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# SHORT COMMUNICATION

# ANALYSIS OF STEROID METABOLITES PRODUCED BY THECA CELLS FROM THE ADULT DOMESTIC HEN

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**Summary**—In a previous study on steroid metabolism by hen ovarian cells we reported on the production of 17-hydroxyprogesterone (17OH), androstenedione (A), testosterone (T), and oestrogens from [$^3$H]progesterone (P) by theca cells. The present study examines further the metabolism of P by theca cells from the preovulatory follicles of the hen. The results show that the major metabolite of P is 20$\beta$-hydroxy-4-pregnen-3-one (20$\beta$-DHP), representing up to 40% of the recovered radioactivity. In addition, 3$\alpha$-hydroxy-4-pregnen-20-one (3$\alpha$-DHP) and 17$\alpha$,20$\beta$-dihydroxy-4-pregnen-3-one (17,20$\beta$) were identified as metabolites of P, comprising 1 and 3% of the recovered radioactivity, respectively. This is the first evidence that the allylic steroid, 3$\alpha$-DHP, can be produced by avian ovaries.

## INTRODUCTION

We have recently reported on the steroidogenesis of theca and granulosa cells during follicular development in the adult domestic hen [3, 4]. In the hen, the granulosa layer of the largest yolk-filled follicle is the source of the preovulatory progesterone (P). P has been shown to be the major product of pregnenolone produced by the granulosa cells, with only negligible amounts of androstenedione (A) and testosterone (T) [4].

In contrast to granulosa cells, the theca cells of the hen's ovarian follicles, particularly the third and fourth largest preovulatory follicles, very extensively convert pregnenolone and P to a variety of C-21, C-19, and C-18 steroids. In a previous study we utilized high performance liquid chromatography (HPLC) analysis to identify A, 17-hydroxyprogesterone (17OH), T, and oestrogens following incubations of theca cells with either [$^3$H]pregnenolone or [$^3$H]P [4]. However, we were unable to identify all of the metabolites of P produced by theca cells. In this study, we present evidence that 20$\beta$-hydroxy-4-pregnen-3-one (20$\beta$-DHP), 3$\alpha$-hydroxy-4-pregnen-20-one (3$\alpha$-DHP), and 17$\alpha$,20$\beta$-dihydroxy-4-pregnen-3-one (17$\alpha$20$\beta$) are produced from P by hen theca cells.

## EXPERIMENTAL

### *Experimental animals*

One- to two-year old white Leghorn hens were housed in individual cages under a 14L:10D light cycle, and their time of oviposition was recorded by an automatic device. Purina Layena ration and water were provided *ad libitum*.

### *Hormones and chemicals*

[1,2,6,7-$^3$H]Progesterone (101 Ci/mmol), [4-$^{14}$C]progesterone (55 Ci/mmol), and [1,2-$^3$H]17-hydroxyprogesterone (50 Ci/mmol) were obtained from New England Nuclear Corp., and when necessary, were purified prior to use by thin-layer chromatography (TLC; benzene–methanol, 19:1, v/v). Other chemicals used for the cell incubations have been reported previously [3]. Baker and Fisher HPLC-grade solvents were used for the HPLC analyses. Reference steroids were obtained from Sigma, St Louis, MO, and Steraloids, Wilton, NH, or were synthesized in the laboratory (Wiebe, unpublished).

### *Tissue collection and cell preparation*

Birds were killed by cervical dislocation 2 h prior to expected oviposition and the theca layers from the third largest follicles were collected. Theca cells were isolated by collagenase digestion, as reported previously [3]. Viability of the cells was determined by exclusion of 0.01% trypan blue and was routinely greater than 90%. Cells were counted with the aid of a hemocytometer.

### *Incubations and extractions*

Incubations were done in 12 × 75 mm polystyrene tubes. Each tube contained $2 \times 10^5$ viable cells and either $8 \times 10^{-7}$ M [$^3$H]P, $8 \times 10^{-7}$ M [$^3$H]17OH, or $8 \times 10^{-6}$ M [$^{14}$C]P in 1 ml Medium 199. The incubations proceeded for 5 h at 37°C, open to the air in a shaking water bath. They were terminated by immersion into an ice bath and frozen until further use.

The cells and media were extracted with diethyl ether (twice with 2 vol). The extracts were evaporated to dryness under a gentle stream of nitrogen in a 45°C water bath and the residue stored at −10°C in HPLC-grade methanol until further analysis.

### *HPLC*

Extracts from incubations containing [$^3$H]P or [$^3$H]17OH were analyzed by HPLC as previously [4]. Samples were eluted from a 15 cm × 4.6 mm $C_{18}$ reversed phase column using tetrahydrofuan–methanol–water (16:28:56, by vol.) at 1 ml/min as the mobile phase. Fractions were collected every 0.5 min for 60 min and counted in a Beckman LS 230 scintillation counter. In some cases, HPLC fractions were collected and extracted twice with 2 vol of diethyl ether and reapplied to HPLC or oxidized in 0.2 ml of a 1:1 mixture of acetic acid and aqueous $CrO_3$ (2% w/v) overnight at 4°C. Water was added to the oxidized sample, the sample was re-extracted with ether, and reapplied to HPLC.

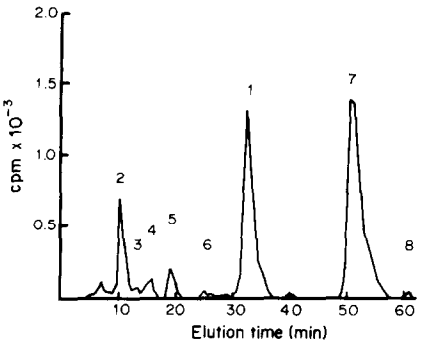


Fig. 1. Profiles of radioactivity collected at 0.5 min intervals from HPLC following 5 h incubations of theca cells with [$^3$H]progesterone as described in Experimental. Peak 1, [$^3$H]progesterone substrate; Peak 2, androstenedione; Peak 3, testosterone; Peak 4, 17α,20β-dihydroxy-4-pregnen-3-one; Peak 5, 17α-hydroxyprogesterone; Peak 6, oestradiol and oestrone; Peak 7, 20β-hydroxy-4-pregnen-3-one; Peak 8, 3α-hydroxy-4-pregnen-20-one. The HPLC solvent system used was tetrahydrofuran–methanol–water (16:28:56, by vol.) at a flow rate of 1 ml/min.

Some extracts from incubations containing [$^{14}$C]P were spotted on 20 × 20 cm Silica gel thin layer chromatographic (TLC) plates and were separated two-dimensionally as described previously [5]. The position of the radioactive metabolites was determined by autoradiography [5]. The radioactive areas were eluted with ether–chloroform (4:1, v/v), solvents were evaporated under nitrogen and the extracts analyzed by HPLC and gas chromatography–mass spectrometry (GC–MS). Radioactive areas comigrating with 20β-hydroxy-4-pregnen-3-one and 3α-hydroxy-4-pregnen-20-one were analyzed by HPLC using a Whatman Partisil 5 OD-3 RAC II/10 column with a $C_{18}$ (70 mm) guard column using tetrahydrofuran–methanol–water (16:28:56, by vol.) and water–methanol (39:61, v/v) at 1.5 ml/min. Fractions were collected every 0.5 min and radioactivity was determined with a Philips 4700/10 microprocessor controlled liquid scintillation counter. In some cases, HPLC fractions were collected and extracted with diethyl ether, evaporated to dryness under nitrogen and then analyzed on GC–MS.

*GC–MS analysis*

Thin-layer chromatography of HPLC fractions were dissolved in heptane–propanol (99:1, v/v). One microliter aliquots were analyzed on a Hewlett–Packard GC–Mass Spectrometer (Model 5970A with 5792A GC, 9825B, 2671G and 9134A Winchester Drive). The GC was fitted with a 15 m, narrow bore (0.25 mm) capillary column bonded wth methyl-3,3-trifluoropropyl-polysiloxane (0.25 micron; J & W Scientific, Inc.). The sample was applied by splitless injection mode and helium was the carrier gas. Temperature programming was from 88 to 188°C at 25°C/min and 188 to 220°C at 6°C/min. Peaks were scanned at 690 amu/s at an electron multiplier setting of 1400 V.

## RESULTS

Figure 1 shows the HPLC profile of radioactivity peaks from [$^3$H]P metabolism by theca cells. In an effort to further characterize the major metabolite from these incubations (Peak 7), which had a retention time of about 53 min in this system and represented as much as 40% of the total recovered radioactivity, a large fraction from several samples was collected between 45 and 58 min. Peak 7 was identified as 20β-DHP by the following procedures: (1) Following ether extraction, samples of Peak 7 were subjected to the oxidation procedure. When reapplied to HPLC, the radioactivity in the oxidized samples eluted with a retention time identical to that of P (34 min). Oxidation of Peak 7 to P indicated that this metabolite must have a hydroxyl group on either the C-3 or C-20. From previous experience, we had ruled out 20α-hydroxy-4-pregnen-3-one based on its shorter retention time (30 min). (2) Extracts from theca cells incubated with [$^{14}$C]P were applied to 2-dimensional TLC (chloroform–ether, 10:3, v/v, in the first direction followed by hexane–ethyl acetate, 5:2, v/v, in the second direction). Authentic 20β-DHP co-migrated with a large spot of radioactivity from theca cell incubations. (3) The spot of radioactivity that co-migrated with authentic 20β-DHP in the 2-dimensional TLC system cocrystallized with unlabelled 20β-DHP as indicated by the constant specific activity in successive crystallizations as shown in Table 1. (4) Authentic 20β-DHP had a retention time of 53 min on HPLC, the same as Peak 7 using tetrahydrofuran–methanol–water (16:28:56, by vol.) at 1 ml/min. (5) Finally, a GC–MS profile of this radioactive metabolite showed that it had the same ion fragmentation pattern as authentic 20β-DHP.

From the 2-dimensional TLC system we also observed [$^{14}$C] radioactivity co-migrating with 3α-DHP. Similarly, the radioactivity in this TLC spot co-eluted with authentic 3α-DHP in 2 HPLC solvent systems: tetrahydrofuran–methanol–water, 16:28:56, by vol. (Peak 8 in Fig. 1), and water–methanol, 29:61, v/v. 3α-DHP represented 1% of the total recovered radioactivity. In addition, the GC–MS profile of this metabolite showed that it had the same ion fragmentation pattern as authentic 3α-DHP, reported previously [6].

Peak 4 (Fig. 1) co-eluted with authentic 17α,20β on HPLC (using tetrahydrofuran–methanol–water, 16:28:56, by vol.) with a retention time of 16 min and was well-separated from the 20α-reduced metabolite of 17OH (17α-20β-dihydroxy-4-pregnen-3-one), which had a retention time of 12 min in this system, and T, which had a retention time of 14 min. Based on these criteria, Peak 4 (Fig. 1) was assumed to be 17α20β.

In additional experiments, in which theca cells were incubated with [$^3$H]17OH, the major metabolite, in addition to A, appeared to be 17α20β when analyzed by HPLC (Fig. 2).

## DISCUSSION

The presence of 20β-hydroxylated products as major metabolites of P in theca cell preparations from the preovulatory follicles of the domestic hen is not surprising in light of previous studies that demonstrated the presence of 20β-DHP from thecal homogenates of the hen incubated with radiolabeled P [7], and the measurement of both free

Table 1. Identification of 20β-hydroxy-4-pregnen-3-one (20β-DHP) produced from [$^{14}$C]progesterone by theca cells from the third largest preovulatory follicle of the domestic hen

| Crystalization No. | Specific activity (dpm/mg) | |
|---|---|---|
| | Crystals | Mother liquor |
| 1 | 246 | 200 |
| 2 | 201 | 397 |
| 3 | 229 | 221 |
| 4 | 246 | 233 |

An aliquot of the radioactive fraction that co-migrated with 20β-DHP in the 2-dimensional TLC system was mixed with 22 mg of authentic 20β-DHP and re-crystallized 4 times. The theoretical specific activity was 230 dpm/mg. Acetone–*n*-pentane was used as the solvent system for the first three crystallizations; methanol–$H_2O$ was used for the fourth crystallization.

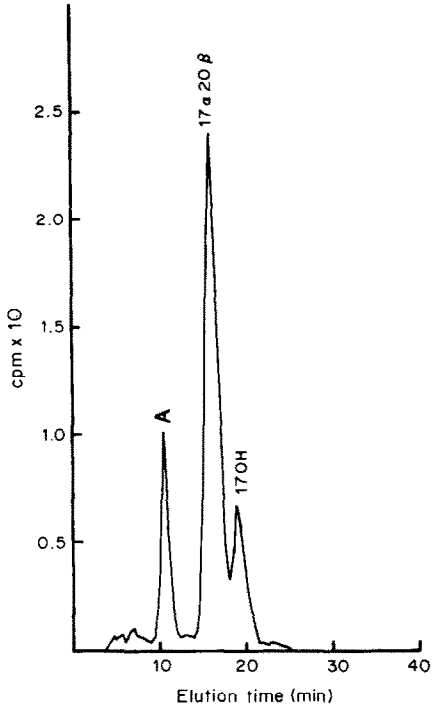


Fig. 2. Profile of radioactivity collected at 0.5 min intervals from HPLC following a 5 h incubation of theca cells with [$^3$H]17-hydroxyprogesterone (17OH) as described in Experimental. The name of the co-eluting steroid appears above each Peak. 17α20β = 17α,20β-dihydroxy-4-pregnen-3-one, a = androstenedione. Tetrahydrofuran–methanol–water (16:28:56, by vol.) at a flow rate of 1 ml/min was used as the solvent system.

and conjugated forms of 20β-DHP in hen plasma [8, 9]. In the bovine ovary, 20β-DHP is also a major metabolite of P [10, 11]. Thus, the ovarian production of 20β-hydroxylated products of P is not unique to avians. However, 20α-DHP appears to be a more common metabolite of P in the mammalian ovary. In the rat, 20α-DHP is produced by granulosa cells and luteal cells [12]. During the preovulatory period, there is a rise in 20α-DHP levels in plasma, coincident with the preovulatory rise in P[13] and 20α-DHP, like P, has been shown to have positive feedback effects on LH and FSH secretion in oestrogen-treated, castrated rats [14].

This is the first report of 3α-DHP formation by ovarian tissue. Its production by rat testicular tissue was recently established [6]. Production of 3α-DHP occurred in the Sertoli cells, but not in the Leydig cells, and its production varied with the age of the rat, being highest at 6–17 days of age round the onset of gametogenesis. The compartmentalization of 3α-DHP production, its variation with age in the rat testis, and some *in vivo* experiments suggest the possibility of a biological function for this allylic steroid. Direct injection of 3α-DHP into testes of 14-day old rats resulted in highly significant increases in number of germ cells in the late pachytene stages, whereas similar treatments with testosterone and dihydrotestosterone had no apparent effect [15].

17α20β is a major metabolite in some fish species [16]. It is produced by the ovarian follicles [17], and it appears to enhance oocyte maturation [18]. Based on the identification of 17α20β as a major metabolite of 17OH in hen theca cells presented in this report, it is tempting to speculate a similar role for 17α20β in oocyte maturation in the hen.

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