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Received January 3, 1967. P.S.E.B.M., 1967, v124.

Effect of Steriods on Germinal Vesicle of Oocytes of the Frog (*Rana pipiens*) *in vitro*.* (31993)

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Germinal vesicle breakdown occurs prior to the reduction divisions characteristic of meiosis in oocytes of the frog *Rana pipiens*. This nuclear disintegration normally takes place at the time of natural ovulation in the spring breeding season, or the ovulation resulting from the injection of pituitary glands or steroids into hibernating frogs(1,2). Steroids or frog pituitary glands are also effective in inducing ovulation *in vitro*(3,4); however, their effects on the process of meiosis are poorly understood. Experiments presented here were conducted to assess and compare effects of various steroids on the breakdown of the germinal vesicle. A standardized *in vitro* assay, utilizing individual ovarian follicles, was perfected to ascertain these effects.

Procedure and methods. Sexually mature large female frogs, *Rana pipiens*, were purchased from a commercial supplier (Shettles, Stillwater, Minn.) and maintained in a 4°C cold room until utilized. The frogs were kept in one-quarter strength Holtfreter's solution which was changed once a week. Experiments presented here were conducted during the months of February and March. Frogs were decapitated, and the ovaries were removed from the body cavity under semi-sterile conditions and immediately placed in finger bowls containing Holtfreter's solution.

Individual follicles containing a single oocyte or ovarian fragments containing 30-80 follicles were obtained by means of blunt dissection using watchmakers' forceps under magnification. Oocytes were freed of investing follicular tissue by tearing the follicular membranes. Gentle pressure applied to the follicle wall opposite the torn membranes caused the oocytes to fall free of the follicular tissues. When sufficient individual oocytes from a single female were obtained, they were mixed together and then randomly assigned (15 individual follicles or oocytes/flask) to 25 Erlenmeyer flasks, each containing 15 ml of Holtfreter's solution. Concentrated stock Holtfreter's solution was diluted immediately prior to use and $NaHCO_3$ added. Although oocytes were found to respond to steroids over a wide range of pH, the pH in these experiments was maintained between 7.8 to 8.1. The flasks were then placed in a Dubnoff metabolic shaker. Steroids were dissolved in 0.1 ml ethanol-propylene glycol (1:1) and then added to the flasks. Each treatment was replicated once (2 flasks) on follicles from an individual animal. Flasks were incubated with shaking for approximately 24 hours at 21-23°C. At termination of incubation, oocytes were pipetted into a watch glass and examined under a dissecting microscope. Shedding or ovulation of oocytes from the ovarian follicle could be established merely by inspection. The effect of test substances on the germinal vesicle was determined by tearing or puncturing the oocyte with watchmakers' forceps. This resulted in the oozing of the oocyte contents into the surrounding medium, and the germinal vesicle was distinguished by its translucent and balloonlike

* Research aided by funds from Nat. Cancer Inst. for the Training Program in Steroid Biochemistry (5 TI-CA 5079).

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TABLE I. Effect of Progesterone on Oocyte Germinal Vesicle in Isolated Ovarian Follicles of *Rana pipiens*.

Treatment	Dose	Total No. of oocytes examined	Total No. of oocytes with intact germinal vesicles
Control		120	120
Vehicle control	.1 ml	120	120
Progesterone	.01 μg	120	120
"	.1 "	120	6
"	1.0 "	120	0
"	10 "	120	0
"	100 "	120	0
"	1000 "	120	60
Progesterone + Versene (.1 M)	10 μg 1.5 ml	120	120
Versene (.1 M)	1.5 ml	120	120

TABLE II. Effect of Various Steroids on Oocyte Germinal Vesicle Breakdown in Isolated Ovarian Follicles of *Rana pipiens*.

	Total No. of oocytes of 60 examined with intact germinal vesicles			
Hormone (μg/15 ml)	0	.1	1.0	10.0
Control	60			
Vehicle control	60			
Estradiol		60	60	60
Androstenedione		30	30	30
Desoxycorticosterone		60	0	0
Dehydroepiandrosterone		60	60	30
Dehydroepiandrosterone sulfate		60	60	60
Pregnenolone		60	30	3
Pregnenolone sulfate		60	60	60
Cholesterol		60	60	60
Testosterone		60	40	1
Hormone (μg/15 ml)	0	1.	10.	100.
Control	60			
Vehicle control	60			
Corticosterone		60	38	0
Hydrocortisone		60	30	18
Androstanedione		60	60	52
Pregnanediol		60	15	0
Progesterone		0	0	0
Aldosterone		60	60	40
17α-OH-pregnenolone		60	35	15

shape. The following steroids were tested: estradiol (1, 3, 5 (10)-estratriene-3, 17-β-diol); androstenedione (4-androstene-3, 17-dione); desoxycorticosterone (4-pregnen-21-ol-3, 20 dione); dehydroepiandrosterone (5-androsten-3 β-ol-17-one); dehydropiandrosterone sulfate (5-androsten-3 β-ol-17-one 3-sodium sulfate); progesterone (4-pregnen-3, 20-dione); pregnenolone (5-pregen-3 β-ol-20-one); pregnenolone sulfate (5-pregnen- 3 β-ol-20-one-3-sodium sulfate); cholesterol (5-cholesten-3 β-ol); testosterone (4-androsten-17 β-ol-3-one); hydrocortisone (4-pregnen-11 β, 17α, 21-triol-3, 20-dione); corticosterone (4-pregnen-11β, 21-diol-3, 20-dione); aldosterone (4-pregnen-18-al-11β, 21-diol-3, 20 dione); androstanedione (5α-androstan-3, 17-dione); pregnanediol (5β-pregnen-3α, 20α-diol); 17α-OH-pregnenolone (5-pregnan-3β, 17α-diol-20-one).

Results. Effect of progesterone on isolated follicles. Individual follicles from the ovaries of one animal were added to flasks. Varying amounts of progesterone were then added. The entire experiment was repeated 4 times utilizing follicles from 4 different animals. The combined data are presented in Table I and demonstrate that progesterone was effective in producing germinal vesicle breakdown over a wide range of doses (0.1-1000 μg)/15 ml. Addition of Versene (divalent chelating agent) to the incubation medium prevented the the action of progesterone. Versene in the presence or absence of progesterone caused the germinal vesicle to swell and migrate to the surface of the oocyte. Breakdown of the germinal vesicle was only rarely accompanied by ovulation of the oocytes from the follicle.

Comparative effect of steroids on isolated follicles. Isolated follicles were exposed to graded doses of various steroids and their relative effectiveness in producing germinal vesicle breakdown was assessed. Follicles obtained from 2 animals were utilized and each treatment was replicated once with the oocytes from each animal. The results of 2 separate series of experiments presented in Table II demonstrate that the level of steroid required to produce germinal vesicle breakdown varied markedly. The compounds progesterone, pregnenolone, testosterone, desoxycorticosterone, and androstenedione all produced some germinal vesicle breakdown at a dose of 1 μg/15 ml. The other compounds tested were either ineffective or only effective at much higher doses. The sulfated steroids were also ineffective in producing nuclear changes. In some cases the level of steroid required to produce germinal vesicle breakdown varied with the

TABLE III. Responsiveness of Oocytes to Progesterone Following the Removal of the Ovarian Follicular Tissue.

Treatment	Total No. of oocytes examined	No. of oocytes with intact germinal vesicles
Ovarian membrane present		
Control	30	30
0.1 μg Progesterone	30	30
1.0 " "	30	0
10 " "	30	0
100 " "	30	0
Ovarian membrane removed		
Control	30	30
0.1 μg Progesterone	30	30
1.0 " "	30	0
10 " "	30	0
100 " "	30	0

animal utilized in testing. Androstenedione was ineffective on oocytes obtained from one animal but was effective on oocytes from the other animal. Desoxycorticosterone produced germinal vesicle breakdown in follicles of both animals at similar doses (Table II).

Effect of progesterone on oocytes freed from the follicle. Removal of the follicle wall to free the oocyte did not produce spontaneous germinal vesicle breakdown. Addition of progesterone was equally as effective in producing nuclear breakdown in the presence or absence of the follicle wall (Table III).

Discussion. The data presented here demonstrate that steroids are effective agents in producing germinal vesicle breakdown of isolated oocytes of *Rana pipiens in vitro.* The physiologically potent steroids estradiol and aldosterone have relatively little effective biological activity when contrasted to the activity of progesterone, pregnenolone, desoxycorticosterone, testosterone, and androstenedione. This marked variation in the biological activity of the steroids suggest that some degree of steroidal specificity is required to produce these changes in the nucleus. The absence of any enhanced activity of the more water soluble sulfated steroids (Table II) furthermore suggests that the differential effectiveness of the steroids is not due to their solubility characteristics.

Although the steroids induce germinal vesicle breakdown in oocytes, with or without the follicular tissue present (Table III), ovulation rarely occurs. Previous investigators, as well as myself, have observed that the steroids used here are effective in producing ovulation when ovarian fragments of whole ovaries are utilized instead of individual follicles(3). Since ovarian tissue normally undergoes contractions prior to and during the process of extruding oocytes, the inability of steroids to produce ovulation from isolated follicles may be due to an interference with this contraction process. Ovulation does not appear, therefore, to be a prerequisite for the induction of germinal vesicle breakdown. These data also strongly suggest that the effect of the steroids is directly upon the oocytes and not mediated by the follicular tissue. The intraoocyte action of the steroid on the germinal vesicle is not known. Data on the effects of Versene (Table I) indicate that the presence of the single divalent ion calcium in Holtfreter's medium is important for these changes to occur.

The demonstration of germinal vesicle breakdown by the addition of steroids to a medium containing fresh frog oocytes suggests a possible relationship to the normal physiological process. The pituitary gland is normally responsible for the induction of ovulation and germinal vesicle breakdown; however, it remains to be demonstrated whether the mediation of its effects may be partially dependent upon steroids. On the basis of the structure-activity relationship, the demonstration that the closely related substances, progesterone, pregnenolone, and 11-deoxycorticosterone, are the most active of those tested, may be of real significance to the problem under study.

Summary. Individual follicles, with their enclosed oocytes, were dissected from the ovary of the leopard frog, *Rana pipiens,* and their response to steroid hormones studied. Biologically and chemically related substance, progesterone, pregnenolone and 11-deoxycorticosterone, were most effective in producing germinal vesicle breakdown. Removal of the follicular tissue did not prevent the action of the steroid progesterone on the germinal vesicle. Steroids appear to act directly on the oocyte and calcium ions appear to be important for the biological action of the steroids.

The author wishes to express his sincere appreciation to The Upjohn Co. for the support which greatly aided this study. The efforts of Dr. Albert Notation in preparation of sulfated steroids are also acknowledged.

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Received January 4, 1967. P.S.E.B.M., 1967, v124.

Metabolism of Estradiol-17β-4-^{14}C in a Non-Pregnant Rhesus Monkey.* (31994)

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Although the rhesus monkey is being used extensively for investigations in reproductive biology, only a few studies of estrogen metabolism have been carried out in this species. Short and Eckstein identified estrone and estradiol-17β in the urine of pregnant monkeys but were unable to detect estriol(1). Recently Laumas reported estriol to be present in the urine of rhesus monkeys (2).

The present study was carried out to determine the metabolism of estradiol-17β-4-^{14}C in a non-pregnant rhesus monkey during the follicular stage of the menstrual cycle.

Materials and methods. A 6 kg *Macaca mulatta* was injected intravenously with 4.9 μC of estradiol-17β-4-^{14}C (specific activity, 8.18 mC/mM; New England Nuclear Corp.) dissolved in 4.5 ml of saline and 0.5 ml of ethanol. The estradiol-17β-4-^{14}C was purified by paper and thin layer chromatography prior to its use in the present experiment.

Injection was carried out on the eighth day of a 29-day menstrual cycle. The animal was housed in a metabolic cage with the collection flask packed in dry ice so as to insure immediate freezing of voided urine throughout three consecutive 24-hour collections.

*This work was supported in part by U.S.P.H.S. Grants HD-01199 and AM,14013 and a grant from the Ford Foundation.

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Five ml of heparinized blood, drawn at 5, 30, 60 and 180 minutes after injection, was centrifuged for 30 minutes at 3600 rpm. One-tenth ml of plasma was diluted to 1 ml with water and extracted 2 × with 5 ml of ethyl acetate. The ethyl acetate was evaporated in scintillation vials and counted. Fifty μg each of carrier estrone, estradiol-17β, estradiol-17α, 16 epiestriol and estriol was added to each 24-hour urine. One ml aliquot of each 24-hour volume was assayed for radioactivity. The remaining total volume of each day's urine was extracted 3 × with 2 volumes of freshly distilled diethyl ether ("free fractions"). The extracted urines were adjusted to pH 6.8 following addition of 1/10 volume of 0.2 M maleate buffer(3). The urine was incubated in the presence of bacterial glucuronidase (300 units/ml; Sigma) for 24 hours at 37°C. Extraction was carried out as before ("glucuronide fractions").

The aqueous phases were adjusted to pH 6.0 with 0.2 M maleic acid and incubated for 24 hours at 50°C in the presence of phenolic sulfatase (10 mg/ml; Nutritional Biochemicals). Following extraction as above ("sulfate fractions"), 15 volumes percent concentrated HCl was added to the extracted urines. They were refluxed for 1 hour, cooled and extracted with ether ("acid fractions").

The ether from each of the above frac-