Breakdown of Germinal Vesicle of Frog Oocytes with 5α-Reduced Products of Progesterone *in Vitro* (37618)

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(Introduced by Roy O. Greep)

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Breakdown of the germinal vesicle of frog oocytes occurs prior to natural ovulation during the spring breeding season. Progesterone induces disintegration of the germinal vesicle in the oocytes of the frog *in vitro* (1–5). The present investigation describes the production of 5α-reduced metabolites from progesterone-4-^{14}C during *in vitro* incubation of oocytes, and the effect of these compounds, from commercial sources, on the disintegration of the germinal vesicle of isolated frog oocytes.

Materials and Methods. Experiments were conducted in mature frogs, *Rana pipiens,* purchased from the Connecticut Valley Frog Farm during Feb.–Mar. They were maintained in water at 4° for a short time in plastic cages. The frogs were decapitated and the ovaries were dissected out. They were immediately placed in freshly made amphibian Ringer solution (pH 7.4), containing approximately 50 mg of streptomycin and 30 mg of penicillin/liter. The oocytes were removed from the ovarian sac using fine watchmaker's forceps under a binocular microscope at 100× magnification.

One hundred oocytes from several frogs were suspended in 2.0 ml of amphibian Ringer solution in a 25 ml Erlenmyer flask. Progesterone-4-^{14}C (New England Nuclear), 1.0 μCi/100 nmoles dissolved in 10 μl of ethanol, was then added to each flask. They were incubated in a Dubnoff shaker at 24° for varying lengths of time, 0, 2, 6, and 21 hr, each in quadruplicate. Two 21-hr incubations received 500 nmoles of nonradioactive 5α-dihydroprogesterone (5α-DHP) in addition to radioactive progesterone. The incubations were terminated by addition of 10 ml of methylene chloride, shaken vigorously, and frozen at −80° until analysis. The content of each flask was extracted twice with methylene chloride and once with ethyl acetate saturated with water, and analyzed by methods described previously (6). The extracts were partitioned between aqueous methanol and hexane(s) several times. The methanol fraction was evaporated to dryness in a vacuum oven at 60°. The methanol extracts were redissolved in 100 μl of ethanol and chromatographed (TLC) on thin layer silica gel plates, 20 × 20 cm (Brinkman, Inc.), in two directions with two different solvent systems: chloroform:ethyl ether (10:3) and hexane:ethyl acetate (5:2). These thin layer plates were then exposed to X-ray film for 24 hr. Black spots on the developed X-ray film indicated the location of the incubation products containing radioactivity (Fig. 1). Silica was scraped from the area of the spots (numbered 1–4) whose R_f values were 0.70 and 0.42; 0.44 and 0.26; 0.36 and 0.22; 0.59 and 0.24 in the two respective solvent systems. They were then eluted with methanol, filtered through a cotton plug, and dried. The eluates were redissolved in ethanol and aliquots were taken for liquid scintillation counting (Beckman, Inc.). The eluates of spots 1–3 from the 21-hr incubation were then recrystallized with 5–10 mg of authentic steroids (Steraloids, Inc.): 5α-DHP,3α-OH-5α-pregnan-20-one, and 3β-OH-5α-pregnan-20-one. The fourth spot was the initial substrate, progesterone-4-^{14}C, and was not recrystallized.

In order to assess the physiological activ-

TABLE I. Temporal Changes in Production of Δ^4-3-keto-Reduced Metabolites During Incubation of Progesterone-4-^{14}C with Oocytes of Frog (dpm).

TLC spot no.	Tentative identification	Incubation (hr)				
		0	2	6	21	21[a]
1	5α-Pregnane-3,20-dione	—	3162	11,647	39,295	23,619
2	3α-OH-5α-Pregnan-20-one	—	1598	3019	11,245	1975
3	3β-OH-5α-Pregnan-20-one	—	2451	5225	20,558	660
4	Progesterone	324,033	281,664	223,670	238,579	324,094

[a] 500 nmoles of nonradioactive 5α-DHP added to the incubations.

ity of these steroids, oocytes were then incubated for 21 hr at room temperature according to the methods outlined by Schuetz (2). Twenty oocytes with intact outer membranes were placed in a 25 ml Erlenmyer flask containing 15 ml of amphibian Ringer solution. Either progesterone, 5α-DHP, 5β-DHP, 3α-OH-5α-pregnan-20-one, or 3β-OH-5α-pregnan-20-one, dissolved in 10 μl of ethanol or 25 μl of propylene glycol:ethanol (1:1), was then added to the incubation flasks in varying concentrations. Control incubations received solvents only. The incubations were terminated by heating the flasks until the solution started to boil. Presence or absence of the germinal vesicle was ascertained by opening the oocytes under a binocular microscope at 100× magnification.

Results and Discussion. A temporal change in the amount of metabolites produced was observed in incubations of oocytes with progesterone-4-^{14}C. The quantity of radioactive steroid formed (dpm) at various times of incubation is shown in Table I (procedural loss not corrected). During shorter periods of incubation, fewer metabolites were produced as observed in the autoradiographs of the TLC plates. The spot, whose R_f in TLC (0.70 and 0.42) compared closely to those of the 5α-DHP standard, was prominent by 2 hr of incubation. Two other metabolites whose R_f corresponded to authentic 3α-OH-5α-pregnan-20-one (R_f 0.44 and 0.26) and 3β-OH-5α-pregnan-20-one (R_f 0.36 and 0.22) were produced in significant amounts in 6 hr. In 21 hr of incubation, approximately 85% of the

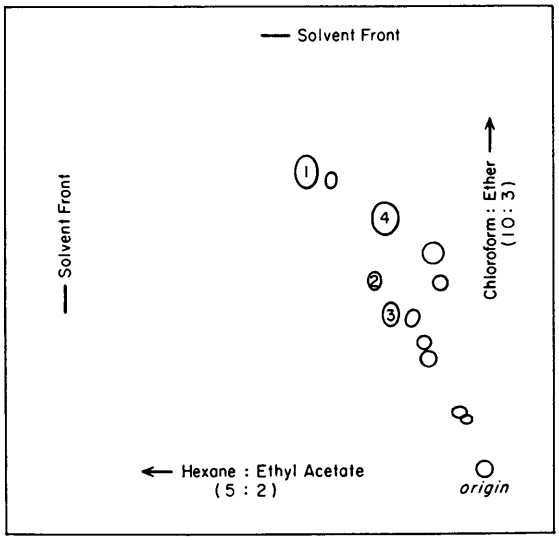

FIG. 1. An autoradiograph tracing of a thin layer chromatograph of extracts from a 21-hr incubation of frog oocytes with progesterone-4-^{14}C. The spots have been tentatively identified as 5α-DHP (1), 3α-OH-5α-pregnan-20-one (2), 3β-OH-5α-pregnan-20-one (3), and progesterone (4).

TABLE II. Isotope Recrystallization of Products of Incubation of Progesterone-4-^{14}C with Oocytes.

TLC spot no.	R_f in TLC (1)[a]	R_f in TLC (2)	Crystallization steroid	Crystal sp act (dpm/mg) *n*-2(3)	Crystal sp act (dpm/mg) *n*-1(4)	Crystal sp act (dpm/mg) *n*(5)
1	0.70	0.42	5α-Pregnane-3,20-dione	3600	3240	3950
2	0.44	0.26	3α-OH-5α-Pregnan-20-one	848	963	895
3	0.36	0.22	3β-OH-5α-Pregnan-20-one	2445	2120	1701

[a] Solvents: (1) chloroform:ether (10:3); (2) hexane:ethyl acetate (5:2); (3) ethanol:H_2O; (4) hexane:benzene; (5) *n*-heptane:methanol.

progesterone substrate was utilized, and many different metabolites were produced as seen in the autoradiographs (Fig. 1). The identity of most of these products remains unknown.

With the addition of 500 nmoles of nonradioactive 5α-DHP to the incubation mixture, the metabolism of progesterone-4-^{14}C into these compounds in 21 hr was significantly retarded (Table I). Formation of 5α-DHP was reduced by 40% compared to the 21-hr incubations which had no additional nonradioactive 5α-DHP. A marked decrease in the formation of 3α-OH- and 3β-OH-5α-pregnan-20-one was observed due to the dilution of radioactive 5α-DHP produced from the initial progesterone-4-^{14}C substrate.

Each of the 3 metabolites (spots 1–3) from the 21-hr incubation of progesterone-4-^{14}C alone was recrystallized in several solvent systems (Table II). The specific activities of the crystals (dpm/mg) after subsequent recrystallizations with different solvents remained essentially unchanged. On the basis of this data, the tentative identifications of these metabolites are: 5α-DHP (spot 1), 3α-OH-5α-pregnan-20-one (spot 2), and 3β-OH-5α-pregnan-20-one (spot 3).

In the second series of incubations conducted for evaluation of the physiological activity of these steroids (Table III), progesterone stimulated germinal vesicle breakdown in a substantial number of oocytes (70–90%) at concentrations ranging from 10^{-6} to 10^{-7}

TABLE III. Breakdown of the Germinal Vesicle (GV) in the Oocytes of the Frog, *Rana pipiens*, with Δ^4-Reduced Progestins.

Steroid	Concn (*M*)	Replicates	Total oocytes incubated	Oocytes without GV (%)
Control	—	6	120	3
Progesterone	10^{-6}	6	120	93
	5×10^{-7}	6	120	87
	10^{-7}	6	120	68
5α-Pregnane-3,20-dione	10^{-6}	5	100	83
	5×10^{-7}	5	100	74
	10^{-7}	5	99	46
3α-OH-5α-Pregnan-20-one	10^{-6}	2	40	65
	10^{-7}	2	40	3
3β-OH-5α-Pregnan-20-one	10^{-6}	2	40	70
	10^{-7}	2	40	3
5β-Pregnane-3,20-dione	10^{-6}	2	40	25
	5×10^{-7}	2	40	20
	10^{-7}	2	40	15

M, which was significantly higher than those of controls (3%). This observation was similar to that described previously (1–5). 5α-DHP also caused disintegration of the germinal vesicle in a significant number of oocytes under identical experimental conditions. The number of oocytes in which germinal vesicle breakdown occurred was slightly lower than that of progesterone, but the values were not markedly different. The further reduced epimers, 3α-OH-5α-pregnan-20-one and 3β-OH-5α-pregnan-20-one, caused disintegration of the germinal vesicle in 65–70% of oocytes at 10^{-6} *M*, but their effectiveness declined sharply as the concentration decreased. The isomer, 5β-dihydroprogesterone, was not as effective in stimulating breakdown of the germinal vesicle (15–25%) at the same dilutions.

Progesterone has been the most effective steroid in causing germinal vesicle breakdown in frog oocytes. Pregnenolone, a precursor of progesterone, has also been shown to induce disintegration of the germinal vesicle (1, 7). Recently it has been shown that frog oocytes possess Δ^5-3β-hydroxy-steroid-dehydrogenase and Δ^5-isomerase enzyme systems which convert pregnenolone into progesterone (1, 7). The biochemical evidence in the present experiments tentatively indicates that progesterone can be further metabolized into 5α-reduced and 3-keto-reduced metabolites.

The reduction of the double bond between C_4 and C_5 of ring A of progesterone is irreversible (8). Since 5α-dihydrotestosterone is a potent androgen in mammals (9), the biological importance of these reduced compounds is of great interest. The physiological role of 5α-dihydroprogesterone is not known, although 5α-DHP has been shown to affect ovulation in rats (10). 5α-Reductase exists in the rat ovaries, but the effect of reduced metabolites on ovum maturation remains to be seen (11, 12). The present experiments show that 5α-reduced progestins can initiate the breakdown of the germinal vesicle of oocytes effectively, and therefore it is likely that the production of 5α-reduced metabolites may be a key step in the mechanism of progesterone action in inducing maturation of frog oocytes.

Summary. The present experiments showed that the oocytes of the frog, *Rana pipiens*, metabolize progesterone-4-^{14}C *in vitro* into several metabolites. Of these 5α-reduced products, 5α-dihydroprogesterone, 3α-OH-5α-pregnan-20-one, and 3β-OH-5α-pregnan-20-one have been tentatively identified by thin layer chromatography and reverse isotope recrystallization. It has been demonstrated that these 5α-reduced steroids effectively induce breakdown of the germinal vesicle of isolated oocytes *in vitro*.

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