J. Steroid Biochem. Molec. Biol. Vol. 37, No. 1, pp. 113–120, 1990
Printed in Great Britain.

0960-0760/90 $3.00 + 0.00

METABOLISM OF PROGESTERONE BY AVIAN GRANULOSA CELLS IN CULTURE

J. P. WIEBE, K. D. BUCKINGHAM, R. L. ZOBELL[1] and F. HERTELENDY[1]

Hormonal Regulatory Mechanisms Laboratory, Department of Zoology, University of Western Ontario, London, Ontario, Canada N6A 5B7 and [1] Department of Obstetrics and Gynecology, St Louis University Medical Center, St Louis, MO 63110-0250, U.S.A.

(Received 5 March 1990)

Summary—Previous studies have demonstrated that progesterone is the primary product of steroidogenesis in avian granulosa cells during short-term incubation. However, during more prolonged culture, lasting several days, the progesterone content in the medium was found to decrease progressively, indicating *in vitro* metabolic conversion. In the present study we have isolated and identified a number of progesterone metabolites. Granulosa cells, isolated from mature ovarian follicles of laying hens, were cultured in medium 199 supplemented with fetal calf serum and containing [^{14}C]progesterone. After 4 days in culture, cells + media were extracted and the radioactive metabolites separated and identified by TLC, HPLC and GC–MS. Several of the metabolites were further characterized by derivatization and crystallization to constant specific activity. A total of 24 radioactive substances was detected. Of these, 15 have been positively identified, 5 tentatively and the remaining 4 are unidentified. The principal metabolite, representing more than 45% of the total radioactivity, was identified as 3α-hydroxy-5β-pregnan-20-one. In addition, significant amounts of 3α-hydroxy-5α-pregnan-20-one (5.76%), 5β-pregnane-3,20-dione (3.05%), and 5α-pregnane-3,20-dione (2.95%) were detected and identified. The results indicate that avian granulosa cells possess 3α-hydroxysteroid dehydrogense (3α-HSD), 17β-HSD, 20α-HSD, 20β-HSD, 17α-hydroxylase, C_{17-20}-lyase and 5α- and 5β-reductase activities. These enzyme activities may convert progesterone to biologically inactive or less active metabolites. However, a functional role for some of these metabolites cannot be ruled out.

INTRODUCTION

Avian granulosa cells, harvested from mature ovarian follicles, produce copious amounts of progesterone during short-term incubations in response to stimulation with LH or other steroidogenic agonists, such as cyclic AMP derivatives and forskolin [1–3]. Previous studies have demonstrated that during a 3-h incubation, 93.6% of [^{3}H]pregnenolone substrate was converted to progesterone by mature chicken granulosa cells [4]. The only other detectable product, representing 1.2% of the total recovered radioactivity, was identified by HPLC as androstenedione [4]. However, preliminary studies [5] have shown that during more extended culture, chicken granulosa cells acquire the ability to metabolize significant amounts of progesterone. By day 4 only 26.3% of added [^{3}H]progesterone could be recovered, with the concomitant appearance of several unidentified metabolites as detected by HPLC. The aim of the present study was to isolate and identify these metabolites of progesterone.

EXPERIMENTAL

Isolation and culture of granulosa cells

The largest ovarian follicle was removed asceptically from White Leghorn hens, 3–4 h before expected ovulation, and placed in cold sterile saline containing 100 mU/ml penicillin and 1 μg/ml streptomycin. The granulosa layer was separated and the cells dispersed with collagenase [6] in medium 199 with Hanks' salts (Gibco, Grand Island, N.Y.). 100,000 viable cells in 1 ml medium M199 containing 10% fetal calf serum and 5 μg/ml gentamycin sulfate were transferred to each well of 24-well plates (Baxter Scientific, McGaw Park, Ill.). To each well, 825 ng [^{14}C]progesterone (57.2 mCi/mmol; DuPont–NEN, Boston, Mass), previously purified by TLC on silica gel G plates (Fisher Scientific, Pittsburgh, Penn.) using benzene:methanol (19:1) as the solvent, was added. The well plates were placed in an incubator maintained at 37°C at high humidity and 5% CO_2/95% air atmosphere. After 96 h in culture, the cells and media

were extracted twice with 2 vols diethyl ether. The pooled extract from two 24-well plates was used for the subsequent isolation and identification of metabolites.

Isolation of metabolites

Methods previously described [7–9] were employed to extract and identify the steroids. Briefly, each of a total of 217 steroid standards was individually spotted on silica gel GF thin-layer (0.25 mm) plates (TLC) and run twice for 15 cm in system 1 (chloroform/ether, 10:3) and the R_f of each was determined. The R_f values were similarly determined by running the steroid standards two times in system 2 (hexane/ethyl acetate, 5:2). The mobility of each steroid was then converted relative to the mobility of progesterone (R_{fp}). The extract from the cell culture was spotted on the lower right hand corner of a 20 × 20 cm TLC plate along with the following standards: 4-androstene-3,17-dione (10 μg), 20α-hydroxy-4-pregnen-3-one (10 μg), 17α-hydroxy-4-pregnene-3,20-dione (10 μg), 4-pregnene-17,20β-diol-3-one (10 μg), 3α-hydroxy-4-pregnen-20-one (30 μg), and 5α-pregnene-3α,20β-diol (50 μg). Each plate was run two times in system 1, then turned 90 deg and run 2 times in system 2. Each plate was then apposed to a sheet of Kodak Medical X-Ray film (S OMAT R film) for a 7-day exposure. Figure 1 shows the developed X-ray film after

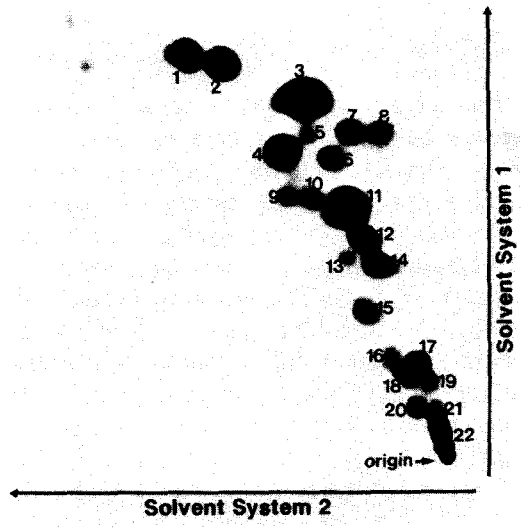

Fig. 1. Autoradiograph of a TLC plate showing the results of incubating avian granulosa cells with [^{14}C]progesterone. The culture contained about 100,000 viable cells. The extracts were spotted on the lower right hand corner (origin) and the plates were run 2 × in system 1 and 2 × in system 2 in the direction indicated. The radioactive spots are numbered and these numbers are applied to the metabolites in subsequent figures and tables.

such an exposure to the metabolites of progesterone formed by the cells under investigation. The relative mobilities (R_f values) were determined from the autoradiographs and the standards were visualized by u.v. (delta-4,3-keto steroids) or by iodine vapour. The R_{fp} values of the radioactive spots were determined and compared with those of 217 known steroids in our library. The spots were then scraped from the plates and the gels extracted with ether/chloroform (4:1). The solvent was evaporated under nitrogen 35°C and the metabolite was taken up in 0.2 ml methanol. An aliquot of each spot was used to determine the radioactivity via scintillation spectrometry in a toluene–ppo–popop system using a Phillips model PW 4700 liquid scintillation counter. Aliquots of each metabolite were then used in various procedures for identification as previously described [8–10] and as outlined under Results.

High-pressure liquid chromatography (HPLC)

A Beckman Model 332 gradient liquid chromatograph with Altex Model 420 microprocessor and Model 155 variable wavelength detector was interfaced with a Hewlett–Packard 5840A GC terminal and was used with a Whatman Partisil 5 ODS-3 RAC II/10 column with a C_{18} (70-mm) guard column using methanol:water (7:3; system A) or tetrahydrofuran:methanol:water (16:28:54 by vol; system B) at 1.0 or 1.5 ml/min. Aliquots of metabolites along with standards were applied and fractions collected very 0.5 or 1.0 min and radioactivity determined by liquid scintillation spectrometry.

GC–MS analysis

To establish capillary GC retention times and fragmentation spectra, a Hewlett–Packard GC–Mass Spectrometer (Model 5970A with 5792A GC) was used with a 12.5-m cross-linked methyl silicone capillary column [11]. The conditions were as follows: splitless mode, 0.7 kg/cm^2 helium, 220°C injection temperature, column temperature at 150°C (initial) to 220°C at 15°C/min, and scan speed of 690 amu/sec at an electron multiplier setting of 1800 V. Steroids were dissolved in methanol and 1 μl aliquots were injected.

Formation of derivatives

Oxidations were performed in 0.2 ml of a 1:1 mixture of acetic acid and aqueous chromium trioxide (2%, w/v) for 10–120 min at room

temperature [8]. The reaction was terminated with 5–10 vols of double distilled water and the steroids were extracted with ether. The extract was washed with 0.1 vol of sodium bicarbonate (50% saturated) and then with water (0.5 vol), dried with sodium sulfate, and evaporated under N_2 at 45°C. Acetylations were performed in 0.2 ml of a 1:1 mixture of pyridine and acetic anhydride, overnight at room temperature. The product was recovered by evaporating the solvents under a stream of N_2 at 45°C. Trifluoroacetylations were carried out at room temperature for 60 min in 0.1 ml of a 1:1 mixture of pyridine and *N*-methyl-bis-trifluoroacetamide (MBTFA; Pierce Chemical Co.).

Crystallizations

An aliquot of ^{14}C-labelled metabolite was placed in a glass tube, the solvent was evaporated, about 20 mg of authentic unlabelled steroid were added and crystallizations occurred from acetone/n-pentane or aqueous methanol.

RESULTS

Figure 1 is a representative autoradiograph showing the metabolites of progesterone produced by the cells. Table 1 shows the radioactivity recovered (% of total) from the 22 spots observed in the autoradiographs and a summary of the methods employed to determine the identity of the metabolites. Ten of the spots were positively identified as 12 different steroids and they represent 83% (in quantity) of all metabolites recovered.

Identified metabolites

Metabolite No. 1 (Fig. 1). The TLC R_{fp} values of metabolite 1 coincided with those of 5α-pregnane-3,20-dione. An aliquot (3890 dpm) was crystallized with authentic 5α-pregnane-3,20-dione (22 mg) using hot acetone/pentane as solvent. The specific activity of the crystals from four crystallizations varied by less than 5% of the initial calculated specific activity (Table 2) and it was concluded that metabolite 1 is **5α-pregnane-3,20-dione**.

Metabolite No. 2. The TLC R_{fp} values of metabolite 2 coincided with those of 5β-pregnane-3,20-dione. An aliquot (4840 dpm) was crystallized with authentic 5β-pregnane-3,20-dione (22 mg) using hot acetone/pentane as solvent. The specific activity of the crystals from 3 crystallizations varied by less than 5% (Table 2) and it was concluded that metabolite 2 is **5β-pregnane-3,20-dione**.

Table 1. Identification and quantitation of metabolites of [^{14}C]progesterone produced by chicken granulosa cells in culture

Metabolite (spot) No.	Steroid	% of total radioactivity	Methods of identification
1	5α-P-3,20-dione	2.95	T, A, Cr
2	5β-P-3,20-dione	3.05	T, A, Cr
3	Progesterone (Substr)	24.36	T, A
4	3α-OH-5α-P-20-one	5.76	T, A, Cr
5	3β-OH-4-P-20-one	0.16	T, A, HP
6	3α-OH-4-P-20-one	0.60	T, A, (ST), HP
7	4-An-3,17-dione	0.86	T, A, (ST), Cr
8	(4-P-3,6,20-trione)	0.13	T, A
9	(5β-P-20β-ol-3-one)	0.33	T, A
10	(5α(β)-P-3β-ol-20-one)	0.48	T, A
11	3α-OH-5β-P-20-one	45.77	T, A, Td, Cr, MSd
12a	20β-OH-4-P-3-one	1.03	T, A, (ST), HP, Cr
12b	20α-OH-4-P-3-one	0.84	T, A, HP
13	(3α-OH-5β-An-17-one)	0.24	T, A
14a	17-OH-4-P-3,20-dione	1.24	T, A, (ST), HP, Cr
14b	Testosterone	0.39	T, A, HP, Cr
15	5α-P-3α,20α-diol	0.39	T, A, Td
16	5β-P-3α,20β-diol	1.24	T, A, (ST), Td
17	(5β-An-3α,17β-diol)	2.18	T, A, Td
18	5β-P-3α,20α-diol	1.09	T, A, Td
19	Unidentified	0.64	T, A
20	Unidentified	1.27	T, A
21	Unidentified	0.69	T, A
22	Unidentified	4.27	T, A

T: TLC in Systems I and II. A: Autoradiography. An; androstene or androstane. Cr: Crystallization with authentic mass standards. HP: HPLC of metabolite along with mass standards in one or more solvent systems followed by radioactivity detection of eluates. MSd: Mass spectrometry of metabolite and standards and of their MBTFA derivatives. P: pregnene or pregnane. (ST): Authentic mass standard was added prior to TLC and autoradiography. Td: TLC of derivatized (oxidized and/or acetylated) metabolite.

Table 2. Crystallization data of eight of the metabolites of [^{14}C]progesterone produced by chicken granulosa cells in culture

Steroid	Spot No.	dpm per mg steroid Crystals (1)	Crystals (2)	Crystals (3)	Crystals (4)
5α-pregnane-3,20-dione	1	167	172	144	164
5β-pregnane-3,20-dione	2	191	198	188	—
5α-pregnan-3α-ol-20-one	4	364	396	346	355
4-androstene-3,17-dione	7	97	90	98	97
5β-pregnan-3α-ol-20-one	11	606	638	591	—
4-pregnen-20α-ol-3-one	12	141	152	130	—
4-pregnen-17α-ol-3,20-dione	14a	75	71	74	85
Testosterone	14b	411	358	326	312

An aliquot of the radioactive metabolite (Spot No.) was crystallized with the authentic steroid (about 20 mg) as outlined under Experimental.

Metabolite No. 3. This was identified as **progesterone** (the unmetabolized precursor), by TLC, HPLC, and by crystallization methods (data not shown).

Metabolite No. 4. The TLC R_{fp} values of metabolite 4 coincided with those of 3α-hydroxy-5α-pregnan-20-one and were similar to those of 17α-hydroxy-5α-pregnane,3,20-dione and 20α-hydroxy-5α-pregnan-3-one. An aliquot (7380 dpm) was crystallized with authentic 3α-hydroxy-5α-pregnan-20-one (18 mg) using hot acetone/pentane as solvent. The specific activities of the crystals from 3 crystallizations varied by less than 5% (Table 2) and it was concluded that metabolite 4 is **3α-hydroxy-5α-pregnan-20-one** (5α-pregnan-3α-ol-20-one).

Metabolite No. 5. The TLC R_{fp} values of metabolite 5 coincided with those of 3β-hydroxy-4-pregnen-20-one and were similar to those of progesterone and 3α-hydroxy-4-pregnen-20-one. An aliquot of metabolite 5 (200 dpm) was run on HPLC (system A) together with these three steroid standards which had retenton times of 33.8, 18.9 and 39.5 min, respectively, and all the radioactivity eluted with the 3β-hydroxy-4-pregnen-20-one peak (33.0–35.5 min) and it was concluded that metabolite 5 is **3β-hydroxy-4-pregnen-20-one** (4-pregnen-3β-ol-20-one).

Metabolite No. 6. The TLC autoradiographic spot of metabolite 6 coincided with the 3α-hydroxy-4-pregnen-20-one mass standard visualized by iodine vapour. An aliquot of metabolite 6 (600 dpm) was run on HPLC (system A), together with the mass standards of progesterone, 3β-hydroxy-4-pregnen-20-one and 3α-hydroxy-4-pregnen-20-one which had retention times of 18.9, 33.8 and 39.5 min, respectively. All of the radioactivity eluted with the 3α-hydroxy-4-pregnen-20-one peak and it was concluded that Metabolite 6 is **3α-hydroxy-4-pregnen-20-one** (4-pregnen-3α-ol-20-one).

Metabolite No. 7. The TLC autoradiographic spot of metabolite 7 coincided with the 4-androstene-3,17-dione mass standard visualized by u.v. An aliquot of metabolite 7 (2660 dpm) was crystallized with authentic 4 androstene-3,17-dione (25 mg) using hot acetone/pentane as solvent. The specific activity of the crystals from three crystallizations varied by les than 5% of the initial calculated specific activity (Table 2) and it was concluded that metabolite 7 is **4-androstene-3,17-dione.**

Metabolite No. 11. Metabolite 11 was the major metabolite (Table 1), constituting over 45% of the total radioactivity. The TLC R_{fp} values of metabolite 11 coincided with those of 3α-hydroxy-5α-androstan-17-one, 5β-hydroxy-5α-androstan-17-one, 17β-hydroxy-5α-androstan-3-one, 3β-hydroxy-5β-pregnan-20-one and 3α-hydroxy-5β-pregnan-20-one. An aliquot of metabolite 11 (3000 dpm) was oxidized and spotted on a 5 × 20 cm TLC plate along with authentic 5α-androstane-3,17-dione and 5β-pregnane-3,17-dione in parallel lanes. The plate was then developed three times in system 2 and apposed to an X-ray film for 1 week. The autoradiographic area of oxidized metabolite 11 coincided with the mass standard of 5β-pregnane-3,20-dione. Acyl derivatives of 3α-hydroxy-5β-pregnan-20-one, 3β-hydroxy-5β-pregnan-20-one (50 μg) and metabolite 11 (3000 dpm), as well as nonderivatized forms were spotted in parallel lanes on a 20 × 20 cm TLC plate. The plate was then developed in system 1 and apposed to an X-ray film for 1 week after which the mass standards were visualized in iodine vapour. Metabolite 11 migrated the same as 3α-hydroxy-5β-pregnan-20-one. An aliquot (14,100 dpm) was crystallized with authentic 3α-hydroxy-5β-pregnan-20-one; radioactivity was retained in the crystals through three separate crystallizations (Table 2). Finally, an aliquot was derivatized

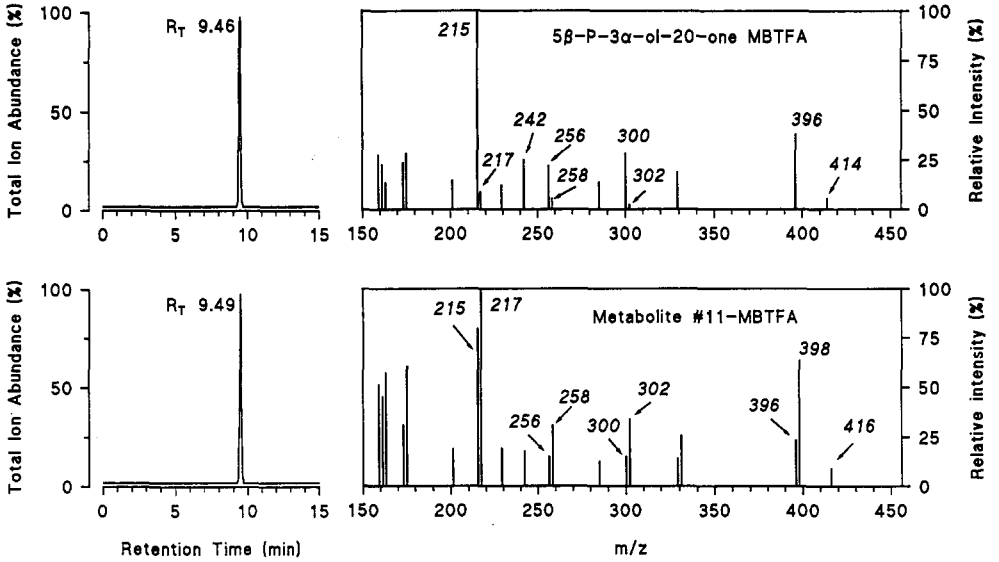

Fig. 2. The GC/MS results of trifluoroacetylated (MBTFA) 5β-pregnan-3α-ol-20-one (top panel) and metabolite No. 11 (bottom panel). The left panels show the retention times on the capillary GC and the righ panels show the relative intensity of the major ions with m/z between 150 and 450. Note that 5 of the major ions in metabolite No. 11 showed a mass of $m/z + 2$, indicating the presence of ^{14}C.

with MBTFA and run on capillary GC/MS: each Trifluoroacetyl group results in the addition of 96 mass units to the molecular mass of a derivatized steroid. The retention time and fragmentation patterns (Fig. 2) were identical to those of MBTFA-derivatized 3α-hydroxy-5β-pregnan-20-one and the major ions ($m/e = 396$, 300, 256 and 121) and molecular mass ($m/e = 318 + 96 = 414$) showed mass of $m/e + 2$ (416, 398, 302, 258 and 123) in the metabolite, indicating the presence of ^{14}C label. Based on these criteria, it was concluded that the major metabolite (No. 11) is **3α-hydroxy-5β-pregnan-20-one** (5β-pregnan-3α-ol-20-one).

Metabolite No. 12. The TLC R_{fp} values of metabolite 12 coincided with those of 20α-hydroxy-4-pregnen-3-one and 20β-hydroxy-4-pregnen-3-one. The autoradiographic spot of metabolite 12 coincided with the u.v. absorbing area of the mass standard of 20β-hydroxy-4-pregnen-3-one. An aliquot of metabolite 12 (3960 dpm) was crystallized with authentic 20β-hydroxy-4-pregnen-3-one (22 mg) and activity was retained in the crystals after 4 crystallizations (Table 2). Another aliquot (365 dpm) was analyzed by HPLC (system B) and showed elution of radioactivity with 20α-hydroxy-4-pregnen-3-one (retention time of 16.6 min) and 20β-hydroxy-4-pregnen-3-one (retention time of 25.7 min) at a ratio of 56 and 44, respectively. Based on these criteria it was concluded that TLC Spot No. 12 (Fig. 1) is a mixture of **20β-hydroxy-4-pregnen-3-one** and **20α-hydroxy-4-pregnen-3-one**. (Crystallizations of 20α-hydroxy-4-pregnen-3-one were not attempted because of insufficient amounts of the authentic steroid.)

Metabolite No. 14. The TLC R_{fp} values of spot No. 14 coincided with those of 17α-hydroxy-4-pregnene-3,20-dione and testosterone (17β-hydroxy-4-androsten-3-one). Spot No. 14 coincided with the u.v. absorbing mass standard of 17α-hydroxy-4-pregnen-3,20-dione. An aliquot (390 dpm) was run on the HPLC (system B) together with the two standards and radioactivity was eluted. Testosterone (retention time of 7.94 min) eluted with 24% of the radioactivity and 76% of the radioactivity eluted with the 17α-hydroxy-4-pregnene-3,20-dione (retention time of 10.33 min). An aliquot of metabolite 14 (2610 dpm) was crystallized with authentic 17α-hydroxy-4-pregnene-3,20-dione and constant specific activity was obtained in crystals through 4 successive crystallizations (Table 2). It was concluded that metabolite 14 is a mixture of **17α-hydroxy-4-pregnene-3,20-dione** and **17β-hydroxy-4-androsten-3-one** (testosterone) at a ratio of 3:1, respectively.

Metabolite No. 15. The TLC (system 1 and 2) R_{fp} values of metabolite 15 coincided with those of 5α-pregnane-3α,20α-diol and were similar to those of 5α-androstane-3α,17β-diol, 5α-androstane-3β,17β-diol, 5α-pregnane-3β,20α-diol, and 17α-hydroxy-5β-androstan-3-one. An aliquot of metabolite 15 and acetylated metabolite 15 were run, along with authentic and

acetylated mass standards, in TLC system 2 and system 16 (hexane/ethyl acetate, 9:1). In each case the R_f value of metabolite 15 coincided with that of 5α-pregnane-3α,20α-diol and it was concluded that spot No. 15 (metabolite 15) is **5α-pregnane-3α,20α-diol**.

Metabolite No. 16. The autoradiographic spot of metabolite 16 coincided with the u.v. absorbing area of the mass standard of 5β-pregnane-3α,20β-diol. The TLC R_{fp} values of metabolite 16 coincided with those of 5β-pregnane-3α,20β-diol and were similar to those of, 3α,17α-dihydroxy-5α-pregnan-20-one, 17α,21-dihydroxy-5α-pregnane-3,20-dione, 18-hydroxy-4-pregnene-3,20-dione. Aliquots of metabolite 16 and acetylated metabolite 16 were run, along with authentic and acetylated mass standards in TLC system 2 and system 16 (hexane/ethyl acetate, 9:1). In each case the R_f values of metabolite 16 coincided with those of 5β-pregnane-3α,20β-diol and it was concluded that spot No. 16 (metabolite 16) is **5β-pregnane-3α,20β-diol**.

Metabolite No. 18. The TLC R_{fp} values of metabolite 18 coincided with those of 5β-pregnane-3α,20α-diol and were similar to those of 3α,17α-dihydroxy-5α-pregnan-20-one, 3α,11β-dihydroxy-5α-androstan-17-one, 6β-hydroxy-4-pregnene-3,20-dione, and 18-hydroxy-4-pregnene-3,20-dione. Aliquots of metabolite 18 and acetylated metabolite 18 were run, along with authentic and acetylated mass standards in TLC system 2 and system 16 (hexane/ethyl acetate, 9:1). In each case the R_f values of metabolite 18 coincided with those of 5β-pregnane-3α,20α-diol and it was concluded that spot No. 16 (metabolite 16) is **5β-pregnane-3α,20α-diol**.

Other metabolites

Metabolites 8, 9, 10, 13 and 17 (Table 1) were only tentatively identified, based on TLC R_f values (systems 1 and 2) of the autoradiographic spots. Spots 19–22 (Table 1) remain unidentified.

DISCUSSION

Progesterone plays a key role in the regulation of ovulation in the domestic hen, and probably other avian species as well. A few hours before ovulation, plasma levels of both progesterone and LH rise simultaneously [12]. Injection of progesterone at an appropriate time during the normal ovulatory cycle causes a premature rise in LH as well as ovulation [13, 14]. It has also been reported recently that progesterone alone can induce ovulation in the isolated perfused chicken ovary [15]. The principal source of progesterone is the granulosa cells [4], the capacity of which to synthesize this steroid hormone increases dramatically during the last 2–3 days of follicular maturation. At the same time, the ability of theca cells to synthesize androgens and estrogens derived from granulosa cell progesterone declines [16]. Previous studies in our laboratories [4, 8, 17] have identified several additional metabolites of progesterone, principally 20β-hydroxy-4-pregnen-3-one and in smaller amounts the allylic steroid, 3α-hydroxy-4-pregnene-3-one, as well as 17α,20β-dihydroxy-4-pregnen-3-one, produced by theca cells isolated from preovulatory follicles of laying hens.

In this study we have confirmed and extended some preliminary observations [5], by identifying for the first time a number of metabolites synthesized by hen granulosa cells in culture. The most abundant of these, representing almost one half of the total radioactivity (including the unmetabolized substrate [^{14}C]progesterone), was identified as 3α-hydroxy-5β-pregnan-20-one. The physiological significance of this metabolite is unknown. Although 3α-hydroxy-5β-pregnan-20-one was identified in the magnum portion of estrogenized chick oviduct, it was devoid of biological activity, as assessed by its inability to induce avidin synthesis [18]. On the other hand, 3α-hydroxy-5α-pegnan-20-one, the second major metabolite of progesterone in the present study, accounting for 5.8% of recovered radioactivity, was found to induce avidin synthesis [18]. The possibility that these 3α-hydroxylated metabolites may play a functional role in the regulation of gonadotropin secretion cannot be ruled out, especially in view of the fact that 3α-hydroxy-5α-pregnan-20-one is a major metabolite of progesterone in mammalian neuroendocrine tissues [19]. Similarly, the hypothalamus, the pituitary and the hyperstriatum dorsale of the laying hen can metabolize progesterone to 3α-hydroxy-5β-pregnan-20-one as well as 5α- and 5β-pregnane-3,20-dione [20]. The latter two metabolites were also produced by granulosa cells in appreciable amounts, accounting for approximately 6% of total radioactivity. However, their possible role as mediators of the positive feedback mechanism regulating LH release has been discounted due to low or no biological activity [20].

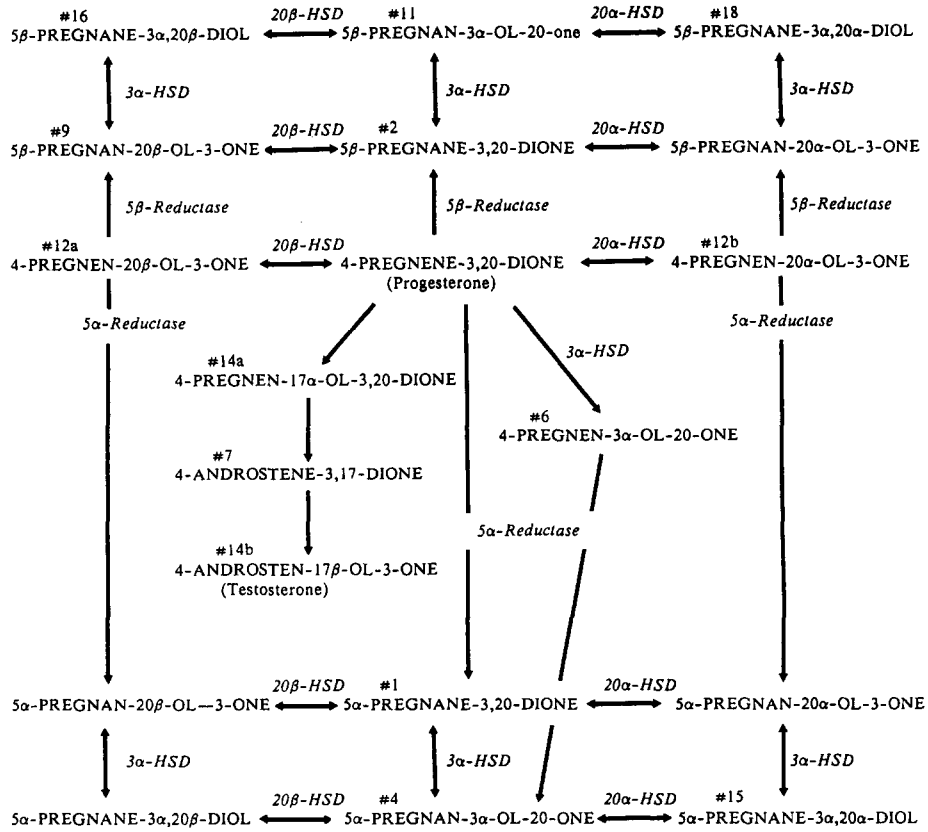

Fig. 3. Diagram showing the metabolic pathways, the metabolites and the enzymes resulting in the metabolism of progesterone in chicken granulosa cells. (Numbers indicate metabolite number.)

The allylic steroid, 3α-hydroxy-4-pregnen-20-one, was identified in the present study as one of the metabolites of progesterone produced by chicken granulosa cells. This allylic steroid has been shown to be produced by rat Sertoli cells [8] and granulosa cells (J. P. Wiebe and M. J. Dallaire, unpublished results). It has been shown to selectively suppress pituitary FSH secretion in the rat *in vivo* [21] and *in vitro* [22] and to act at the hypothalamic level via opioids [23]. No experiments have been conducted to determine the biological effects in chickens of progesterone metabolites such as 3α-hydroxy-4-pregnen-20-one.

It remains unclear why and how granulosa cells acquire the enzymatic machinery to convert progesterone to the various metabolites (Fig. 3). Because progesterone substrate during short-term culture (3 h) was found to remain essentially unchanged [4], the present study indicates that the acquisition of steroid metabolic activity *in vitro* of granulosa cells may be a time-dependent process, suggestive of enzyme induction.

One may speculate that the metabolic conversion of progesterone to biologically inactive or less active products by granulosa cells exposed for extended periods to high levels of progesterone, as in the present study, represents a "detoxification" mechanism. Such a mechanism may also become operative when granulosa cells are stimulated to produce large amounts of progesterone in response to prolonged stimulation with LH. Whether the production of 3α-hydroxy-5β-pregnan-20-one is a unique feature of avian granulosa cell metabolism, or is typical also of mammalian granulosa cells in culture, remains to be clarified.

In summary, this study has demonstrated that mature chicken granulosa cells maintained for several days in culture, efficiently metabolize progesterone to a number of steroids, the principal of which is 3α-hydroxy-5β-pregnan-20-one.

Acknowledgements—We gratefully acknowledge the support of NSERC of Canada grants A6865 and OGP6865 (JPW) and NSF grant DCB8820550 (FH). K. Barr provided valuable technical assistance.

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