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Metabolism of C_{19}-steroids in testicular tissue of the newt *Triturus vulgaris*, during and after breeding

T. Teräväinen, E. Antila and A. Saure

Department of Medical Biology, University of Helsinki, Siltavuorenpenger 20 A, SF-00170 Helsinki 17 (Finland), 18 April 1983

Summary. Incubation studies with testicular homogenates of *Triturus vulgaris* showed that testosterone was quantitatively the most important metabolite of dehydroepiandrosterone and androstenedione during breeding. After breeding the recovery of testosterone declined. This suggests that 17β-hydroxysteroid dehydrogenase may be regulated for the needs of testicular androgen production.

The presence of $\Delta^5 3\beta$-hydroxysteroid dehydrogenase ($\Delta^5 3\beta$HSD), 17α-hydroxylase, 17–20 desmolase and 17β-hydroxysteroid dehydrogenase (17βHSD) has been shown in anuran and urodelan testis[1–7]. Furthermore 5α-reductase activity has been demonstrated in anuran testicular tissue[1,4,6,7].

Studies on seasonal variation of plasma steroid concentrations have shown that testosterone levels were maximal from December to March in *Rana esculenta*[8]. In *Bufo mauritanicus* both testosterone and 5α-dihydrotestosterone (5α-DHT) were high during breeding[9]. Incubation studies have indicated that testes of *Rana temporaria*[1,7], *Discoglossus pictus*[4] and *Bufo marinus, Rana catesbeiana* and *R. esculenta*[6] are capable of forming 5α-DHT in considerable amounts. The 5α-DHT/testosterone ratio seems to rise during the breeding season, 5α-DHT being the major testicular androgen from exogenous precursors[7]. However, in urodele *Amphibia* the major in vitro product of the testis has been found to be testosterone[2,3]. We wanted to study how the testicular androgen formation varies in the newt, *Triturus vulgaris* during the reproductive cycle.

Materials and methods. In southern Finland breeding of newts is in May. Their phenotype simultaneously changes to fit aquatic life and males turn colored. Breeding is completed during July. Newts used in the present study were caught at the end of May or at the beginning of June.

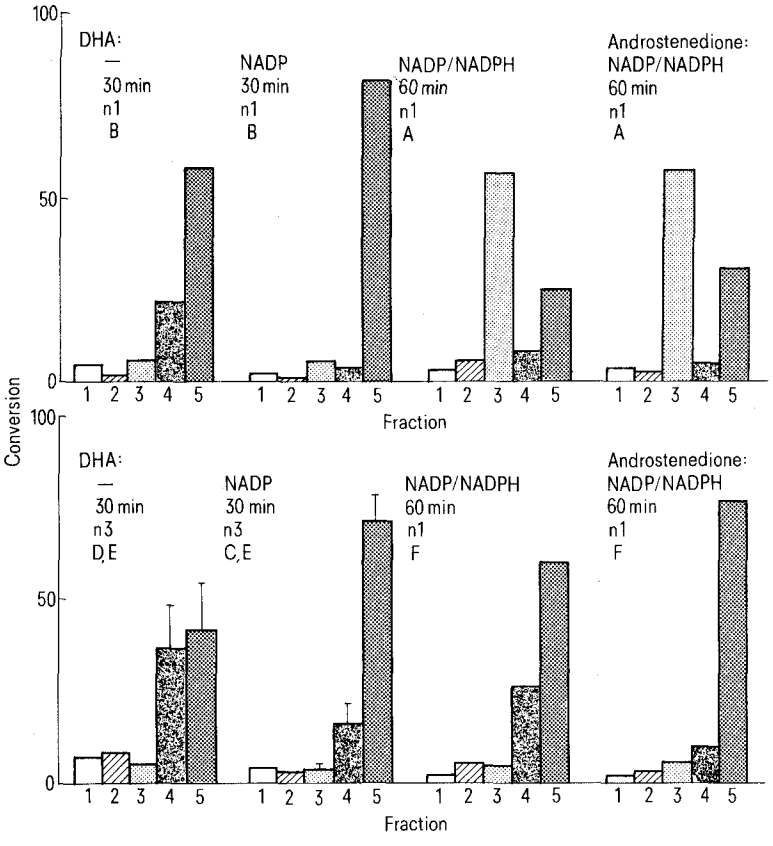
The animals were kept in groups of 50 individuals of both sexes in a half-filled aquarium with a bottom area of 15 dm^2, at +15 °C and exposed to a normal photoperiodic cycle. The dates of the experiments with number of animals and gross gonadosomatic indexes are given in table 1. The first males were sacrificed when the females were laying eggs (groups A and B). After breeding the water was reduced and newts were allowed to creep onto sand. The subsequent samplings (groups C–E) were made after the breeding when the phenotype of the newts started to turn to the terrestrial form. The last males (group F) were used after feeding for a month on natural living food. Testes were homogenized in frog-Ringer medium at pH 7.5 buffered by Trizma® (Sigma) and each of the homogenates (10% w/v) derived from 23–25 individuals were incubated as 5-ml portions at +15 °C for 30 min or 1 h, under oxygen with 14C-DHA (dehydroepiandrosterone, 0.12 µCi, 0.46×10^{-3} mmol/l) or 14C-androstenedione (4-androstene-3,17-dione, 0.12 µCi, 0.40×10^{-3} mmol/l) as the substrate. As indicated in the figure, NADP and/or NADPH (1.35 mmol/l) was used. An acetone denaturated homogenate was used as a control. The extraction and separation of steroids to give the free neutral and phenolic fractions and further studies on the neutral steroids by ascending TLC were carried out as described earlier[7,10]. Chromatographic separation in the first TLC was done by chloroform-diethylether, 3:1 (system A). All solvent systems used are described in the footnote to table 2. The radiochemical purity and identification of fractions obtained in the first TLC was confirmed by derivative formation, by repeated TLCs, by chromatography, or by recrystallizations to constant specific activity (c.s.a.) or to constant isotope ratio (c.i.r.)[11].

Results and discussion. Recovery of the ether extractable (free) radioactivity was 85.7 ± 4.4% for DHA and 86.2 ± 3.6% for androstenedione. Radioactivity recovered in the phenolic fraction amounted to 2.2 ± 0.2% after 30 min and 6.9 ± 1.5% after 1 h for both substrates. Recovery in the water phase was 5.7 ± 1.7%, which was significantly different from the control (2.4 ± 0.6%). Although the radioactivity in the phenolic steroid fraction increased with increasing incubation time, suggesting the possibility of estrogen formation, this fraction was not studied in more detail due to the low level of radioactivity present. The same applies to the water phase; the absolute amount of radioactivity remained too low for possible conjugates to be analyzed. Five peaks (fractions) numbered in the order of decreasing polarity could be recorded by scanning after the first TLC of each analysis from the free neutral steroid fraction (fig. 1).

The radioactive steroids found in TLC fractions were characterized as follows:

Fraction 1 was isopolar with 4-androstene-19-ol-3,17-dione (TLC systems A, D, I/see table 2, footnote). After reduction with $NaBH_4$ it behaved like authentic 4-androstene-3β,17β,19-triol (I,E).

Fraction 2 separated in further TLCs from authentic 4-androstene-11β-ol-3,17-dione and 4-androstene-17β-ol-3,11-dione. It was isopolar with 5-androstene-3β,17β-diol but in recrystallization the radioactivity separated from the carrier.



Percentage conversion of the labelled substrates, DHA and androstenedione to main metabolites by 10% testicular homogenate of the newt *Triturus vulgaris*. Incubation at +15 °C, time, co-factors and number of incubations (n) indicated.
I (A,B), experiments done during breeding; II (C,D,E,F), experiments done after breeding. Each group (A–F) consists of material pooled from 23–25 animals as indicated in table 1.
1, 19-hydroxyandrostenedione; 2, unidentified; 3, testosterone; 4 (striped), DHA; 5, androstenedione.

Table 1. Experimental groups

Group	Number of animals	Date of experiment	Mean body weight ± SD (g)	Gross testis weight (mg/animal)	gGSI[a]
A	25	27 May	1.3 ± 0.2	21.5	1.65
B	25	6 June	2.3 ± 0.4	63.1	2.74
C	25	14 June	1.5 ± 0.3	45.4	3.03
D	25	25 June	2.1 ± 0.3	59.4	2.83
E	25	21 July	1.3 ± 0.3	59.2	4.55[b]
F	23	1 August	1.3 ± 0.3	37.3	2.87[c]

[a] gGSI, gross gonadosomatic index. [b] Body weight strongly reduced because of water loss during landing. [c] Body weight increased due to feeding.

Fraction 3 behaved like authentic testosterone (K,F) and after oxidation with CrO_3 it was isopolar with 4-androstene-3,17-dione (G,D). Constant specific activity was obtained (table 2). Neither 5α-nor 5β-DHT was found in this fraction.
Fraction 4 was isopolar with the unchanged substrate, DHA and with 5α-DHT but not with 5β-DHT. The presence of DHA in the pool was confirmed (G,B). Part of the rest of the radioactivity separated from authentic 5α-DHT in recrystallizations.
Fraction 5 appeared as a single and homogenous peak associated with authentic androstenedione. After reduction it was isopolar with 4-androstene-3β,17β-diol (H,C). The substrate, androstenedione was chromatographed to c.s.a. The metabo-

Table 2. Identification of fractions by TLC and recrystallization

Substrate	Carrier	Fraction	Specific activity (cpm/µg)				Solvent systems in TLC*/in recrystallization	Identification
A. Chromatography			Before	1st	2nd	3rd		
DHA	Androstenedione	5	600	568	577	552	C, F, H	+
DHA	Androstenedione	5	199	190	194	186	C, F, H	+
DHA	Testosterone	3	153	150	144	144	C, F, H	+
Androstenedione	Androstenedione	5	149	124	121	120	C, F, H	+
Androstenedione	Testosterone	3	71.7	80.3	83.5	82.7	C, F, H	+

B. Recrystallization			dpm/mg in crystals		Isotoperatio or specific activity in crystals					
			Before	after 3rd	0	1st	2nd	3rd		
DHA	Androstenedione	5	3405	3641	0.714	0.632	0.620	0.591	I	+
Androstenedione	5α-DHT	4	831	21	831.3	78.7	24.8	20.5	II	–
DHA	5-androstene-3β,17β-diol	2	3120	5817	3120	792	5817	–	III	–

* Solvent systems in TLC: A, Chloroform-diethylether, 3:1; B, Chloroform-diethylether, 2:1; C, Chloroform-ethanol, 19:1; D, Benzene-ethanol, 9:1; E, Benzene-ethanol, 4:1; F, Benzene-ethylacetate, 1:1; G, Cyclohexane-ethylacetate, 1:1; H, Cyclohexane-ethylacetate-ethanol, 9:9:2; I, Ethylacetate-*n*-hexane-ethanol-acetic acid, 144:27:9:20; K, Dichlormethane-acetone, 5:1.
Solvent systems in recrystallization: I Acetone-pentane, Acetone-water, Methanol-water; II Acetone-pentane, Acetone-water, Methanol-water; III Acetone-pentane, Chloroform-n-hexan.

lite of DHA, fr. 5, was in addition recrystallized to constant isotope ratio (table 2).

In all 30-min DHA incubations carried out either without cofactors or with NADP the main metabolite was androstenedione. The utilization of the substrate by $\Delta^5 3\beta$HSD was increased when NADP was added in both experimental groups. The recovery of androstenedione, however, was higher in the incubations performed during breeding, suggesting increased $\Delta^5 3\beta$HSD activity in the testicular tissue of these animals.

When the incubation time was doubled to 1 h and NADP/NADPH added in equimolar concentrations, testosterone was found as the main metabolite of DHA during breeding but not after breeding.

Comparable observations were made when androstenedione was used as the substrate. In the 1-h incubations with NADP/NADPH testosterone was produced in the same proportions as in DHA incubations and the production declined similarly after breeding.

These observations suggest that the activity of 17βHSD may be regulated for the needs of testicular androgen production leading to the increased testosterone formation during breeding.

Previous studies in other urodele Amphibia *(Pleurodeles waltlii, Triturus cristatus carnifex)* have also demonstrated that testosterone is the major androgen produced in vitro by the testis[2,3]. In peripheral plasma of *Ambystoma tigrinum, Pseudoeyrycea smithi*[12] *Necturus maculosus*[13] 5α-DHT can be found in relatively minor amounts compared to testosterone. In accordance with these findings 5α- or 5β-DHT were not found in our studies. On the other hand testicular tissue of anuran species has been found to form large amounts of 5α-DHT from exogenous precursors during breeding[1,4,6,7]. This ability differs therefore from that observed in urodele Amphibia and among all other orders of vertebrates[6,14].

In conclusion the formation of biologically active androgens from DHA and androstenedione in testicular homogenates of *T. vulgaris* showed a clearcut variation between breeding, aquatic newts and non-breeding, terrestrial newts. The difference was most distinct in relation to 17βHSD activity. Testosterone was quantitatively the most important metabolite of both substrates during breeding.

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The presence of β-phenylethylamine, *p*-tyramine, *m*-tyramine and tryptamine in ganglia and foot muscle of the garden snail *(Helix aspersa)*[1]

A.V. Juorio and C.W. Kazakoff[2]

Psychiatric Research Division, Cancer and Medical Research Building, University of Saskatchewan, Saskatoon (Saskatchewan S7N OWO, Canada), 2 August 1983

Summary. The concentrations of β-phenylethylamine, *p*-tyramine, *m*-tyramine, *m*-octopamine and tryptamine in the ganglia or foot muscle of *Helix aspersa* range from < 0.6 to 11 ng/g. *p*-Octopamine levels are higher in ganglia (327 ng/g) than in foot muscle (4.1 ng/g). Dopamine and 5-hydroxytryptamine range from 840 to 2710 ng/g while their acid metabolites, 3,4-dihydroxyphenylacetic acid, 3-methoxy-4-hydroxyphenylacetic acid and 5-hydroxyindoleacetic acid range from < 20 to 130 ng/g.

It has been known for quite some time that the ganglia of gastropods contain relatively high concentrations of dopamine (DA) or 5-hydroxytryptamine (5-HT)[3-5], and this has been confirmed by a histochemical fluorescence technique[6]. In addition, snail ganglia contain relatively large concentrations of *p*-octopamine and/or *m*-octopamine[7].

The snail ganglia possess low monoamine oxidase (MAO) activity[8-11] and it has been suggested that the effects of DA or 5-HT in these snail ganglia are ended by their re-uptake into nerve terminals or by diffusion into the blood[12,13]. The low concentration of 5-hydroxyindoleacetic acid (5-HIAA) observed in ganglia of *Helix*[14] support these interpretations.

In this work, we report the identification of β-phenylethylamine (PE), *p*-tyramine (*p*-TA), *m*-tyramine (*m*-TA), *p*-octopamine (*p*-OA), *m*-octopamine (*m*-OA) and tryptamine (T) in garden snail *(Helix aspersa)* ganglia and foot muscle. For comparison, the concentrations of DA, 5-HT and those of their respective acid metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC), 4-hydroxy-3-methoxyphenylacetic acid (HVA) and 5-HIAA) were also determined.

Materials and methods. Helix aspersa (body weight including shell 7–10 g) were supplied by College Biological Supplies, Bothel, Washington, USA. They were fed on fresh lettuce leaves and kept active for at least 7 days before an experiment. Experiments were conducted throughout the year and hibernating animals were induced to become active by keeping them at room temperature, in a moist atmosphere, until they emerged from their shells.

The determinations were carried out using the whole circumoesophageal ganglia (COG) including the cerebrosuboeso-