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# Biosynthesis of 17α-hydroxy,20α-dihydroprogesterone by Ovaries of the Spotted Flounder (*Verasper variegatus*)

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To examine the production of steroids with potential oocyte maturation-inducing activity in the spotted flounder, *Verasper variegatus*, we have incubated post-vitellogenic oocytes (0.82~0.95 mm in diameters) with radiolabeled pregnenolone and 17α-hydroxyprogesterone. The resulting metabolites were analyzed by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). The two main metabolites (progestogens) found in both incubations co-migrated with 17α-hydroxy,20α-dihydroprogesterone (17α20αOHP) and 17α-hydroxy,20β-dihydroprogesterone (17α20βOHP). Additional chromatography by HPLC and TLC confirmed the presence of radioactive 17α20αOHP and a large amount of unknown metabolite. The present study did not reveal *in vitro* formation of 17α20βOHP. Although 17α20αOHP was found in a small amount, the synthesis of this steroid suggests that it may play a role in regulating the oocyte maturation process in the spotted flounder.

Key words: Biosynthesis, Maturation-inducing steroid, Metabolism, Oocyte, Progestogens, Spotted flounder

## Introduction

In teleosts, as in most of the other vertebrates, oocyte maturation (leading to ovulation) is under the control of gonadotropin released by the pituitary and is mediated by steroids secreted by the follicular cells (Goetz, 1983). For the oocytes to be fertilized they must undergo final oocyte maturation involving germinal vesicle break down (GVBD). Many studies were made on oocyte maturation in Salmoniform and Cypriniform fishes showing that the progestogens, especially those with 20β-, 20α- and 21-hydroxylated steroids, are very effective in inducing oocyte maturation (Scott and Canario, 1987; Nagahama, 1987). One of the most effective steroids so far known is 17α-hydroxy, 20β-dihydroprogesterone (17α20βOHP), which is believed to be a maturation-inducing steroid (MIS) in Salmoniform and Cypriniform fishes (Baek, 1990; Nagahama and Adachi, 1985; Scott and Canario, 1987) and in Siluriformes (Canario and Scott, 1988).

Following our previous study (Baek and Kim, 1996) in which 17α20βOHP and 17α20αOHP were found to be effective in inducing germinal vesicle migration (GVM) and break down (GVBD), the present study examined the biosynthesis of 17α20βOHP, 17α20αOHP and other related steroids from radiolabeled pregnenolone (P5) and 17α-hydroxyprogesterone (17αOHP) in the oocyte of mature female spotted flounder, *V. variegatus*. This flounder, a marine flatfish mainly found off the coasts of the southern regions of Korea and Japan has an asynchronous ovary and is a serial spawner at intervals of approximately 3~4 days over a period of 4 to 6 weeks (Kim et al., 1998). Resulting steroidal metabolites were analyzed using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC).

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## Materials and Methods

Ovaries were taken from two mature females with 0.82~0.95 mm in oocyte diameter. After ovaries were dissected into small pieces in ice-cold trout balanced salt solution (TBSS, Jalabert and Fostier 1984), approximately 40 oocytes were incubated in 24-well culture plates containing 1 mL of Leibovitz L15 medium (Gibco) with the precursor steroids (1.5 μCi [$^3$H]pregnenolone or 4 μCi [$^3$H]17α-hydroxyprogesterone). Incubations were maintained for 24 hrs at 13℃ with constant gentle shaking. At the end of incubations, the steroid carriers were added to each incubation. Medium and oocytes from each incubation were homogenized and extracted twice in 80% ethanol and dichloromethane. The organic phase (free steroids) was evaporated to dry, dissolved in 200 μL ethanol and stored at -20℃ until analysis.

### Reagents

[7-$^3$H(N)]pregnenolone (sp. act. 21.1 Ci/mmol) and 17α-[1,2,6,7-$^3$H]hydroxyprogesterone (sp. act. 66 Ci/mmol) were purchased from NEN and Amersham, respectively. Unlabelled steroids were purchased from Sigma, and solvents were of analytical grade (Merck).

### Thin-layer chromatography (TLC)

One or two-dimensional separations of steroids on silica gel TLC plates 60F$^{254}$ (Merck) were carried out in saturated tanks. Two solvent systems were utilized. System II (chloroform:ethanol=9:1) was run only once in a perpendicular orientation to system I (benzene:acetone=8:2). Carrier and reference steroids were detected by UV absorption (at 254 nm) or by spraying with antimony trichloride.

### Detection of radioactive metabolites

Both autoradiography and plate scanning determined the distribution of radioactivity on the plates. Autoradiograms were obtained using Hyperfilm MP (Amersham). The films were exposed for 72 hrs at -70℃. Radioactive peaks were detected using a Packard Model 7220 TLC scanner.

### Identification of radioactive metabolites

The extract from the TLC plate was analyzed by reversed-phase HPLC (Waters Associates) using a Nucleosil 5 μ $C_{18}$ column (4.6×250 mm) and acetonitril:water 40:60 at a flow rate of 1 mL/min. Absorbance was measured at 254 nm and radioactivity was monitored using a FlowOne (Packard).

## Results

### Incubation of folliculated oocytes with [7-$^3$H (N)]pregnenolone and 17α-[1,2,6,7-$^3$H]hydroxyprogesteron

The experiment was carried out with post-vitellogenic oocytes (0.82~0.95 mm in diameters). The initial separation of the steroids on TLC system I (benzene:acetone=8:2) showed that most of the radioactivity was concentrated in one peak (not including the origin), coincident with the 17α20β OHP standard from the radioactive pregnenolone incubation (Fig. 1). An autoradiogram of the TLC profile of the metabolites was shown in Fig. 3A. Six distinct bands of radioactivity (not including the origin) were observed; pregnenolone (P5, precursor), 17α-hydroxyprogesterone (17αOHP), 17α-hydroxy, 20β-dihydroprogesterone (17α20βOHP), 17α-hydroxy,

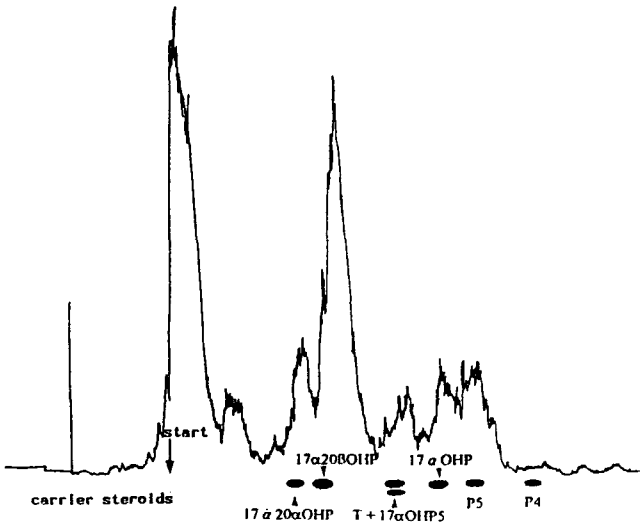


Fig. 1. TLC radiochromatogram of the metabolites produced by isolated oocytes (40 oocytes/mL/well) after exposure to [$^3$H] pregnenolone for 24 hrs. 17α20αOHP (17α-hydroxy,20α-dihydroprogesterone); 17α20βOHP (17α-hydroxy,20β-dihydroprogesterone); T (testosterone); 17αOHP5 (17α-hydroxypregnenolone); 17αOHP (17α-hydroxyprogesterone); P5 (pregnenolone); P4 (progesterone).

20α-dihydroprogesterone (17α20αOHP), mixture of testosterone (T) and 17α-hydroxypregnenolone (17α OHP5) and unknown compound (? was not studied further).

The main metabolites synthesized from radioactive 17α-hydroxyprogesterone coincided with 17α20βOHP, 17α20αOHP and testosterone standards (Fig. 2, 3B).

Progestogen productions, comprising 17α20αOHP and 17α20βOHP were major components in both incubations. These steroids were resolved by HPLC.

When TLC was used for steroid separation, total recovery (radioactivities in the organic and aqueous phases) was calculated as 62~80%.

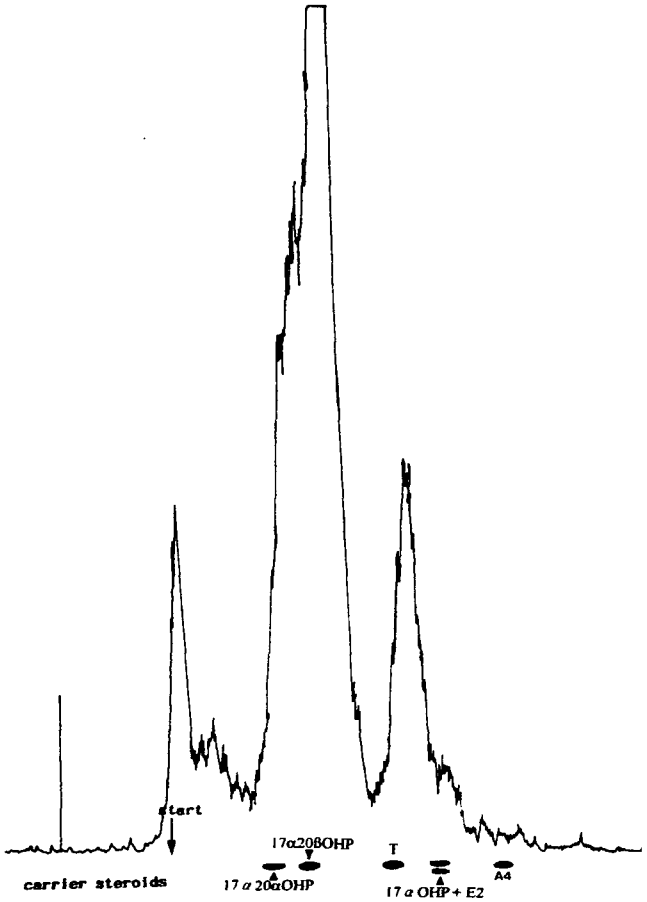


Fig. 2. TLC radiochromatogram of the metabolites produced by isolated oocytes (40 oocytes/mL/well) after exposure to [$^3$H] 17α-hydroxyprogesterone for 24 hrs. 17α20αOHP (17α-hydroxy,20α-dihydroprogesterone); 17α20βOHP (17α-hydroxy,20β-dihydroprogesterone); T (testosterone); 17αOHP (17α-hydroxyprogesterone); E2 (estradiol-17β); A4 (androstenedione).

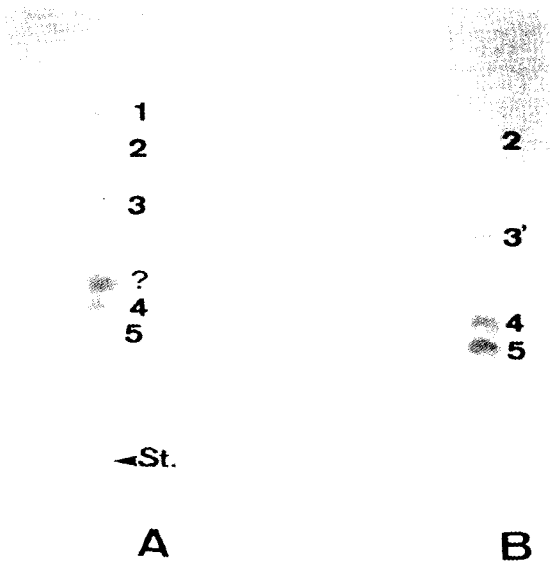


Fig. 3. Autoradiographs of the thin-layer chromatograms of the metabolites formed from [$^3$H] pregnenolone (A) and [$^3$H] 17α-hydroxyprogesterone (B) after 24 hrs incubation with isolated oocytes (40 oocytes/mL/well). 1=pregnenolone, 2=17α-hydroxyprogesterone, 3=testosterone+17α-hydroxypregnenolone, 3=testosterone, 4=17α-hydroxy,20β-dihydroprogesterone, 5=17α-hydroxy,20α-dihydroprogesterone, ? =Unknown compound, St.=start.

### Identification of 17α20αOHP/17α20βOHP metabolites

To identify the metabolites (progestogens) by HPLC and/or TLC, two main bands 4, 5 were collected as individual or combined fractions. The results (Fig. 4) showed a small amount of activity was associated with 17α20αOHP. Radioactivity presumed to be 17α20βOHP did not coincide with its standard. Unknown radiolabeled peak was found at 29.40 min. The identity of this fraction was not investigated further. However, its polarity suggests that it may be a pregnanes metabolite.

## Discussion

It is generally accepted that final oocyte maturation in teleosts is induced by $C_{21}$ steroid hormones, especially those with 20β-, 20α- and 21-hydroxylated steroids (Scott and Canario, 1987). Steroids such as 17α20βOHP, a major ovarian steroid in salmoniform fish (Goetz et al., 1987; Baek, 1990), 17α20α

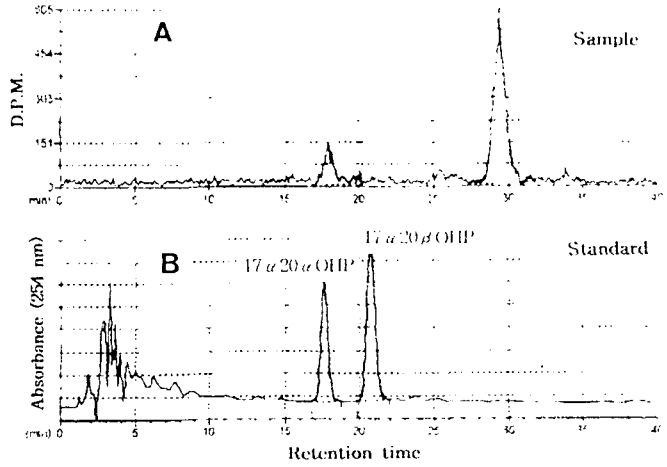


Fig. 4. HPLC elution profile of the bands 4 and 5 showing (A) radioactive metabolite and (B) authentic steroid. 17α20αOHP (17α-hydroxy,20α-dihydroprogesterone), 17α20βOHP (17α-hydroxy,20β-dihydroprogesterone).

OHP, a major ovarian steroid in the dab, *Limanda limanda* (Canario and Scott, 1989) and 17α20β21P, a major ovarian steroid in the Atlantic croaker, *Micropogonias undulatus* (Trant et al., 1986) are all relatively minor components of female plaice plasma (Scott and Canario, 1990).

In the present work, we have investigated the biosynthesis of these and other related steroids from radiolabeled pregnenolone (P5) and 17α-hydroxyprogesterone (17αOHP) in the oocyte of mature female spotted flounder. The results showed that postvitellogenic oocytes of the spotted flounder contain the enzyme 20α-hydroxysteroid dehydrogenase (20α-HSD) and produce 17α20αOHP when incubated with P5 and 17αOHP for 24 hours *in vitro*. In addition, the other steroid, unknown metabolite was also produced at a large amount of activity. We did not detect 20β-hydroxysteroid dehydrogenase (20β-HSD) activity. From *in vivo* experiment, however, lowed plasma levels of 17α20βOHP (<20 pg/mL) were detected with radioimmunoassay after the administration of HCG (data, not shown).

17α20αOHP is reported to be synthesized *in vitro* in the ovarian tissues of a marine flatfish, the dab (*Limanda limanda*) (Canario and Scott, 1989). In the dab, high levels of this steroid were detected after HCG treatment of both female and male fishes (Canario and Scott 1990a, 1991). No traces of any 20β- or 21-hydroxylated steroids among the radioactive steroids produced by the dab ovaries (Canario and Scott, 1989). It is similar to that in the spotted flounder. Synthesis of 17α20αOHP has also been reported in the ovaries of goldfish (*Carassius auratus*) (Kime et al., 1992, 1994), yellow perch (*Perca flavescens*) (Theofan and Goetz, 1983) and African catfish (*Clarias gariepinus*) (Schoonen et al., 1988).

In plaice ovaries 17α20βOHP and 17α20β21P are potent steroids in inducing oocyte final maturation. However, incubations of ovaries of maturing/ovulating plaice with radioactively labelled precursors yield no 20β-reduced steroids (Canario and Scott, 1990b, c). Inbaraj et al. (1997) suggested that 17α20β OHP is the maturation-inducing steroid (MIS) in plaice but that it is rapidly metabolized to inactivated. The same hypothesis has been presented to explain the low levels of 17α20βOHP in plasma of *Fundulus heteroclitus* (Petrino et al., 1993).

In medaka, *Olyzias latipes*, 17α20βOHP is a major MIS, 17α, 20β-dihydroxy-5β-pregnan-3-one appeared immediately after the appearance of 17α20βOHP, suggesting that 5β-reductase may be activated in medaka follicles immediately after the activation of 20β-HSD (Fukada et al., 1994).

The present result provide little evidence that 17α20αOHP is a major MIS in spotted flounder. However, it suggests that 17α20αOHP plays a role (s) in regulating the oocyte maturation process together with unknown metabolite, which would be of considerable interest. Its polarity suggests that it may be a pregnane metabolites.

The identification of unknown metabolite and assay of 17α20αOHP in plasma of the spotted flounder remain to be tested.

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