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Steroidogenesis in the Ovary of the Rainbow Trout, *Salmo gairdneri*, During the Reproductive Cycle

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Homogenates of ovaries of the rainbow trout were incubated with pregnenolone-7α-^{3}H and androstenedione-7α-^{3}H in four different stages of the reproductive cycle. During previtellogenesis delta-4 steroids predominated, but during vitellogenesis a shift caused mainly delta-5 steroids to be present at the end of exogenous vitellogenesis. Conversion of androstenedione into oestrone and oestradiol-17β was observed mainly during the period of endogenous vitellogenesis and at the beginning of the exogenous vitellogenesis.

Introduction

For oviparous vertebrates it has been suggested that ovarian steroids (e.g. oestradiol-17β) may trigger the liver to synthesize yolk proteins (for review: Clemens 1974, Tata 1976). There are indications that this also applies to teleosts. Physiological studies have revealed that in some teleosts E_2* increases the yolk protein content of plasma and liver. The same effect of E_2 on yolk protein synthesis has also been observed in *Salma gairdneri* (Van Bohemen; in prep.).

Although E_2 was demonstrated in the plasma of the rainbow trout (Schreck et al. 1973, Fostier et al. 1978, Lambert et al. 1978), no data are available on oestrogen synthesis by the ovary. For this reason, the steroid synthesis has been studied by incubating ovarian homogenates with tritiated pregnenolone to assess Δ-5 and Δ-4 pathway steroids,

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*The following abbreviations and trivial names are used in this paper.

P_5	: (pregnenolone)	3β-hydroxypregn-5-ene-20-one
17α-P_5	: (17α-hydroxypregnenolone)	3β, 17α-dihydroxypregn-5-ene-20-one
DHA	: (dehydroepiandrosterone)	3β-hydroxyandrost-5-ene-17-one
A-diol	: (androstenediol)	3β, 17β-dihydroxyandrost-5-ene
P_4	: (progesterone)	pregn-4-ene-3,20-dione
17α-P_4	: (17α-hydroxyprogesterone)	17α-hydroxypregn-4-ene-3,20-dione
A-dione	: (androstenedione)	androst-4-ene-3, 17-dione
T	: (testosterone)	17β-hydroxyandrost-4-ene-3-one
DOC	: (desoxycorticosterone)	21-hydroxypregn-4-ene-3, 20-dione
17α, 20β-dihydro-P_4	: (17α-hydroxy-20β-dihydroprogesterone)	17α, 20β-dihydroxypregn-4-ene-3-one
E_1	: (oestrone)	3-hydroxyoestra-1,3,5(10)-triene-17- one
E_2	: (oestradiol-17β)	3, 17β-dihydroxyoestra-1, 3, 5 (10)-triene

and with tritiated androstenedione to determine the oestrogen-synthesizing capacity. These incubations were carried out in triplicate in four different months of the reproductive cycle, namely March, the period of previtellogenesis, May, the period of endogenous vitellogenesis, August, during exogenous vitellogenesis, and November, at the end of the vitellogenesis.

Materials and Methods

Animals

Adult female specimens of the trout (*Salmo gairdneri*) were obtained from a hatchery in Vaassen (the Netherlands). After anaesthesia with MS 222, blood samples were taken to be assayed for oestradiol-17β (Lambert et al. 1978), and the ovaries removed and prepared for *in vitro* incubations to study steroidogenesis.

Radioactive steroids

Pregnenolone-7α-^{3}H (spec. act. : 15 Ci/mmol) and androstenedione-7α-^{3}H (spec. act. : 8.2 Ci/mmol) from The Radiochemical Centre, Amersham (UK) were purified by thin-layer chromatography before use.

Reagents

All chemicals were of analytical grade; organic solvents were redistilled once just before use. Co-factors used during the incubtion 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 and Macherey-Nagel) in saturated tanks, with the following systems: (*I*) toluene-cyclohexane (1 : 1); (*II*) benzene-ethylacetate (3 : 1); (*III*) diisopropylether-chloroform-hexane (7:2:1); (*IV*) dichloromethane-methanol (97 : 3), (*V*) chloroform-acetic acid (10 : 1); (*VI*) toluene-ethylacetate (3 : 1); (*VII*) chloroform-ethanol (9 : 1). After developing the plates, the 3-keto-Δ^4-steroids were located by UV absorption, whereas the other steroids were detected by spraying with primuline (Wright 1971).

Gas-liquid chromatography (GLC) was applied in the quantification of the carrier steroids to determine losses during the isolation and purification procedures by using a Hewlett-Packard gaschromatograph with a flame ionization detector and a 6ft 3% OV 1 column.

Microchemical reactions

Formylation was carried out by dissolving the dry steroid in formic acid 98% (0.5 ml). After 2 hr at room temperature the formylation is completed.

Measurement of radioactivity

Samples were assayed by using a Searle Analytic 92 scintillation counter with a scintillator of PPO (4 g) and POPOP (40 mg) in toluene (1 *l*). Radioactive areas on TLC plates were located by means of a Berthold thin-layer radiochromatogram scanner.

Recrystallization

Recrystallizations to constant specific activity were carried out according to Axelrod et al. (1965) by using aqueous methanol as solvent. Quantification of the crystals was carried out with a Mettler ME 30 balance.

Calculation of yields

The yields were calculated with two methods:

(le) *Percentage distribution method*—With the aid of the radiochromatograms it is possible to determine the percentage distribution of the tritiated compounds and the relative value of each of these compounds after each TLC. Subsequent calculations of these relative values result in the percentage

yield of the steroid fractions before crystallization. From a comparison of the specific activity before and after crystallization the degree of purity can be calculated, and the percentage yield of the purified ^{3}H-steroids can be determined.

(2e) *Recovery determination method*—The percentage yields have been calculated on the basis of precursor initial radioactivity and corrected for procedural losses by recovery determination by GLC of the corresponding cold carriers.

Incubation procedure

The ovaries were removed, weighed and homogenized at 0°C in 0.1M phosphate buffer *p*H 7.4 containing 0.25M sucrose (0.25 g tissue/ml; in November this ratio was corrected to 2.5 g/ml to compensate for the increased amount of yolk proteins). Following centrifugation (800 g) 1 ml of the supernatant was transferred into a 25 ml Erlenmeyer flask containing ^{3}H-androstenedione (3 μCi in 0.5 ml propyleneglycol) or ^{3}H-pregnenolone (3.5 μCi in 0.5 ml propyleneglycol), the cofactors NAD, NADP and NADPH (final concentration 2 mm of each) and 5 ml phosphate buffer (0.1 M, *p*H 7.4). Incubations were carried out at 25°C in an air atmosphere under continuous shaking, After 2 hr the enzyme reactions were terminated by adding dichloromethane (10 ml.)

Extraction

Known amounts of unlabelled carriers (± 100 μg) were added to the incubation mixture before extraction with dichloromethane (3×10 ml). The combined dichloromethane extracts were evaporated *in vacuo* and the residue was subjected to TLC in system I (3×) to separate apolar compounds (triglycerides) from steroids, In this system the steroids remain localized on the base line, so it is possible to use the same plate for a first separation of the steroids.

Results

Incubation with pregnenolone 7α-^{3}H as precursor

After incubation the following steroids were added as carriers; P_5, P_4, 17α-P_5, 17α-P_4, DHA, A-dione, A-diol, T and DOC. The first separation of steroids was carried out by TLC in system II (3×). The areas corresponding with the added carriers representing more than 90% of the total radioactivity, were eluded, e.g. (*A*) 17α-P_5, 17α-P_4, A-diol, T and DOC; (*B*) P_5, DHA and A-dione; (*C*) P_4. The remaining 10% of the total radioactivity consists of polar compounds. Fraction A could be separated with TLC in system IV into 5 fractions: (*i*) 17α-P_5 and A-diol, (*ii*) T, (*iii*) 17α-P_4, (*iv*) DOC, and (*v*) unknown. After formylation and TLC in system IV, it was possible to separate 17α-P_5 formiate and A-diol diformiate Fraction B was separated with TLC in system IV into 2 fractions: (*i*) P_5 and DHA and (*ii*) A dione. TLC in system VI effected a separation of P_5 and DHA. Finally, the isolated ^{3}H-compounds corresponding with the added carriers were purified by repeated crystallization to a constant specific activity. The method of isolation and purification as well as the percentages of yield after each purification step, as calculated by the percentage distribution method, are summarized in figure 1. The percentages of yield in figure 1 are based on the test-animal in each investigated period. The individul values of the three test-animals, studied in each period, have been averaged. The final percentages of yield of the synthesized steroid, during the four investigated periods, were calculated by averaging the results of the percentage distribution method and the recovery determination method (table 1).

During this experiment the conversion of pregnenolone into corticosteroids and 17α-20β-dihydro-P_4 has also been studied. With the exception of DOC, the corticosteroids e.g.

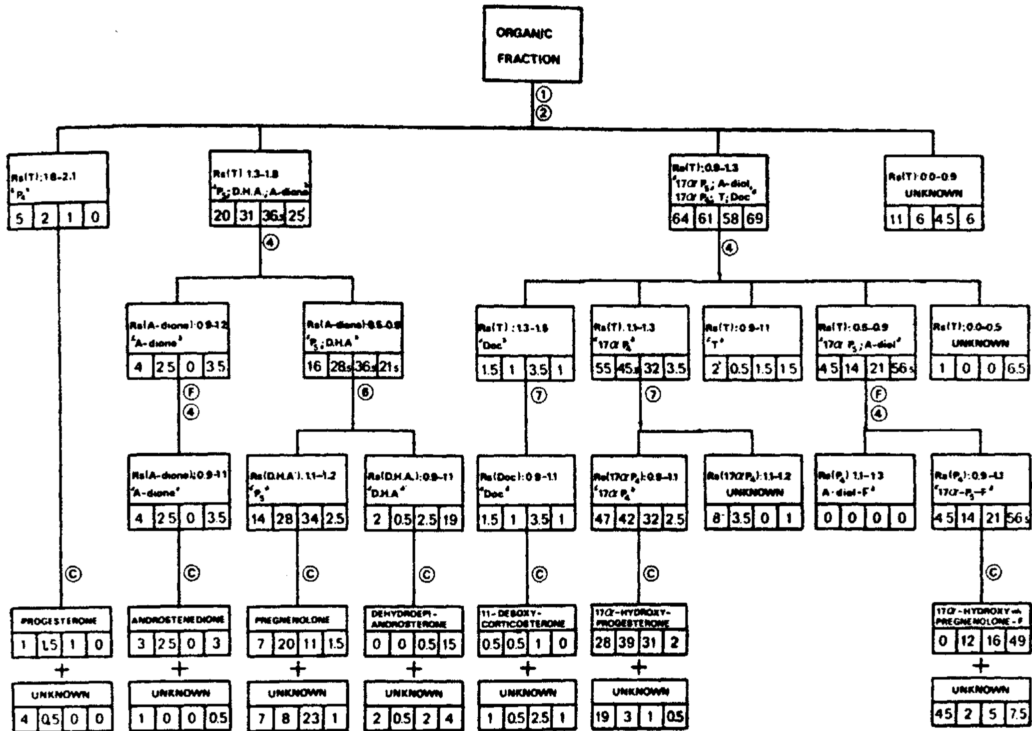

Figure 1 Schematic representation of the isolation and purification procedure of the ³H-pregnenolone incubation. The results of the percentage distribution method for the consecutive TLC's are given for one animal in each investigated period—March, May, August and November respectively. 1, 2...TLC system 1,2 etc; *F*, formylation; *C*, crystallization.

Table 1 *Percentages of yield of steroids isolated from an incubation of ovarian tissue of* Salmo gairdneri *with pregnenolone-7α-³H*

	Percentage of yield			
	March	May	Aug.	Nov.
Pregnenolone	9.5	20.5	14	2.5
17α-Hydroxypregnenolone	0.5	14.5	15	46.5
Dehydroepiandrosterone	0.5	1	1.5	16
Androstenediol	0	0	0	0
Progesterone	1	1.5	1	0
17α-Hydroxyprogesterone	28	41.5	32	1.5
Androstenedione	3	3.5	3.5	2
Testosterone	2	0.5	1	0.5
Desoxycorticosterone	2.5	0.5	0.5	0.5
Unknown	53	16.5	31.5	30.5

corticosterone, desoxycortisol, cortisol and cortisone, as well as 17α, 20β-dihydro-P_4 could be expected to be present in the polar fraction after the first TLC in system II. From these steroids, only traces of cortisone have been detected, mainly in the November animals.

Incubation with androstenedione-7α-³H as precursor

After the incubation the following steroids were added as carriers : A-dione, T, E_1 and E_2. The first separation was carried out with TLC in system III and effected a separation of the four added carriers. The fractions containing the carriers of A-dione and T were recrystallized to a constant specific activity. The separate fractions of E_1 and E_2

were consecutively subjected to TLC in systems IV and V. Finally, the fractions were purified by repeated crystallization up to a constant specific activity. After averaging the results of both quantification methods, the final percentages of yield are presented in table 2.

Table 2 *Percentage of yield of steroids isolated from an incubation of ovarian tissue of* Salmo gairdneri *with androstenedione-7α-^{3}H*

	Percentage of yield			
	March	May	Aug.	Nov.
Androstenedione	56.5	66.5	70	20
Testosterone	22	8	3.5	3
Oestrone	0	2.5	6.75	0.25
Oestradiol-17β	0	7	3.25	2.5
Unknown	21.5	14.5	13.5	74.25

Discussion

Enzyme cytochemical data show that in the ovary of the trout, *Salmo gairdneri*, steroidogenesis takes place in the granulosa cells of oocytes in the beginning of exogenous vitellogenesis, and in interstitial (stromal) cells (Lambert et al. 1978). To investigate the possible pathways, incubation experiments *in vitro* have been carried out. Ovarian homogenates were incubated with ^{3}H-P_5 and ^{3}H-A-dione, respectively, in four different months during the annual reproductive cycle; in March during previtellogenesis, in May during endogenous vitellogenesis, in August at the beginning of exogenous vitellogenesis, and in November at the end of vitellogenesis.

After P_5 incubation, it was found that the ovary was capable of synthesizing the main Δ-5 and Δ-4 steroids during all physiological stages. A-diol production, however, has not been detected. Although these steroids were produced throughout the year, it appeared that production was different during the various stages. The conversion to progesterone is low in the four periods. This may be due to a rapid conversion to other products, for instance to 17α-P_4, or to a lack of P_5-dependent 3β-hydroxysteroid dehydrogenase (3β-HSD). Since shorter incubations do not increase the yield of P_4, a lack of 3β-HSD activity is the most plausible explanation. During the first three periods the Δ-4 steroids, i.e. 17α-P_4 and A-dione, predominated, but at the end of vitellogenesis, Δ-5 steroids were predominant, i.e. 17α-P_5 and DHA. The beginning of this change can be noticed in May-August, when 17α-P_5 and DHA are present in minor amounts. The ratio between Δ-5 steroids and Δ-4 steroids increases five-hundred times during vitellogenesis. This is probably due to a decreasing activity of the enzyme 3β-HSD.

It has been suggested that 21-hydroxylated steroids, especially DOC (Goswami & Sundararaj 1971) and 17α, 20β-dihydro-P_4 (Jalabert 1976) may act as oocyte maturation inducing substances. In some species the synthesis of 21-hydroxylated steroids could be established (Colombo et al. 1973, Colombo et al. 1978), in other species, however, the 21-hydroxylating enzymes seem to be absent (Colombo & Colombo-Belvedere 1976, Ungar et al. 1977, Lambert 1978). In the trout, *Salmo gairdneri*, a DOC synthesis can be detected, but the conversion to DOC is so low, especially towards the end of vitellogenesis, that a physiological function during maturation is not likely. A synthesis to 17α, 20β-dihydro-P_4 has not been detected.

With regard to oestrogen synthesis, it may be mentioned that the ovary of the trout contains aromatization enzymes—as E_2 and E_1 were both synthesized—but this is not so during all physiological stages. The highest conversion rate into oestrogens (11–13%) was noticed from May through August. In March, during previtellogenesis, oestrogen synthesis is hardly noticeable. In May, during endogenous vitellogenesis, the main oestrogen is E_2 (7%) but in August, at the beginning of exogenous vitellogenesis, the

ratio between E_2 and E_1 changed to the advantage of E_1 (6.75%). In November minor amounts of E_2 (2.5%) were detected, this might be due to a dilution of the labelled precursor with endogenous A-dione. But since ^{3}H-A-dione is converted very rapidly, the reduction in oestrogen synthesis towards the end of vitellogenesis is probably due to a decreasing activity of the aromatization enzymes. In addition, the activity of the enzyme 3β-HSD decreases, as in the same period mainly Δ-5 steroids are synthesized. The decrease in 3β-HSD activity is also confirmed by the results of *in vitro* incubations with ^{3}H-DHA as precursor. Conversion into A-dione was low in the November period (unpublished data).

From these results it may be concluded that the ovary of the trout is capable of synthesizing oestrogens mainly during the onset of vitellogenesis. As the granulosa cells contain 3β-HSD during this period only, it is likely that these cells are involved in the oestrogen synthesis. These findings are in agreement with the hypothesis that oestrogens may trigger the liver to synthesize yolk proteins (for review: Clemens 1974, Tata 1976).

Remarkable, however, is that during the period of low oestrogen synthesis in the ovary (November) the plasma levels of E_2 are high (Lambert et al. 1978). From January to June, during previtellogenesis, plasma levels are low (1.3 ± 0.2 ng/ml). At the beginning of the period of exogenous vitellogenesis, the E_2 level rose and a maximum (16.9 ± 0.2 ng/ml) was reached at the end of vitellogenesis in November some weeks before spawning. The above contradiction leads to the assumption that in trout other organs are involved in oestrogen synthesis too. Preliminary results have demonstrated that interrenal tissue, liver, fat tissue, blood and muscle tissue do not contain aromatization enzymes. The brain, however, shows *in vitro* a rapid conversion of A-dione into oestrogens, E_2 as well as E_1. This means that the brain might be responsible for the high level of E_2; an explanation that is only valid if the brain is capable of E_2 synthesis and also of E_2 secretion. The secretion of E_2 by the brain is a subject for further investigation.

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