (Düren,
Germany) in the system petroleum ether (40–60°C)–
methanol–water (5 : 4 : 1). Steroids were detected by
absorption of u.v. light.

Gas–liquid chromatography (GLC) was applied in the
quantification of the steroids during the recrystallization
procedure, by using a Hewlett–Packard 402 gas chro-
matograph with a flame ionization detector and a 4 ft
3% SE 30 column.

Microchemical reactions

Oxidation was brought about with chromium trioxide
according to Eckstein (1970).

Methylation was carried out with dimethyl sulphate
as described by Brown *et al.* (1957).

Formylation was performed by dissolving the dry
steroid in formic acid 98% (0·5 ml). After 2 hr at room
temperature the formylation is completed.

Saponification of steroid formates was carried out
with sodium hydroxide according to Bush (1961).

Measurement of radioactivity

Samples were essayed using a Nuclear Chicago Mark I
scintillation counter with a scintillator of PPO (4 g)
and POPOP (40 mg) in toluene (1 l.). Radioactive areas
on TLC plates and PC papers were located by means of
a Berthold thin layer and paper radiogram scanner.

Incubation procedure

The ovaries were removed from decapitated guppies,
pooled, weighed and homogenized at 0°C in a medium
(1 ml/100 mg tissue) containing per litre aqua dest.:
NaCl (11·69 g), KCl (0·37 g), $CaCl_2$ (0·44 g), $MgCl_2$
(0·41 g), $NaHCO_3$ (0·08 g), $KHCO_3$ (0·14 g), glucose
(0·10 g), nicotinamide (0·12 g) and fumaric acid (0·01 g).
Appropriate quantities of labeled precursors and co-
factors (ATP, NAD, NADP, NADH and NADPH;
2 μMol of each) were prepared in 25 ml incubation vials,
to which the homogenates were added. Incubations were
carried out at 25°C in an air atmosphere or in a 95%
O_2 and 5% CO_2 gas phase, with continuous shaking.
After 1 hr, additional quantities (2 μMol) of cofactors
were added and after a total of 2½ hr the enzyme reactions
were terminated by the addition of dichloromethane
(10 ml).

Extraction and fractionation

Labeled and unlabeled carriers were added to the
incubation mixture before extraction with dichloro-
methane (3 × 10 ml). The combined dichloromethane
extracts were evaporated *in vacuo* and the residue
dissolved in aqueous methanol (70%; 8 ml) and stored at
−18°C overnight to precipitate triglycerides. Following
centrifugation the supernatant (the steroid fraction) was
evaporated to dryness, and then fractionated into a
phenolic and a neutral fraction (see Lambert *et al.*, 1971).

RESULTS

I. *Incubation with pregnenolone-7α-³H and Pro-
gesterone-4-¹⁴C as precursors*

The homogenate of the ovaries (190 mg) of eight,
4-month-old virgin guppies was incubated with ³H-
pregnenolone (4·99 μCi; 10·1 μg) and ¹⁴-C-progeste-
rone (1·15 μCi; 6·3 μg) in the presence of cofactors
in an atmosphere of 95% O_2 and 5% CO_2. After the
incubation the following steroids (100 μg of each)
were added as carriers: pregnenolone, progesterone,
17α-hydroxypregnenolone,   17α-hydroxyprogeste-
rone,   dehydroepiandrosterone,   androstenedione,
testosterone, oestradiol and oestrone. Following
the extraction and removal of triglycerides the
steroid extract was fractionated into a phenolic
fraction and a neutral fraction.

Phenolic fraction

Following TLC in system 3 the oestradiol and
oestrone fractions were methylated. After TLC in
system 8 it appeared that the ³H as well as the ¹⁴C

activity, remaining in the methylated carriers of oestradiol and oestrone, were too low to permit further examination.

Neutral fraction

For a first separation the neutral fraction was subjected to PC. After developing, only the areas corresponding with the added carriers were eluted i.e. (1) 17α-hydroxypregnenolone, 17α-hydroxyprogesterone and testosterone; (2) dehydroepiandrosterone; (3) androstenedione; (4) pregnenolone; (5) progesterone. TLC in system 2 of the first fraction effected a separation in a combined 17α-hydroxypregnenolone and testosterone fraction and in a 17α-hydroxyprogesterone fraction. After TLC in system 1, it was possible to separate 17α-hydroxypregnenolone from testosterone. The individual steroid fractions were further purified by TLC, derivative formation and crystallization.

17α-Hydroxyprogesterone. The fraction containing the carrier 17α-hydroxyprogesterone was subjected to the procedure of formylation. The ^{3}H,^{14}C-compound remained unaffected and showed an R_f identical to authentic 17α-hydroxyprogesterone after TLC in system 1. Finally, the ^{3}H,^{14}C-compound showed a constant specific activity after repeated crystallizations (Table 1).

17α-Hydroxypregnenolone. The fraction was formylated and the radioactive material showed the same mobility on TLC in system 1 as authentic 17α-hydroxypregnenolone formate. After saponification and a renewed TLC in the same system, the tritium active area appeared to correspond with carrier 17α-hydroxypregnenolone.

Dehydroepiandrosterone. The dehydroepiandrosterone fraction obtained after PC was separated into three radioactive areas after a TLC in system 1. One of these active areas corresponds with carrier dehydroepiandrosterone. Following a second TLC in system 7, the active dehydroepiandrosterone fraction was eluted and divided into three portions for oxidation, formylation and crystallization. After oxidation and formylation, and after TLC in system 1 the tritium active products showed an R_f

identical to androstenedione and dehydroepiandrosterone formate respectively. Moreover, a constant specific activity was reached after repeated crystallizations (Table 1).

Androstenedione. TLC in system 1 of the androstenedione fraction resulted in at least three radioactive areas, one of which corresponds with authentic androstenedione. The androstenedione area was eluted and the active compound appeared to resist oxidation and formylation. Finally, the substance showed a constant specific activity after repeated crystallizations (Table 1). The purified androstenedione was mainly tritium labeled; the ^{3}H/^{14}C ratio is 33.

Testosterone. Formylation of the fraction containing the carrier testosterone yielded a compound with the same mobility as authentic testosterone formate after TLC in system 1. This formate was saponified, and after TLC in system 10 the radioactive product showed a similar chromatographic behaviour as authentic testosterone. After recrystallization to constant specific activity (Table 1), a ^{3}H activity was the only one to be detected.

II. Incubation with androstenedione-1,2-^{3}H as precursor

Homogenized ovarian tissue (400 mg) of six, 12-month-old virgin guppies was incubated with ^{3}H-androstenedione (6·7 μCi) in the presence of cofactors in an air atmosphere. After incubation the following labeled carriers were added: 11β-hydroxytestosterone-4-^{14}C (8·93 nCi, 50 μg); 11-ketotestosterone-4-^{14}C (7·63 nCi, 50 μg); 11β-hydroxyandrostenedione-4-^{14}C (7·89 nCi; 50 μg); adrenosterone-4-^{14}C (6·21 nCi, 50 μg); oestrone-4-^{14}C (6·73 nCi, 50 μg); oestradiol-4-^{14}C (8·04 nCi, 50 μg).

Following extraction and removal of triglycerides, the steroid extract was subjected to a phenolic separation procedure.

Conversion to ^{3}H-oestrogens

The phenolic fractions could be separated with TLC in system 3 into two major radioactive areas corresponding with oestradiol and oestrone.

Table 1. The last three crystallizations of steroids isolated from an incubation of ovarian tissue of *P. reticulata* with pregnenolone-7α-^{3}H and progesterone-4-^{14}C

Steroid		Specific activity (dis/min per mg)		
		Crystallization 1	Crystallization 2	Crystallization 3
17α-Hydroxyprogesterone	^{3}H	4038	3304	3377
	^{14}C	2603	2214	2282
Dehydroepiandrosterone	^{3}H	2347	1830	1804
	^{14}C	—	—	—
Androstenedione	^{3}H	1478	1386	1391
	^{14}C	53	47	42
Testosterone	^{3}H	712	643	625
	^{14}C	—	—	—

Table 2. ^{3}H/^{14}C ratios of steroids and their derivatives from an incubation of ovarian tissue of
P. reticulata with androstenedione-1,2-^{3}H

Steroid	^{3}H/^{14}C ratios			
	Free	Formylated	Oxidized	Methylated
Oestradiol	7·98	7·83		7·61
11β-Hydroxytestosterone	0·66	0·57	0·55	
11-Ketotestosterone	2·13	1·96	2·04	

Oestradiol. Part of the ^{3}H-^{14}C material, behaving chromatographically like oestradiol, was methylated. On TLC in system 6 the ^{3}H-^{14}C product had the same mobility as authentic oestradiol monomethyl ether. Another portion of the "oestradiol" fraction was formylated and after TLC in system 5 the ^{3}H-^{14}C product showed an R_f value identical to authentic oestradiol diformate. The ^{3}H/^{14}C ratios of the two derivatives appeared to correspond (Table 2), and from the average of these ratios the total production was calculated. The percentage of androstenedione converted to oestradiol ($\pm 0.2\%/100$ mg tissue) was 0·9.

Oestrone. The "oestrone" fraction was also partly formylated and partly methylated. The radioactivity in the areas corresponding with the derivatives after TLC in system 3 (oestrone formate) and in system 6 (oestrone–methyl ether) appeared to consist mainly of ^{14}C activity. The amount of ^{3}H-oestrone was too low to allow further examination.

Conversion to 11β-hydroxy- and 11-ketosteroids

The neutral fraction was first subjected to TLC in system 4 in order to remove fatty material, and then separated into four fractions by TLC in system 1. The four fractions, corresponding with the four added labeled carriers, were eluted and further purified as follows.

11β-hydroxytestosterone. The fraction was purified first by TLC in system 9 (running twice) and then by TLC on cellulose plates in system 12. The ^{3}H,^{14}C material, corresponding in R_f value with authentic 11β-hydroxytestosterone, was then partly oxidized. After TLC in system 1, the ^{3}H,^{14}C product had the same mobility as authentic 11β-hydroxyandrostenedione. Another portion was formylated and after TLC in system 1 the chromatographic behaviour was the same as authentic 11β-hydroxytestosterone formate. From the free steroid and both derivatives, the ^{3}H/^{14}C ratios were established (Table 2); from the average of the ratios it was calculated that ± 0.02 per cent of androstenedione was converted to 11β-hydroxytestosterone.

11-Ketotestosterone. This fraction was chromatographed first by TLC in system 10 and then by partition chromatography on cellulose plates (system 12). Oxidation of the ^{3}H,^{14}C material, eluted after partition chromatography, yielded a ^{3}H,^{14}C com-

pound with the same mobility as authentic adrenosterone on TLC in system 1. Formylation of the above-mentioned ^{3}H,^{14}C material and subsequent TLC in system 1 showed a ^{3}H,^{14}C product with an R_f identical to that of authentic 11-ketotestosterone formate. From the ^{3}H/^{14}C ratios (Table 2) the total amount of produced 11-ketotestosterone was calculated; 0·2 per cent of androstenedione was converted to 11-ketotestosterone.

11β-Hydroxyandrostenedione, adrenosterone. These fractions were purified by TLC in system 11. A second chromatography was carried out on cellulose plates in system 12. After these two purification steps, the levels of ^{3}H activity remaining with each steroid appeared to be too low to permit further examination.

DISCUSSION

In the ovary of the guppy, *P. reticulata*, steroidogenesis takes place in the granulosa cells of growing follicles (Lambert, 1970; Lambert & van Oordt, 1974). Two different incubation experiments were carried out to investigate some pathways in steroidogenesis. Firstly, homogenized ovarian tissue of 3-month-old virgin guppies was incubated with ^{3}H-pregnenolone and ^{14}C-progesterone, to study which metabolic pathway is functioning the delta-5 route, or the delta-4 route. A second incubation was carried out with ovarian homogenates of 12-month-old virgin guppies, with ^{3}H-androstenedione as the precursor, to determine whether the ovary is able to synthesize steroids with functional groups in the 11-position.

The results of the ^{3}H-pregnenolone and ^{14}C-progesterone incubation demonstrate that the ovary of young guppies contains enzyme systems for synthesizing steroids by the delta-5 as well as the delta-4 pathway. The synthesis by the delta-5 route follows from the isolation of tritiated dehydroepiandrosterone. The isolation of ^{14}C-labeled androstenedione indicates the possibility of synthesizing steroids by the delta-4 route. The delta-4 route does not seem to be the principal pathway in the ovary of the guppy, for the amount of ^{14}C-androstenedione is low in comparison with the amount of ^{3}H-androstenedione (the ^{3}H/^{14}C ratio is 33). Moreover, in the testosterone fraction, tritium activity was the only one to be detected.

From the differences of the $^3H/^{14}C$ ratios of 17α-hydroxyprogesterone ($^3H/^{14}C$: 1·5), and androstenedione ($^3H/^{14}C$: 33) it may be concluded that conversion of 17α-hydroxyprogesterone to androstenedione seems to be the rate-limiting step. Thus, in the ovary of the guppy, steroidogenesis mainly operates by the delta-5 pathway. Eckstein (1970) postulated that in the ovary of *Tilapia aurea*, steroidogenesis may take place via three different routes, i.e. a presumably predominating delta-5 route, the delta-4 route and a third route, where progesterone may be converted to testosterone without androstendione as an intermediate. In the guppy, there are no indications for this third pathway. Although we have not succeeded in demonstrating a synthesis of oestradiol in this experiment, it may be concluded that ovaries of 3-month-old guppies should be able to synthesize oestradiol with pregnenolone as precursor, since it not only transforms the latter into androstenedione but it is also able to convert androstenedione to oestradiol (Lambert *et al.*, 1971).

The results of the androstenedione incubation seem to prove that the ovaries of old guppies are capable of synthesizing 11β-hydroxy- and 11-ketosteroids; tritiated 11β-hydroxytestosterone as well as tritiated 11-ketotestosterone have been isolated. A synthesis of 11β-hydroxy-androstenedione and adrenosteron, however, has not been demonstrated. Although an interpretation of quantitative findings of *in vitro* studies is difficult, the obvious predominance of 11-ketotestosterone indicates that it is the most important one from all steroids with a functional group in the 11-position synthesized in the ovaries of 12-month-old guppies. A transformation of androstenedione to oestradiol by homogenates of the ovaries from older guppies was also established; per 100 mg of ovarian tissue 0·2 per cent of androstenedione appeared to be converted to oestradiol. The recent results obtained with ovaries of 12-month-old guppies are different from the results of the androstenedione incubation with homogenates of ovaries from 3-month-old animals (Lambert *et al.*, 1971). None of the above-mentioned steroids with a functional group in the 11-position has been demonstrated after incubation of young ovaries with ^3H-androstenedione; the conversion of androstenedione into oestradiol was as high as 0·5% per 100 mg tissue. These differences seem to prove that steroid metabolism in the ovary changes during aging of virgin guppies. Oestrogen synthesis (oestradiol) decreases, whereas androgen synthesis (11-ketotestosterone) increases. The production of typical fish androgens, such as 11-ketotestosterone and 11β-hydroxytestosterone, in the ovaries of old guppies might be the explanation of sex conversion, occasionally observed in older female guppies.

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