EARLY STEROID METABOLISM BY CHICK BLASTODERM IN VITRO

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ABSTRACT

Yolk free blastoderms of chick embryo were incubated 3 or 22 hours with labeled pregnenolone, progesterone, 17-hydroxyprogesterone, dehydroepiandrosterone, androstenedione, testosterone and estradiol-17β. Metabolites and unconverted substrates were found both in the incubation medium and in the cells. Enzymes responsible for identified conversions were: 17α-hydroxylase, 17-20-desmolase, $\Delta^5$3β- and 3α-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase and 5α- and 5β-reductase. The results suggest that the steroid metabolizing enzyme activities found may reflect a more general ability of early embryonic cells.

INTRODUCTION

Estrogens and progesterone elaborated by hen ovaries (1) induce selective alterations in the gene transcription of oviduct cells so that avidin and ovalbumin are produced to meet the needs of the developing egg (2). Could the egg and/or the early embryo itself respond to these steroids ? Parsons (3) has shown that blastoderms from 48 h chick embryos are able to convert exogenous testosterone to 5β-androstane derivatives. These may be needed for stimulation of hemoglobin synthesis (4). Whether the early chick embryo possesses endogenous testosterone is not known. The membranes of the large follicles have been reported to contain about 33 ng/g of progesterone and about 1 ng/g of estradiol-17β(5).

Previous studies have shown that oocytes and early embryos of amphibians (6-10) and blastocysts of some mammals (11,12) metabolize steroids too. At the time of trophoblast differentiation blastocysts become steroidogenic (13-16). Dickmann and co-workers have claimed that this occurs before implantation in rat and mouse (17) but biochemical studies with mouse have not confirmed this (13-16,18). However, a weak conversion of

pregnenolone to progesterone has been demonstrated in early embryos of amphibians: *Triturus*, *Rana* and *Xenopus* (10).

The aim of the present study was to investigate whether steroid metabolism occurs also in avian embryos, amniots which develop outside the influence of maternal environment.

MATERIALS AND METHODS

Animals

Fertilized eggs of the White Leghorn hen were obtained from a commercial breeder. The eggs were kept about one day in a refrigerator at 4°C or 16 h in an incubator at 37°C before preparing them for incubation with steroids. Blastoderms were removed from the yolk and classified by developmental stage according to Hamburger and Hamilton (19). The stages used in this study were 0 (early blastula) and 2-4 (late blastula). 22 h incubation of prepared blastoderms resulted in abnormal gastrulation.

Chemicals

All solvents were of analytical grade and were redistilled before use. Unlabeled reference and carrier steroids were purchased from Sigma Chemical Co. (St.Louis,Mo.), Research Plus Inc. (Bayonne,N.J.), Steraloids Inc. (Croydon,England) or were obtained as gifts from The M.R.C. Steroid Reference Collection (London,England) by courtesy of Prof.D.N.Kirk. The following tritium labeled steroids were obtained from The Radiochemical Centre (RC, Amersham,England) or from New England Nuclear (NEN, Boston,Mass.): [7-^{3}H]pregnenolone, 10 Ci/mmol, [1,2,6,7-^{3}H]progesterone, 81 Ci/mmol, [1,2-^{3}H]5α-pregnanedione, 55.7 Ci/mmol, [1,2-^{3}H]17-hydroxyprogesterone, 60 Ci/mmol, [1,2,6,7-^{3}H]androstenedione, 87 Ci/mmol, [1,2-^{3}H]dehydroepiandrosterone, 40.2 Ci/mmol, [1,2,6,7-^{3}H]testosterone, 93.9 Ci/mmol and [2,4,6,7-^{3}H]estradiol-17β, 115.0 Ci/mmol.

The 4-^{14}C-labeled steroids were: pregnenolone, 57.2 mCi/mmol (NEN), progesterone,57.2 mCi/mmol (NEN), 17-hydroxyprogesterone, 61.0 mCi/mmol (RC), androstenedione, 60.0 mCi/mmol (RC), dehydroepiandrosterone, 55.0 mCi/mmol (RC), testosterone, 58.0 mCi/mmol (RC) and estradiol-17β, 52.0 mCi/mmol (RC). The purity of labeled steroids was checked by thin layer chromatography before use.

Incubation

Blastoderms, 5 per each flask, were incubated with different steroids for 3 h in 5 mL of Dulbecco phosphate buffer at 37°C. Longer incubations (22h) were used in order to achieve a sufficient amount of metabolites for analysis. These incubations were performed in the minimal essential medium or in the Dulbecco phosphate buffer containing 1 mg/mL of glucose. For a control, yolk was incubated similarly. The substrates were dissolved in 25-50 µL of ethanol-propylene glycol solution (1:1, v/v). Final concentration of substrates in incubations was 2.8-3.1 nmol/5mL (0.012 nmol/5mL for tritiated progesterone). After incubation, medium and blastoderms were treated separately. Before denaturation by acetone, the blastoderms were washed three times with 2 mL of buffer. Both aliquots were acetone-treated and frozen after separation.

Extraction, purification and identification of steroids

Extraction was done three times with 20 mL of diethylether from a sample volume of 10 mL. After extraction, the recovery was determined by liquid scintillation counting using a Wallac Decem NTL^{314} counter (Turku, Finland) which showed counting efficiency in Omnifluor^{r} (NEN) in two separate channels for ^{14}C 80% and 24% and for ^{3}H 9% and 40% respectively.

Ether-soluble radioactivity was thereafter chromatographed in the ascending manner up to 14 cm front using thin layer chromatography (TLC) on a 0.25 mm layer of Silicagel G (Merck, Darmstadt,Germany). Solvent systems were A for pregnanes and M for androstanes and estradiol-17β(see appendix). Chromatograms were obtained by scanning the radioactivity on TLC-plates using a TLC-scanner (LB2721, Berthold,Wildbad,Germany).

Radioactive metabolites were scraped off the plates and purified by multiple and two-dimensional TLC with different carrier steroids. 4-Ene-3-ketosteroids were visualized in the TLC under short-wave UV. Other reference steroids were visualized after colorization with *p*-toluene-sulphonic acid spray (20% in ethanol) (20). Derivatization was done by reduction with sodium borohydride (21). Identification was made by repeated TLC (21) and by recrystallization to constant specific activity (CSA) or to constant isotope ratio (CIR) (22).

RESULTS

a) Pregnenolone incubations

Pregnenolone and its metabolites accumulated in the blastoderms as indicated in fig.1 and table 1. A longer incubation period resulted in a higher yield of metabolites and recovery of radioactivity in blastoderms. The developmental stage had no observable effect to the pattern of steroid metabolism. Three apolar metabolites (R_f in system A, 0.70 - 0.95) were formed to about 5 %. The presence of 5α-pregnanedione and progesterone was confirmed, that of 5β-pregnanedione remained tentative as only repeated TLC (rTLC) was carried out. The substrate fraction (R_f 0.69) was radiochemically homogenous and cochromatographed with pregnenolone in rTLC.

b) Progesterone incubations

Progesterone and its metabolites accumulated in the blastoderms as indicated in fig.1 and table 1. The effect of substrate concentration, 0.012 mmol/5mL versus 2.9 mmol/5mL was hardly reflected in the metabolic

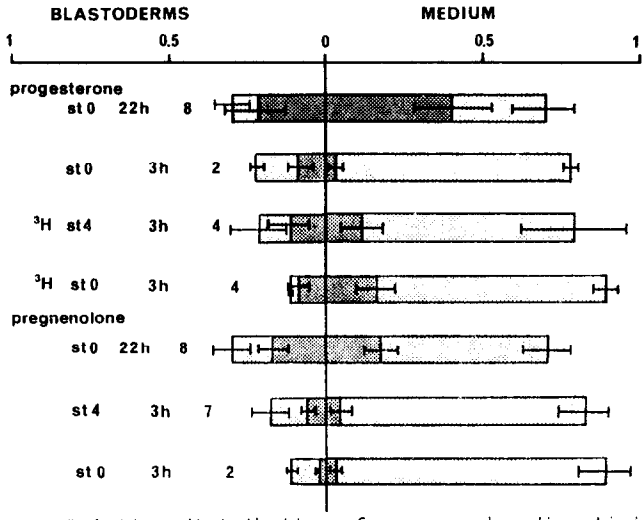

Fig.1. Relative distribution of recovered radioactivity in pregnenolone and progesterone incubations. Dark area represents the metabolites and light area the substrate respectively. Developmental stage (st), incubation time (h) and number of incubations indicated.

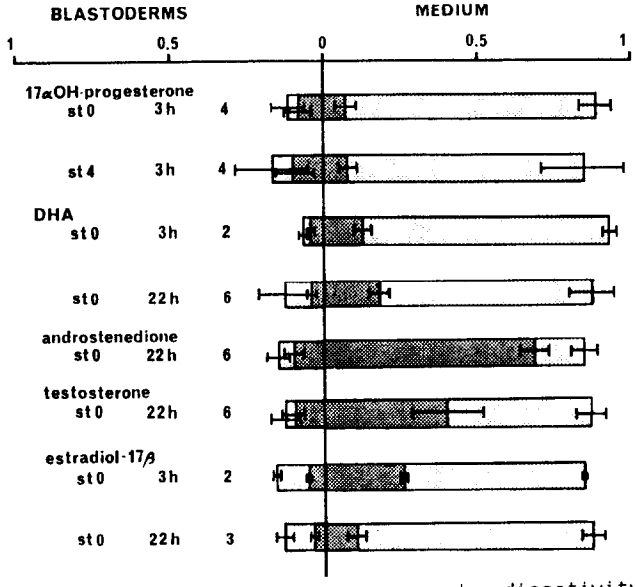

Fig.2. Relative distribution of recovered radioactivity in 17-hydroxyprogesterone, DHA, androstenedione, testosterone and estradiol-17β incubations. Symbols, see legend in fig.1.

pattern between different developmental stages. Apolar metabolites were similar to those of pregnenolone incubations in R_f. 5α- and 5β-pregnanedione were crystallized to CSA and to CIR. The substrate, progesterone (R_f in system A, 0.75) was purified by rTLC from 5β-pregnanedione and androstenedione. This latter $4^{14}C$-labeled metabolite cochromatographed in rTLC with authentic tritiated androstenedione (R_f in system A, 0.70). The most significant metabolite of progesterone (Rf in system A, 0.61), about 13% of total, was identified as 17-hydroxyprogesterone by rTLC.

c) 17-hydroxyprogesterone incubations

Accumulation and amount of metabolism is shown in fig.2 and table 1. Most metabolites were very polar unknown compounds. These were not identified, so the androstenedione, Rf 0.70, remained the only known metabolite of 17-hydroxyprogesterone.

d) Dehydroepiandrosterone incubations

In contrast to other substrates, DHA accumulated poorly in blastoderms (fig.2 and table 1). Most metabolites were more apolar than DHA, of these androstenedione was identified by rTLC. Other apolar metabolites cochromatographed with 5α-and 5β-androstanedione in two-dimensional TLC and rTLC. In polar fractions , testosterone and 5β-androstane-3α,17β-diol were found and identified by rTLC and by recrystallization to CSA, respectively.

e) Estradiol-17β incubations

In spite of accumulation and moderate metabolism of estradiol-17β , estrogen interconversions were not definitively identified. Unconverted estradiol-17β was found (R_f in system M, 0.58), about 77% of total, in the incubation of 3 h. The substrate fraction was radiochemically homogenous and comigrated in rTLC with authentic estradiol-17β.

f) Androstenedione and testosterone incubations

Both androstenedione and testosterone were extensively converted in 22 h incubations to polar metabolites of which 5β-androstane-3α,17β-diol was identified by recrystallization to CSA. Androstenedione was shown to be converted to testosterone, but this was scanty, probably due to further metabolism of testosterone to polar metabolites.

TABLE 1. DISTRIBUTION OF RADIOACTIVITY

SUBSTRATE METABOLITES	n	R_f	IDENTIFICATION	% DISTRIBUTION IN TLC		% TOTAL RECOVERY
				MEDIUM	BLASTODERMS	
Pregnenolone	9	0.47	rTLC	96.3 ±3.9	70.3 ±8.4	
progesterone		0.59	rTLC			
5α-pregnanedione		0.75	rTLC	0.6 ±0.8	20.6 ±15.0	
5β-pregnanedione		0.63	rTLC			17.5 ±5.3
unknown polar				3.1 ±3.1	10.5 ±4.9	
Progesterone	8	0.59	rTLC	85.2 ±9.2	9.7 ±3.1	
5α-pregnanedione		0.75	CSA by CR	nd	18.4 ±4.2	
5β-pregnanedione		0.63	CSA by CR	nd	0.5 ±0.1	
androstenedione		0.54	rTLC	nd	6.4 ±2.4	15.7 ±12.9
17-hydroxyprogest.		0.46	rTLC	7.5 ±2.0	43.9 ±5.2	
unknown polar				5.3 ±1.6	17.3 ±8.4	
17-hydroxyprogest.	8	0.46	rTLC	91.5 ±2.5	30.3 ±11.7	
androstenedione		0.54	rTLC	2.4 ±0.4	6.0 ±5.0	14.1 ±9.4
unknown polar				6.8 ±1.5	62.8 ±15.6	
DHA	2	0.50	rTLC	79.5 ±5.0	51.6 ±5.2	
unknown apolar		0.76		2.9 ±0.7	25.4 ±4.1	
androstenedione		0.67	rTLC	2.1 ±0.8	16.2 ±2.4	
testosterone		0.28	rTLC	6.5 ±1.2	6.8 ±0.8	5.9 ±0.4
5β-A-3α,17β-diol		0.20	CSA by CR	3.3 ±0.5	nd	
unknown polar				5.7 ±1.8	nd	

n= number of incubations, R_f values of pregnanes in system B, of DHA in system M (see Appendix), nd= not determined, rTLC= repeated TLC, CR= crystallization

DISCUSSION

Our results show that blastoderms of chick embryo are capable of metabolizing, at the earliest stages, neutral (pregnenolone, progesterone, 17-hydroxyprogesterone, DHA, androstenedione,testosterone) and phenolic (estradiol-17β) steroids. Metabolites were found both in the medium and in the blastoderms. Enzymes responsible for obtained conversions were 17α-hydroxylase, 17-20-desmolase, $\Delta^5$3β- ($\Delta^5$3βHSDH)and 3α-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase and 5α- and 5β-reductase.

Parsons's observation (3) that reduction of testosterone leads to 5β-androstane-3α,17β-diol which may stimulate hemoglobin synthesis in 22 h old chick blastoderm (4) is consistent with the idea that some metabolites may be biologically active. However, Ozon *et al* (23) have shown that progesterone metabolism (5α-reductive pathway) in *Pleurodeles waltlii* oocytes can be interpreted as an inactivation process which functions in an intracellular regulation mechanism. Progesterone was partly metabolized in chick blastoderm in a similar manner to 5α- and to 5β-pregnanedione. 5β-Androstane-3α,17β-diol was found as the main metabolite of added testosterone.

Previous studies have shown that the ability to metabolize a variety of exogenous steroids is a common feature of early vertebrate embryos (9, 10,14-16). Part of the enzymes responsible for the steroid metabolism in the early embryo may be derived already from the oocyte. It is noteworthy that the same applies also to mammalian spermatozoa (24-27) and to germline tumor cells (28,20,29). Parsons (3) has, however, observed that unincubated eggs are not capable of converting testosterone to 5β-androstane compounds. We did not study possible differences between the unincubated, stage 0 and incubated eggs stage 2-4 in relation to 5α/5β-reduction,

but TLC analyses did not show any significant differences in the metabolism of pregnanes.

Woods and Weeks (30) demonstrated histochemically $\Delta^5$3βHSDH activity in 2-day-old chick embryos before gonadal differentiation. Our observation that a weak $\Delta^5$3βHSDH activity can be found before any organ differentiation (in stage 0) means that this may be a more common feature of early embryonic cells. As Antila (10) has earlier hypothesised, appearance of steroid-metabolizing enzymes may represent basal levels of genetic expressions.

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APPENDIX

Solvent systems in TLC

A acetone-chloroform, 15:85 ,
B benzene-ethanol, 9:1
M chloroform-diethylether, 3:1

TRIVIAL AND IUPAC NAMES

Trivial name	IUPAC name
androstenedione	4-androstene-3,17-dione
dehydroepiandrosterone (DHA)	3β-hydroxy-5-androsten-17-one
estradiol-17β	1,3,5,(10)-estratriene-3,17β-diol
estrone	3-hydroxy-1,3,5(10)-estratrien-17-one
17-hydroxyprogesterone	17-hydroxy-4-pregnene-3,20-dione
5α-pregnanedione	5α-pregnane-3,20-dione
5β-pregnanedione	5β-pregnane-3,20-dione
pregnenolone	3β-hydroxy-5-pregnen-20-one
progesterone	4-pregnene-3,20-dione
testosterone	17β-hydroxy-4-androsten-3-one

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