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STEROID METABOLISM IN GONADS OF TURTLE EMBRYOS AS A FUNCTION OF THE INCUBATION TEMPERATURE OF EGGS

GISÈLE DESVAGES and CLAUDE PIEAU*
Laboratoire de Biochimie du Développement, Institut Jacques Monod, C.N.R.S. et Université Paris 7, Tour 43, 2 Place Jussieu, 75251 Paris Cedex 05, France

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Summary—In embryos of many reptiles, the sexual differentiation of gonads is temperature-dependent. In the turtle *Emys orbicularis*, all individuals become phenotypic males at 25°C, whereas 100% phenotypic females are obtained at 30°C. Steroid metabolism in embryonic gonads was studied at both temperatures, during and after the thermosensitive period for sexual differentiation. Pools of gonads were incubated for various times, with 3β-hydroxy-5-pregnen-20-one (pregnenolone), progesterone, dehydroepiandrosterone or 4-androstene-3,17-dione as substrates. The analysis of metabolites combined two successive chromatographies (HPLC and TLC) and autoradiography. Conversion of pregnenolone to progesterone and of dehydroepiandrosterone to 4-androstene-3,17-dione was more important in testes at 25°C than in ovaries at 30°C. In ovaries, a large amount of 5-pregnene-3β,20β-diol was formed from pregnenolone, and 5-androstene-3β,17β- diol was produced from dehydroepiandrosterone. In both testes and ovaries, 5α-pregnane and 5α-androstane derivatives were the main metabolites obtained from progesterone and 4-androstene-3,17-dione, respectively. Progesterone was also converted to 20β-hydroxy-4-pregnen-3-one. Dehydroepiandrosterone and 4-androstene-3,17-dione were also metabolized into 11β-hydroxy-4-androstene-3,17-dione (only in testes), testosterone, 11β,17β-dihydroxy-4-androstene-3-one, 17β-hydroxy-4-androstene-3,11-dione (low amounts in testes, traces in ovaries), 17α-hydroxy-4-androstene-3-one, estrone and estradiol-17β (traces).

INTRODUCTION

Gonadal steroid synthesis has been investigated in adult reptiles and has been shown generally to resemble that in mammals [1–3]. Very little is known about steroidogenesis in gonads of embryos. Data are chiefly based on histochemical detection of the Δ^5-3β-hydroxysteroid dehydrogenase activity in lizards [4–8], snakes [7, 9] and turtles [10–12]. In the European pond turtle (*Emys orbicularis*), the sexual differentiation of gonads is temperature-dependent [10, 12]. Incubation of eggs below 28°C yields 100% phenotypic males, whereas above 29.5°C all individuals become phenotypic females. Both sexes and also intersexes (with ovotestes) are obtained in various proportions according to the clutches, within the transitional range of temperature 28–29.5°C [13]. Histochemical study of the Δ^5-3β-hydroxysteroid dehydrogenase activity was performed in embryos incubated at 25°C (all males) or at 30°C (all females) [10–12]. The activity of this enzyme was already detectable in the medullary epithelial cords of undifferentiated gonads (stages 15 and 16) at both temperatures, but it was weaker at 30°C than at 25°C. It was then shown to increase in differentiating testicular cords at 25°C with a maximum during the regression of Müllerian ducts (stages 22–24) while at 30°C it disappeared at the beginning of histological differentiation of the ovaries (stage 18) and remained undetectable until at least one week before hatching [12]. These results indicated that either the steroid synthesis in gonads was less active at 30°C than at 25°C or that the steroid pathways at these temperatures were different. Both phenomena could also occur. The level of seven steroids (progesterone, dehydroepiandrosterone, testosterone, androstenedione, dihydrotestosterone, estrone and estradiol) was subsequently measured by radioimmunoassay in gonads of embryos (stage 24), hatchlings and young (3 and 9 months). In embryos and hatchlings, the total level of these steroids was found to be low and even lower at 30°C than at

*To whom correspondence should be addressed.

25°C [14]. This result agreed with a weaker steroid synthesis at the feminizing temperature but did not provide any information about the steroid pathways followed for each temperature.

In the present study, conducted over three years, we have investigated steroid metabolism in embryonic gonads of *E. orbicularis* by incubating them with radiolabeled precursors and analyzing the biosynthesized steroids with a method combining two successive chromatographies (HPLC and TLC) and autoradiography.

EXPERIMENTAL

Chemicals

Δ^5[7(n)-^{3}H]pregnenolone (3β-hydroxy-5-pregnen-20-one) (21 Ci/mmol; 0.777 TBq/mmol), [1,2(n)-^{3}H]progesterone (53.4 Ci/mmol), dehydro [1,2,6,7-^{3}H]epiandrosterone (80 Ci/mmol; 2.96 TBq/mmol) and [1,2,6,7-^{3}H]androstenedione (84 Ci/mmol; 3.11 TBq/mmol) were purchased from Amersham International (Amersham, Bucks. U.K.) and repurified by HPLC prior to use. Steroid standards were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.), acetonitrile was from Baker (Deventer, Holland) and all other chemicals and solvents were of the highest purity available.

Animals and incubation procedures

Gravid females of *E. orbicularis*, the European pond turtle, were captured in Brenne (France) during the month of June. Oviposition was induced by an intracoelomic injection of 4 or 5 IU oxytocin [15]. The eggs, 5–16 per clutch (average $\simeq$ 8), were then incubated as described previously [12], either at 25°C (giving 100% phenotypic males) or at 30°C (giving 100% phenotypic females). With all substrates, assays were performed at stages 22 or 23 of the embryonic development (weight: 1.1–1.8 g; age: 44–50 days at 25°C, 27–31 days at 30°C) [16]. At these stages, which follow the thermosensitive period for sexual differentiation, the gonads are differentiated and in phenotypic males, the Müllerian ducts degenerate. The Δ^5-3β-hydroxysteroid dehydrogenase activity, carried out by histochemical method on gonad sections, appears to be at a maximum in testes (in epithelial cells of medullary cords or tubes, i.e. in future Sertoli cells), whereas it is not detectable in ovaries [12]. With dehydroepiandrosterone and androstenedione as substrates, assays were also performed at stages 18 and 19 (weight: 0.270–0.380 g; age: 31–34 days at 25°C, 18–20 days at 30°C), i.e. during the thermosensitive period for gonad differentiation [16].

For each assay, the gonads of 20 embryos were removed and placed in cold Minimum Eagle's Medium (MEM) until incubation. They were then incubated at 25 or 30°C for 30 min, 3, 16 or 20 h, in 0.2 ml MEM containing the radiolabeled substrate (5 μCi; 5 nmol, 500 or 250 pmol; final concentration 25, 2.5 and 1.25 μM, respectively). Blanks without gonads were incubated in the same manner. Frozen samples were stored at −20°C until steroid extraction and analysis.

Extraction and analysis of steroids

Unlabeled 20α-dihydroprogesterone (80 pmol) was added to each sample to control extractions and procedural losses of steroids. After homogenization of gonads, steroids were extracted with ethylether. More than 80% of the steroid hormones were generally recovered after extractions with 0.8 ml ethylether repeated four times. Extracts were evaporated to dryness in a stream of nitrogen, dissolved in 25 μl methanol and centrifuged at 10,000 *g* for a few minutes. The steroids in the supernatant were then analyzed by two successive chromatographies (HPLC and TLC) followed by autoradiography.

For HPLC, we used a Beckman Model 332 gradient system equipped with two 110 A pumps, a 420 system controller, a 210 A sample injection valve (Altex, Berkeley, CA, U.S.A.), a fixed-wave length (254 or 280 nm) u.v. detector (model 160 Beckman) and a model 201 fraction collector (Gilson Medical Electronics, France). Sensitivity of the u.v. detector was routinely set at 0.01 AUFS (absorption unit full scale). With absorbance at 254 nm, 4-ene-3-one steroids were detected with a sensitivity of 3.5–7 pmol (1 and 2 ng)/peak. The organic extracts (20 μl) were injected into the apparatus. Steroids were separated on an ultrasphere ODS reverse-phase column (5 μm, 4.6 mm × 250 mm) from Altex, at room temperature, at a flow rate of 1 ml/min with a linear gradient H_2O/acetonitrile from 40 to 80% acetonitrile in 30 min, and then maintained for 5 min at 80%. Among several gradients tested, this gradient gave the best results. Fractions were collected at 1.0 min intervals and lyophilized. Then, they were dissolved in 3 μl of methanol, 2 of which were used for TLC. The remainder of each fraction was counted in scintillation fluid (Econofluor ™NEN Research

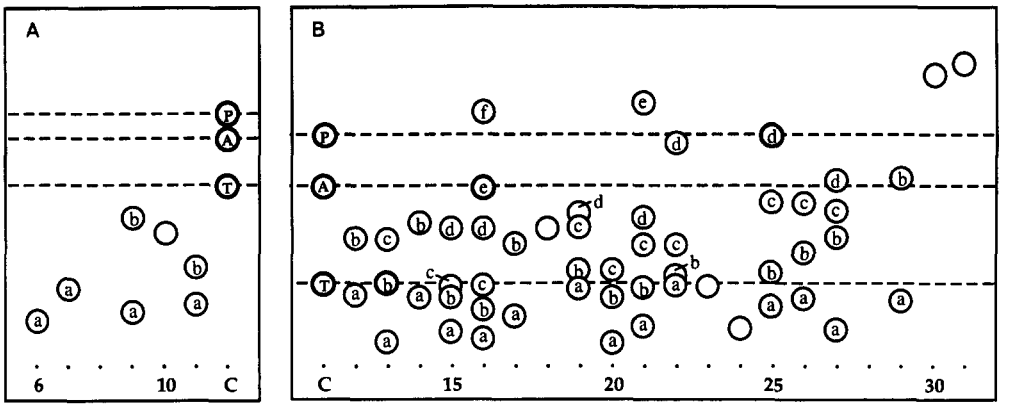

Fig. 1. Steroid mapping used for identification of metabolites produced by embryonic gonads of *E. orbicularis* after incubation with different precursors. Horizontally: HPLC fractions, 6–11 in cyclohexane–ethylacetate (1:1, v/v) solvent (A), 12–31 in chloroform–ethylacetate (5:1, v/v) solvent (B); C, steroid standards used as controls (P, progesterone; A, androstenedione; T, testosterone). Vertically: migration in TLC (10 cm high).

Products Boston, MA, U.S.A.) with an Intertechnique model SL 3000 scintillation counter.

For TLC, the 2 μl aliquots of each fraction were spotted onto 20 × 20 thin layer chromatogram sheets (10 cm high) (Silicagel, $60F_{254}$, E. Merck) which were developed either in chloroform–ethylacetate (5:1, v/v) or in cyclohexane–ethylacetate (1:1, v/v). To establish the steroid mapping, steroid standards (15 nmol) were chromatographied, in the same conditions as products of incubations, on HPLC and TLC. Sheets were sprayed with a solution of acetic acid 9.8 ml, sulfuric acid 0.2 ml and anisic aldehyde 4 drops, and heated for a few minutes at 100°C. All steroids were revealed with different colors. 4-Ene-3-one steroids could be visualized directly by their u.v. absorption. For the experiments, to be quite sure of radiometabolite identification, each spot was run with authentic steroids (10 nmol), testosterone, androstenedione and progesterone, which were used as references. Radioactive metabolites were located by fluorography (EN^{3}HANCE ™Spray, New England, Nuclear, Boston, MA, U.S.A.; Kodak X-Omat X-Ray Film). The times of exposure varied from 4 to 16 h for the detection of most metabolites and could last up to 10 days to detect traces of minor metabolites. Autoradiograms of sheets were scanned at 600 nm with a Schimadzu densitometer model CS-930 at these different times of exposure.

In successively using both HPLC and TLC to separate steroids, radioactive metabolites were characterized by their retention times on the column of HPLC, and by their mobilities on the thin-layer chromatogram compared with those of the authentic preparations (Fig. 1, Table 1). Combining the data given by radioactivity counting in fractions from HPLC and those given by densitometer scanning of autoradiograms from TLC, the production of metabolites was expressed as a percentage of total recovered radioactivity. Moreover, for the 4-ene-3-one steroids (which absorb at 254 nm), the radioactivity percentage was compared to the quantity of metabolic calculated from HPLC peaks, with known quantities of standards taken as references. Out of 56 metabolites looked for, 34 were identified. In all incubations, a number of products collected in the first tubes, slowly migrating on TLC, could not be identified.

The reliability of the method was tested by studying the metabolism of [^{3}H]androstenedione and [^{3}H]testosterone in the left ovaries and paired testes of 15-day-old chicken embryos and comparing the results with those obtained by Imataka *et al.* [17]. We confirmed that 5β-androstanes were predominantly produced; we also detected production of estrogens in ovaries but not in testes.

RESULTS

Metabolism of pregnenolone

Incubation of gonads was carried out with 0.5 nmol tritiated pregnenolone for 3 and 20 h, at stage 22 (Table 2). After incubating testes with pregnenolone for 3 h at 25°C, progesterone and a low amount of 5-pregnene-3β,20β-diol were produced. In ovaries incubated at 30°C, 5-pregnene-3β,20β-diol was the main metabolite; 3β,17α-dihydroxy-5-pregnen-20-one (17α-hydroxypregnenolone), 5-pregnen-3β,20α-diol

Table 1. List of steroids mapped on Fig. 1

Ref.	Retention time (min)	Steroid designation	
6a	5′8	11β,17β-Dihydroxy-4-androsten-3-one	*11β-Hydroxytestosterone*
7a	6′45	17β-Hydroxy-4-androstene-3,11-dione	*11-Ketotestosterone*
9a	8′4	16α-Hydroxy-4-pregnene-3,20-dione	
9b	8′5	11β-Hydroxy-4-androstene-3,17-dione	*11β-Hydroxyandrostenedione*
10	9′4	4-Androstene-3,11,17-trione	
11b	10′	11α-Hydroxy-4-pregnene-3,20-dione	*11α-Hydroxyprogesterone*
11a	10′7	17α,20α-Dihydroxy-4-pregnen-3-one	
12a	—	5-Androstene-3β,17β-diol	
12b,13c	12′	Estradiol-17β	*Estradiol*
13a	12′5	17α,20β-Dihydroxy-4-pregnen-3-one	
13b	12′9	17β-Hydroxy-4-androsten-3-one	*Testosterone*
14a	—	5α-Androstane-3β,17β-diol	
14b	13′25	Estradiol-17α	
15a	14′1	11β-Hydroxy-4-pregnene-3,20-dione	*11β-Hydroxyprogesterone*
15b	—	3β-17α-Dihydroxy-5-pregnen-20-one	*17α-Hydroxypregnenolone*
15c	—	5β-Androstane-3β,17β-diol	
15d-16d	—	3β-Hydroxy-5-androsten-17-one	*Dehydroepiandrosterone*
16a	—	5β-Androstane-3α,17β-diol	
16f	15′1	Estrone	*Estrone*
16e	15′2	4-Androstene-3,17-dione	*Androstenedione*
16b	15′4	17α-Hydroxy-4-androsten-3-one	*Epitestosterone*
16c	15′5	17α-Hydroxy-4-pregnene-3,20-dione	*17α-Hydroxyprogesterone*
17a	—	5α-Androstane-3α,17β-diol	
17b	16′8	3β-Hydroxy-5α-androstan-17-one	*Epiandrosterone*
18,19c	17′9	17β-Hydroxy-5α-androstan-3-one	*5α-Dihydrotestosterone*
19d	18′	3β-Hydroxy-5β-androstan-17-one	
19b	18′1	17β-Hydroxy-5β-androstan-3-one	*5β-Dihydrotestosterone*
19a	—	5-Pregnene-3β,20α-diol	
20b	19′3	3β-Hydroxy-5β-androstan-17-one	*Aetiocholanolone*
20c	19′4	20α-Hydroxy-4-pregnen-3-one	*20α-Dihydroprogesterone*
20a	—	3α,17α-Dihydroxy-5β-pregnan-20-one	
21d	20′3	17α-Hydroxy-5α-pregnane-3,20 dione	
21c,22c	20′8	3α-Hydroxy-5α-androstan-17-one	*Androsterone*
21e,22	20′8	5α-Androstane-3,17-dione	
21a	—	3α,17α-Dihydroxy-5α-pregnan-20-one	
21b	—	5α-Pregnane-3β,20α-diol	
22a	—	5β-Pregnane-3β,20α-diol	
22d	21′2	5β-Androstane-3,17-dione	
22b	21′8	17α-Hydroxy-5β-pregnan-3,20-dione	
23	—	5-Pregnene-3β,20β-diol	
24	—	5β-Pregnane-3α,20α-diol	
25a	—	5α-Pregnane-3α,20α-diol	
25b	24′	20β-Hydroxy-4-pregnen-3-one	*20β-Dihydroprogesterone*
25c	24′2	3β-Hydroxy-5-pregnen-20-one	*Pregnenolone*
25d	24′45	4-Pregnene-3,20-dione	*Progesterone*
26a	—	5α-Pregnane-3β,20β-diol	
26c	25′2	20α-Hydroxy-5α-pregnan-3-one	
26b	25′4	20α-Hydroxy-5β-pregnan-3-one	
27a	—	5β-Pregnane-3α,20β-diol	
27c	26′65	3β-Hydroxy-5α-pregnan-20-one	
27b	26′8	3α-Hydroxy-5β-pregnan-20-one	
27d	26′8	3β-Hydroxy-5β-pregnan-20-one	
29a	—	5α-Pregnane-3α,20β-diol	
29b	28′8	3α-Hydroxy-5α-pregnan-20-one	
30	29′2	5β-Pregnane-3,20-dione	*5β-Dihydroprogesterone*
31	30′1	5α-Pregnane-3,20-dione	*5α-Dihydroprogesterone*

and progesterone were found as traces. The conversion of pregnenolone to progesterone thus appears to be much more active in testes than in ovaries.

After 20 h of incubation, more metabolites were synthesized. Progesterone, 3β-hydroxy-5α-pregnan-20-one and 5α-pregnane-3β,20β-diol were measured in similar amounts in testes and ovaries. 5-Pregnene-3β,20β-diol was also found in both types of gonads but in a much higher amount in ovaries than in testes. Moreover, low levels of 5-pregnene-3β,20α-diol, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone and 5α-pregnane-3β,20α-diol in ovaries and 20β-dihydroprogesterone in testes were detected.

Metabolism of progesterone

This study was performed at stage 22 after 20 h of incubation of gonads with 0.5 nmol tritiated progesterone. In both testes and ovaries, 3β-hydroxy-5α-pregnan-20-one, 5α-pregnane-3β,20β-diol and 20β-dihydroprogesterone were the main metabolites, and low amounts or traces of 20α-dihydroprogesterone, 5α-pregnane-3β,20α-diol, androstenedione and epitestosterone were detected. A small quantity of progesterone appeared to be converted to

Table 2. Metabolism of pregnenolone in testes (T) and ovaries (Ov) of *E. orbicularis* embryos at stage 22 for 3 and 20 h incubation at 25°C (testes) or at 30°C (ovaries), with 0.5 nmol [^{3}H]pregnenolone as the substrate

	Radioactivity (%) at stage 22			
	3 h		20 h	
Metabolites	T	Ov	T	Ov
Residual substrate	56.3	58.3	34	22.4
5-Pregnene-3β,20α-diol	—	Traces	—	1
5-Pregnene-3β,20β-diol	$\leq$1	4.8	1.8	29
17α-Hydroxypregnenolone	—	Traces	—	1
Progesterone	7.8	Traces	1	1.5
17α-Hydroxyprogesterone	—	—	—	Traces
20β-Dihydroprogesterone	—	—	1	—
3β-Hydroxy-5α-pregnan-20-one	—	—	1.7	1.9
5α-Pregnane-3β,20β-diol	—	—	3.2	2.9
5α-Pregnane-3β,20α-diol	—	—	—	$\leq$1

Table 3. Metabolism of progesterone in testes (T) and ovaries (Ov) of *E. orbicularis* embryos at stage 22 for 20 h incubation at 25°C (T) or at 30°C (Ov) with 0.5 nmol [^{3}H]progesterone as the substrate

	Radioactivity (%) at stage 22	
Metabolites	T	Ov
Residual substrate	20	17
Pregnenolone	$\leq$1	—
20α-Dihydroprogesterone	Traces	Traces
20β-Dihydroprogesterone	6	4.5
5α-Pregnane-3,20-dione	1	—
3β-Hydroxy-5α-pregnan-20-one	16	24.9
3α-Hydroxy-5α-pregnan-20-one	1.5	—
5α-Pregnane-3β,20β-diol	6.2	11.6
5α-Pregnane-3β,20α-diol	Traces	1.3
3α-Hydroxy-5β-pregnan-20-one	4	—
5β-Pregnane-3α,20β-diol	4	—
Androstenedione	Traces	Traces
Testosterone	Traces	—
Epitestosterone	Traces	Traces

pregnenolone in testes but not in ovaries, and 5α-pregnane-3,20-dione, 3α-hydroxy-5β-pregnan-20-one, 5β-pregnane-3α,20β-diol and testosterone were found only in testes (Table 3).

Metabolism of dehydroepiandrosterone

Incubations of gonads were carried out either with 0.25 or 5 nmol of tritiated dehydroepiandrosterone. The experiments using 0.25 nmol of this substrate were performed at stages 18 and 19 for 30 min and 3 h and at stage 22 for 3 h. At stages 18 and 19, after both periods of incubation, >85% of dehydroepiandrosterone was metabolized by testes at 25°C, but <50% by ovaries at 30°C (Table 4). Androstenedione levels were higher in testes than in ovaries, showing that during the thermosensitive period for sexual differentiation, the reaction, dehydroepiandrosterone→androstenedione, in gonads is more active at a male-producing temperature than at a female-producing temperature (Figs 2 and 3). 11β-Hydroxyandrostenedione was detected in testes, but not in ovaries (Fig. 3). Just after the thermosensitive period (stage 22), androstenedione was found at the same levels in both testes and ovaries although the residual substrate in ovaries remained higher than in testes.

At stages 18 and 19, testosterone, 11β-hydroxytestosterone, 11-ketotestosterone and epitestosterone were found in both testes and

Table 4. Metabolism of dehydroepiandrosterone in testes (T) and ovaries (Ov) of *E. orbicularis* embryos at stages 18, 19, 22 and 23 at different times of incubation at 25°C (T) or at 30°C (Ov)

	Radioactivity (%)									
	Stages 18 and 19[a]				Stage 22				Stage 23[b]	
	30 min		3 h		3 h[a]		20 h[b]		20 h	
Metabolites	T	Ov	T	Ov	T	Ov	T	Ov	T	Ov
Residual substrate	13.6	65	13	52	22	32	9.8	91	8.3	92
Androstenedione	53.3	17.4	56	15.3	21.7	20.2	3.2	Traces	2.5	Traces
11β-Hydroxyandrostenedione	1.5	—	1.5	—	—	—	ND[c]	ND	ND	ND
Testosterone	5	$\leq$1	1.2	1	3.3	1.8	$\leq$1	—	2.2	—
11β-Hydroxytestosterone	$\leq$1	Traces	Traces	Traces	—	—	ND	ND	ND	ND
11-Ketostestosterone	$\leq$1	Traces	Traces	Traces	—	—	ND	ND	ND	ND
5α-Dihydrotestosterone	—	—	—	—	—	—	1.5	—	2.2	—
Epitestosterone	1	$\leq$1	1	$\leq$1	1	1	$\leq$1	—	$\leq$1	—
5-Androstene-3β,17β-diol	—	$\leq$1	—	1.4	—	1.9	—	$\leq$1	—	$\leq$1
5α-Androstane-3,17-dione	2	1	4	Traces	4	Traces	Traces	—	Traces	—
Androsterone	0.5	Traces	1.5	Traces	2	$\leq$1	30	—	30	—
3β-Hydroxy-5α-androstan-17-one	6.5	5	6	15.4	11	9.1	2.5	—	2.5	—
5α-Androstane-3α,17β-diol	—	—	—	—	—	—	30	—	32	—
5α-Androstane-3β,17β-diol	—	—	—	1.9	—	12.9	2	$\leq$1	3	$\leq$1

[a]Incubation with 0.25 nmol [^{3}H]dehydroepiandrosterone as the substrate.
[b]Incubation with 5 nmol [^{3}H]dehydroepiandrosterone as the substrate.
[c]Not detected.

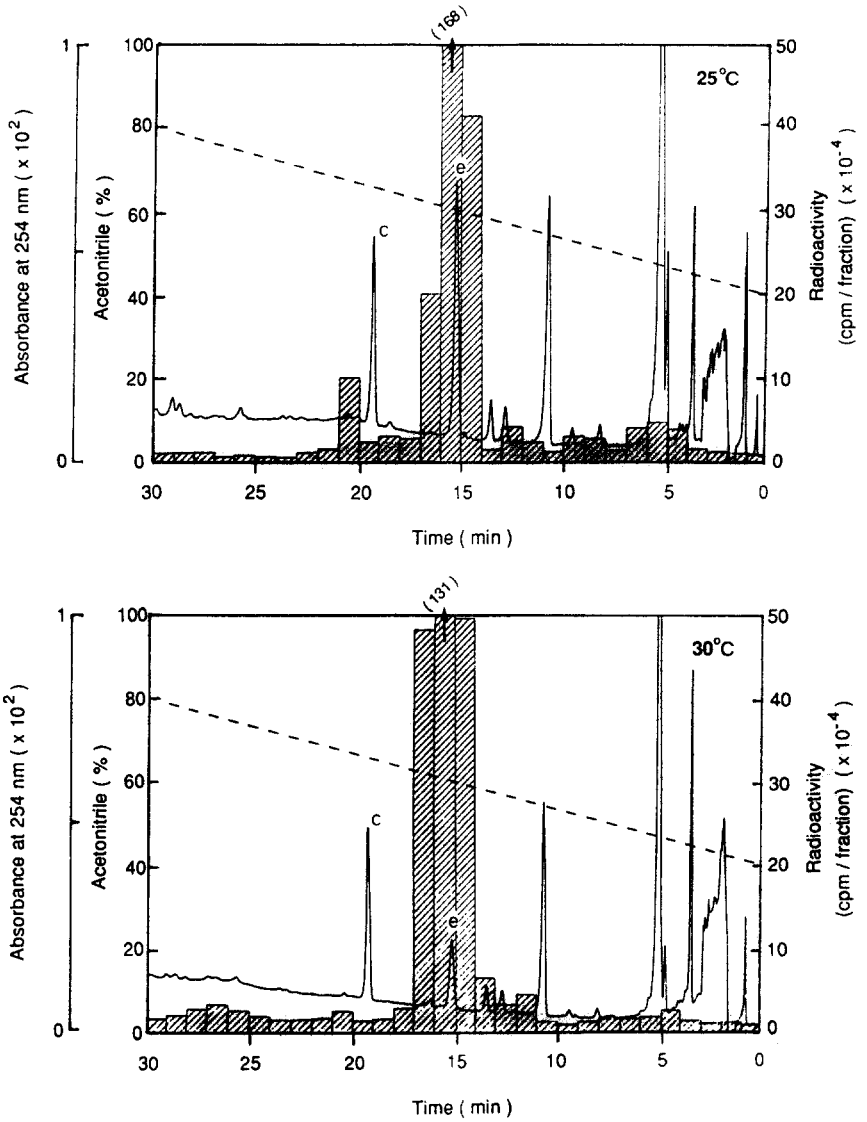

Fig. 2. HPLC profiles of gonadal extracts of *E. orbicularis* embryos at stage 18, after 3 h of incubation with [^{3}H]dehydroepiandrosterone. Chromatographic conditions are given under Experimental. Peak e in fraction 16 corresponds to androstenedione. Peak c in fraction 20 corresponds to 20α-dihydroprogesterone used as a marker. ▨; radioactivity of each fraction including that of minor metabolites and of steroids which do not absorb at 254 nm (see TLC separation in Fig. 3).

ovaries. Although in low amounts or as traces, they appeared somewhat higher in testes (Figs 2 and 3). At stages 18 and 19 as well as at stage 22, the other metabolites were 5α-androstanes derived from androstenedione, 3β-hydroxy-5α-androstan-17-one being the major one. After 3 h of incubation, 5α-androstane-3β,17β-diol was also found in ovaries (chiefly at stage 22), but not in testes (Table 4).

Incubations of gonads with 5 nmol of tritiated dehydroepiandrosterone were performed for 20 h, at stages 22 and 23. At each of these stages, results were very similar (Table 4). The most striking one was that >90% of dehydroepiandrosterone was metabolized by testes at 25°C but <10% by ovaries at 30°C. Androsterone and 5α-androstane-3α,17β-diol were the two major metabolites produced by testes, each representing approx. 30% of the recovered radioactivity. The other metabolites detected in testes at a low level, were: androstenedione; testosterone; 17β-hydroxy-5α-androstan-3-one (5α-dihydrotestosterone); epitestosterone; 3β-hydroxy-5α-androstan-17-one and 5α-androstane-3β,17β-diol. In ovaries, only three metabolites were identified: androstenedione (traces); 5-androstene-3β,17β-diol (not found in testes) and 5α-androstane-3β,17β-diol. In these incubations, we did not

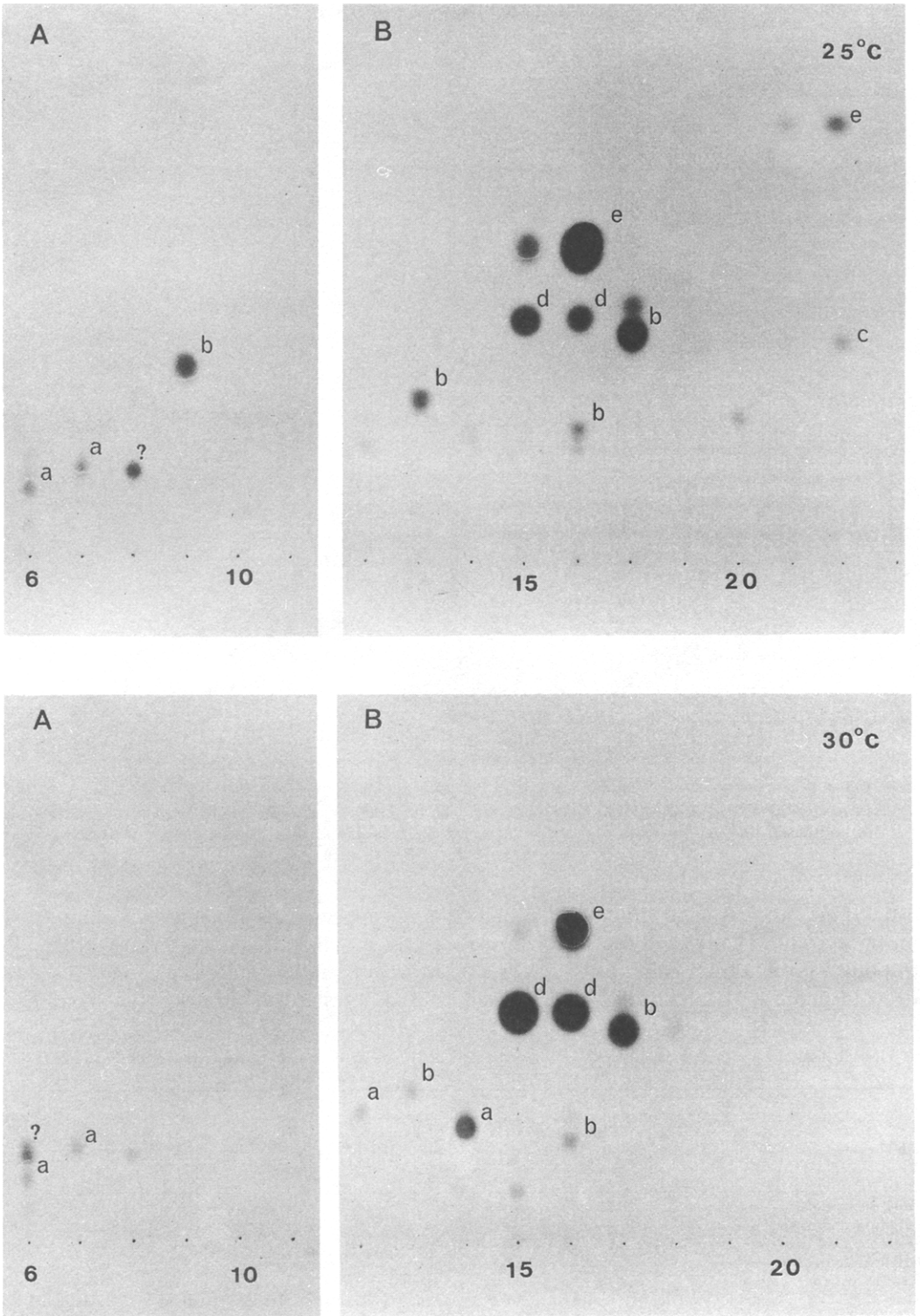

Fig. 3. Autoradiography of steroids analyzed by TLC after fractioning by HPLC (Fig. 2). For identification of metabolites, see Fig. 1 and Table 1.

carry out TLC in the cyclohexane–ethylacetate system and therefore, we could not identify the 11β-hydroxy derivatives and 11-ketotestosterone.

Metabolism of androstenedione

Four incubations were performed for 3 h with 0.25 nmol tritiated androstenedione as

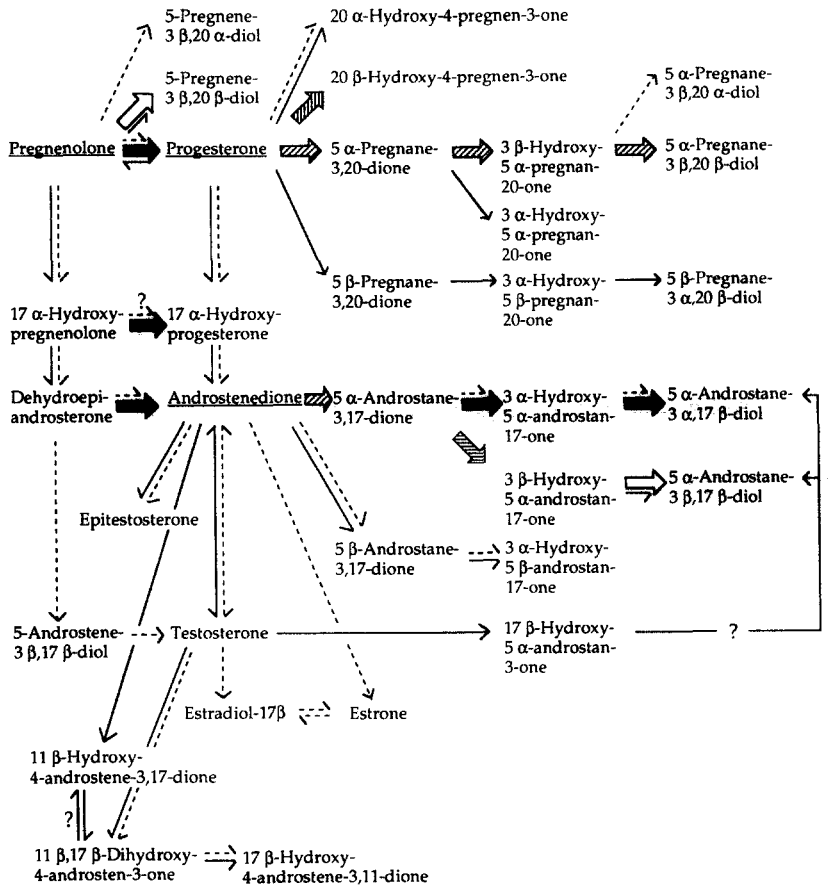

Fig. 4. Postulated metabolic pathways of steroids by the testes and ovaries of *E. orbicularis* embryos issued from eggs incubated at 25 and 30°C, respectively. The thick arrows indicate the main pathways: ▨▶, in both testes and ovaries; ➡, in testes; and ⇨, in ovaries. The thin arrows indicate the minor pathways: ⇌, in testes; and ⇢, in ovaries.

substrate, the two first ones with pooled gonads from embryos of stages 18 and 19, the other two with pooled gonads from embryos of stages 22 and 23 (Table 5). Metabolism of androstenedione appeared to be somewhat more active at stages 22 and 23 than at stages 18 and 19. Moreover, more androstenedione was metabolized in testes than in ovaries at stages 18 and

Table 5. Metabolism of androstenedione in testes (T) and ovaries (Ov) of *E. orbicularis* embryos at stages 18 and 19, 22 and 23 at different times of incubation, at 25°C (T) or at 30°C (Ov)

Metabolites	Radioactivity (%)							
	Stages 18 and 19[a] 3 h		Stages 22 and 23[a] 3 h		Stage 22[b] 20 h		Stage 23[b] 20 h	
	T	Ov	T	Ov	T	Ov	T	Ov
Residual substrate	61.4	70	55.3	46.1	5.6	4.4	8	5.1
11β-Hydroxyandrostenedione	Traces	—	Traces	—	ND[c]	ND	ND	ND
Testosterone	1.1	1.5	3.8	2.7	1	1.4	⩽1	3.5
11β-Hydroxytestosterone	Traces	Traces	⩽1	Traces	ND	ND	ND	ND
11-Ketostestosterone	⩽1	Traces	1	Traces	ND	ND	ND	ND
Estrone	Traces	Traces	Traces	Traces	Traces	Traces	Traces	Traces
Estradiol-17β	Traces	Traces	Traces	Traces	Traces	Traces	Traces	Traces
5α-Dihydrotestosterone	—	—	—	—	2.5	—	1.5	Traces
Epitestosterone	1	Traces	Traces	Traces	1	8.8	1.5	11.9
5α-Androstane-3,17-dione	4	2	9	Traces	1	Traces	1	Traces
Androsterone	5	1	4	Traces	32	⩽1	34	⩽1
3β-Hydroxy-5α-androstan-17-one	3.8	7.1	5	16.8	1	15	1	16
5α-Androstane-3α,17β-diol	—	—	1.7	—	32	11	30	13
5α-Androstane-3β,17β-diol	Traces	Traces	1	1.5	Traces	1.5	—	11.9
3α-Hydroxy-5β-androstan-17-one	2.5	1.4	Traces	Traces	—	—	—	—

[a]Incubation with 0.25 nmol [^{3}H]androstenedione as the substrate.
[b]Incubation with 5 nmol [^{3}H]androstenedione as the substrate.
[c]Not detected.

19, whereas at stages 22 and 23 the reverse was observed. In all cases, the same metabolites were identified, 5α-androstanes being the major ones. Among these derivatives, production of androsterone was higher in testes whereas that of 3β-hydroxy-5α-androstan-17-one was higher in ovaries. In both types of gonads, 3α-hydroxy-5β-androstan-17-one was also found and testosterone was measured in similar amounts. 11β-Hydroxyandrostenedione was detected as traces only in testes. 11β-Hydroxytestosterone and 11-ketotestosterone were found in low amounts (~1%) or as traces in testes and always as traces in ovaries. Estrone and estradiol-17β were identified in both testes and ovaries, but as they were only traces, significant differences between gonads could not be established.

The other incubations were carried out at stages 22 and 23 for 20 h, with 5 nmol tritiated androstenedione. Although at high concentration, approx. 95% of this substrate was metabolized in both testes and ovaries. As in the incubation with 5 nmol of dehydroepiandrosterone for the same time, androsterone and 5α-androstane-3α,17β-diol were the two major metabolites in testes, each representing 30–34% of the recovered radioactivity. In ovaries, 3β-hydroxy-5α-androstan-17-one, 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol (at stage 23) were produced in relatively high amounts. In both testes and ovaries, low amounts of testosterone and traces of estrone and estradiol-17β were detected. In these long incubations with androstenedione as in those with dehydroepiandrosterone, 5α-dihydrotestosterone was produced at a low level in testes (traces were detected in ovaries at stage 23). 11β-Hydroxy derivatives and 11-ketotestosterone were not determined.

DISCUSSION

The present results confirm that embryonic gonads of the turtle *E. orbicularis* synthesize steroid hormones as early as the first stages of their histological differentiation. Indeed, steroid metabolism was already active at stages 18 and 19 when seminiferous cords (at 25°C) and ovarian cortex (at 30°C) were just beginning to develop. Using dehydroepiandrosterone or androstenedione as substrates, the same metabolites as those found at stages 22 and 23 were formed. Therefore, metabolic pathways of steroids in testes and ovaries of turtle embryos appear to be similar at these different stages. The postulated pathways deduced from our results are shown in Fig. 4.

Conversion of pregnenolone to progesterone and of dehydroepiandrosterone to androstenedione was more active in testes than in ovaries, showing a higher activity of Δ^5-3β-hydroxysteroid dehydrogenase in testes. In ovaries, the main metabolite of pregnenolone was 5-pregnene-3β,20β-diol, whereas 5-pregnene-3β,20α-diol was formed in very low amounts. In both ovaries and testes, 20β-dihydroprogesterone and chiefly 5α-pregnanes were the major products obtained from progesterone, and high amounts of 5α-androstanes were measured from dehydroepiandrosterone or androstenedione. Therefore, both 20β-hydroxysteroid oxidoreductase and 5α-reductase enzymes appeared to be very active. Embryonic gonads of *E. orbicularis* also synthesized 11β-hydroxyandrostenedione, testosterone, 11β-hydroxytestosterone, 11-ketotestosterone, 5α-dihydrotestosterone, epitestosterone, 5-androstene-3β,17β-diol and estrogens as minor products. 11β-Hydroxyandrostenedione was produced only by testes. Testosterone was formed in larger amounts in testes than in ovaries with dehydroepiandrosterone as the substrate, but not with androstenedione. 11β-Hydroxytestosterone and 11-ketotestosterone were found in low amounts or as traces in testes and always as traces in ovaries. 5α-Dihydrotestosterone, when detected, was higher in testes. Epitestosterone was generally produced in low amounts, however, it increased in ovaries after a long incubation with androstenedione. 5-Androstene-3β,17β-diol was only synthesized by ovaries from dehydroepiandrosterone and estrogens were found only as traces.

The higher activity of Δ^5-3β-hydroxysteroid dehydrogenase in testes confirms previous results using dehydroepiandrosterone as the substrate for the histochemical detection of this enzyme in embryos of *E. orbicularis* and *Testudo graeca* [10–12]. However, in the present work, the enzyme activity at 30°C appeared to be blocked by a high concentration of substrate, whereas it was not by a lower one (Table 4). Since the levels of endogenous steroids in gonads are very low [14], the difference between Δ^5-3β-hydroxysteroid dehydrogenase activity at 25°C and that at 30°C could be less important in normally differentiating gonads than in gonads incubated with precursors.

The important activity of both 20β-hydroxysteroid oxidoreductase and 5α-reductase that we have observed in *E. orbicularis* embryos agrees with the findings in adult reptiles. Occurrence of 20β-hydroxysteroid oxidoreductase activity was demonstrated in the kidney and liver of the lizard *Tiliqua rugosa* [18] and its predominance over the 20α-activity was subsequently shown in mature testes of both turtle species, *Pseudemys scripta* and *Sternotherus odoratus* [19]. 5α-Reduced steroids were identified in testes of the lizard *Lacerta viridis* [20] and the turtle *S. odoratus* [19]. However, in these species, 5α-androstane-3β,17β-diol was a major product, whereas in *E. orbicularis* it is 5α-androstane-3α,17β-diol. Results in *S. odoratus* showed changes in proportions of testosterone and of 5α-androstane-3β,17β-diol suggesting an enzymatic switch in the steroid pathways that could be associated with the annual testicular cycle of the adult turtle [19]. Embryonic gonads of turtles are not submitted to such a cycle. In the rat, synthesis of 5α-androstane-3α,17β-diol was shown to predominate in immature testes, and to represent an alternative to the 4-ene pathway leading to production of testosterone in mature testes [21]. In the chicken, embryonic gonads, irrespective of sexes and age, converted progesterone, 17α-hydroxyprogesterone, androstenedione and testosterone to 5β-reduced derivatives, showing a marked activity of 5β-reductase rather than 5α-reductase [17 and this paper]. Altogether, these results indicate that the high production of 5α- or 5β-reduced compounds by embryonic, immature or, some times, by mature gonads, could represent a catabolic pathway for the excess of Δ^4-3-oxo steroids, and therefore could be involved in regulating the level of androgens and subsequently of estrogens in the gonads.

In the present study, 5α-dihydrotestosterone was detected after long incubations (20 h) with dehydroepiandrosterone or androstenedione as substrates, but not after shorter (30 min and 3 h) incubations. Therefore, as found in adults of other reptilian species including turtles [19, 22], this hormone does not appear to be a major product and we have considered that 5α-androstanediols mainly derive from androstenedione with 5α-androstane-3,17-dione and androsterone as intermediates (Fig. 4). Epitestosterone was previously found in the testes of the lizard, *T. rugosa* [23, 24], but was not identified in the testes of the turtles *P. scripta* and *S. odoratus* [19]. In embryonic gonads of *E. orbicularis*, it appears as a minor metabolite, except in the case of a large excess of androstenedione.

Although it is limited, production of 11β-hydroxyandrostenedione, 11β-hydroxytestosterone and 11-ketotestosterone by gonads of a reptilian species is a remarkable feature. Indeed, these steroids have been shown to be important metabolites in fish [25, 26]. As in *E. orbicularis*, 11β-hydroxyandrostenedione was found only in testes, while the two other metabolites were detected in both testes and ovaries, testosterone rather than 11β-hydroxyandrostenedione is the presumed intermediate in the synthesis of 11β-hydroxytestosterone and 11-ketotestosterone. Both pathways have been shown in teleosts [25, 26].

Synthesis of 5-androstene-3β,17β-diol by ovaries but not by testes might correspond to an alternative pathway associated with the attenuation of the 4-ene pathway (due to the weak activity of Δ^5-3β-hydroxysteroid dehydrogenase) in ovaries of *E. orbicularis* embryos. As shown in the chicken [17], this could lead to testosterone synthesis and finally contribute to increase the production of estrogens. The amounts of estrone and estradiol-17β were too low to conclude significant differences between ovaries and testes. In other work, estrogen content, measured by radioimmunoassay, was found to be higher in ovaries as early as the beginning of the thermosensitive period (stages 17 and 18). Moreover, study of the effects of estrogens and tamoxifen (an antiestrogen) clearly showed that estrogens are involved in gonad differentiation, ovaries differentiating above a certain level of estrogens at higher temperatures and testes below this level at lower ones [27]. Comparison at different developmental stages of cytochrome-*P*450 aromatase activity in the gonads, at masculinizing and at feminizing temperatures, is thus required to define the role of this enzyme in the sexual differentiation of gonads.

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