

STEROID INHIBITION OF PROTEIN INCORPORATION BY ISOLATED AMPHIBIAN OOCYTES

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ABSTRACT

The relationship between blood protein (vitellogenin) incorporation and nuclear maturation was studied in individual amphibian oocytes after in vitro exposure to desoxycorticosterone acetate (DOCA). Isolated *Rana pipiens* oocytes were incubated in vitro with radioactively labeled oocyte yolk precursor ($[^3\text{H}]$ vitellogenin) obtained from estrogenized *Xenopus laevis*. Incorporation of labeled vitellogenin into the oocytes continued over a 24-h period. Oocytes simultaneously exposed to DOCA and to labeled vitellogenin exhibited both inhibition of vitellogenin incorporation and stimulation of nuclear maturation and cortical changes. Inhibition of vitellogenin incorporation was observed after approximately 9 h of incubation and was correlated with the time of nuclear breakdown. Preincubation of oocytes in steroid for 9 h essentially terminated vitellogenin incorporation. Incorporation of vitellogenin occurred after removal of follicle cells from the oocyte by a short treatment with EDTA. These results demonstrate the macromolecular vitellogenin transport system remains operative in oocytes which can undergo nuclear maturation and that the steroid DOCA can affect its function. Evidence suggests that the mechanism of steroid inhibition is in part the result of inhibition of the micropinocytotic process in the oocyte cortex.

INTRODUCTION

The female gamete undergoes a complicated process of cytoplasmic and nuclear differentiation within the ovarian follicle in preparation for fertilization and embryonic development. In amphibians, cytoplasmic accumulation of yolk proteins (vitellogenesis) is a major factor contributing to the growth of these highly specialized cells. Considerable evidence indicates that the major yolk precursor (vitellogenin) is formed in the liver in response to estrogenic hormones and then released into the blood stream (Wallace and Dumont, 1968; Follett et al., 1968; Wallace, 1972).

Subsequently, the vitellogenin is transported through the ovarian follicle and into the oocyte by a process of micropinocytosis where it undergoes transformation and is incorporated into yolk platelets (Wallace and Dumont, 1968; Wallace and Jared, 1969; Redshaw and Follett, 1971). Experiments have shown that selective incorporation of labeled vitellogenin into isolated ovarian oocytes can occur under in vitro conditions (Jared and Wallace, 1969; Wallace et al., 1970). Incorporation of vitellogenin by oocytes under both in vivo and in vitro conditions, how-

ever, typically occurs or is studied before disintegration of the nucleus and the initiation of the meiotic maturation process. Thus it is not clear whether the cytoplasmic process of growth (vitellogenin incorporation) and the nuclear process of meiotic maturation are associated or mutually exclusive (Wallace and Ho, 1972). Recent in vitro studies in amphibians have shown that particular steroid hormones initiate a structural rearrangement of the cellular elements within the ovarian follicle, as well as inducing meiotic maturation to proceed in the enclosed oocyte (Schuetz, 1967, 1972 *a, c*; Masui, 1967; Smith et al., 1968). These oocyte and follicular alterations appear to be necessary prerequisites for the formation of a haploid gamete as well as for the extrusion or ovulation of the oocyte. Although the physiological consequences of these structural alterations are poorly understood, it has been suggested that these changes may interfere with normal metabolic exchange between the developing oocyte and its maternal environment (Schuetz, 1972 *a, b*).

We have directly examined this question by studying the incorporation of the maternally derived vitellogenin into isolated oocytes and by evaluating the extent and mechanism by which hormones influence the cytoplasmic incorporation of labeled vitellogenin and the nuclear maturation process. The data presented here clearly demonstrate that the steroid, deoxycorticosterone, simultaneously inhibits macromolecular (vitellogenin) incorporation and stimulates nuclear maturation. Thus macromolecular protein incorporation into oocytes is sensitive to steroidal regulation, and the evidence suggests that the steroid is directly affecting the micropinocytotic process in the cortex of the oocyte.

MATERIALS AND METHODS

Animal and Oocyte Collection

Female *Rana pipiens* were obtained from Bay Biologicals, Ontario, Canada, and maintained under conditions of artificial hibernation at 4°C. Females used as oocyte donors were killed and the gonads were removed and maintained in vitro in amphibian Ringer's solution containing added antibiotics, penicillin G and streptomycin sulfate (Schuetz, 1967). Experiments were carried out on oocytes from which the outer follicular membranes (ovarian epithelium and theca) were dissected in order to facilitate vitellogenin incorporation (Wallace et al., 1970).

Media Preparation and Culture Procedure

After the injection of [³H]leucine into animals, radioactive vitellogenin (134 dpm/μg) was obtained by chromatography of the serum from estrogenized female *Xenopus laevis* (Wallace and Jared, 1968, 1969). Before use, frozen samples of labeled vitellogenin (47 mg/ml) were thawed and mixed 1:1 with unlabeled male *X. laevis* blood serum. The mixture was dialyzed overnight at 4°C against amphibian Ringer's solution, collected, and centrifuged at 4°C in a clinical centrifuge to remove any small amounts of precipitated material. The labeled mixture was diluted with amphibian Ringer's (1:1 or 1:3), filtered (0.45 μm Millipore filter, Millipore Corp., Bedford, Mass.), and divided into two aliquots. Desoxycorticosterone acetate (DOCA) was dissolved in propylene glycol:ethanol (1:1) vehicle and added to one aliquot of vitellogenin (1.0 μg DOCA/ml). An equal volume of steroid vehicle alone was added to the other aliquot. Individual denuded oocytes were incubated in a depression dish (Linbro Chemical Co., New Haven, Conn., IS-MRC-96) in 50 μl of steroid or control media containing radioactive vitellogenin. In all cases treatment groups consisted of five oocytes distributed into dishes from a pooled sample of oocytes collected from an individual animal. Depression dishes were covered and placed in a closed container with water-saturated toweling to prevent dehydration, and incubated for varying periods of time at room temperature. After incubation, oocytes were washed briefly for 1 h (three times in 15 ml of amphibian Ringer's), and individually examined under the dissecting microscope for evidence of damage. Oocytes were transferred directly to scintillation vials or were fixed (heat 100°C) and dissected to determine whether nuclear maturation (germinal vesicle breakdown) and/or separation of the vitelline envelope from the oocyte had occurred (Schuetz, 1972 *a*). After dissection, the oocyte fragments were collected and transferred to a scintillation vial for counting. Before counting, oocytes were digested overnight in 0.5 cc Protosol (New England Nuclear, Boston, Mass.) at 50°C. Scintillation cocktail (0.6% 2, 5-diphenyloxazole [PPO] in toluene) was then added. Sample radioactivity was counted in a Beckman L-250 scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). In most experiments, counts were converted to disintegrations per minute (100% efficiency) with the aid of an external standard measurement. Statistical analyses were carried out using the paired *t* test (Steel and Torrie, 1960). Oocyte diameter was measured with the micrometer fitted into the eyepiece of a dissecting microscope.

For electron microscopy, tissues were fixed in 3% glutaraldehyde in 0.066 M phosphate buffer (pH

7.4) for 2 h, washed in the same buffer, postfixed in 1% osmium tetroxide, and infiltrated and embedded in Epon. Thin sections stained in uranyl acetate and lead citrate were examined with an Hitachi 11-E electron microscope.

RESULTS

Effect of DOCA on Vitellogenin Incorporation

Oocytes from three animals were collected and exposed to labeled vitellogenin (diluted 1:1 with amphibian Ringer's) and incubated with or without DOCA for 27 h. Subsequent to washing, oocytes were fixed, individually examined to determine whether nuclear maturation had occurred, and then processed for measurement of radioactivity. Fixing and dissection of individual oocytes was not found to significantly affect the amount of radioactivity recovered from oocytes. Table I summarizes these data and shows that significantly less ($P < 0.01$) radioactive vitellogenin was incorporated by oocytes exposed to DOCA than by control oocytes in all the animals tested. Differences in the amount of labeled vitellogenin incorporated by oocytes from different animals, both in the presence and the absence of DOCA, were observed. Oocytes from animal C exposed to DOCA contained less than half the radioactivity seen in similarly treated oocytes from animal B, even though the oocyte incorporated similar amounts of radioactivity in the absence of DOCA. The data from animal A are not directly comparable because of difficulties arising from the use of a different scintillation cocktail for these oocytes. Time studies further demonstrated that incorporation of labeled vitellogenin occurred over the 27-h period of incubation in oocytes not treated with the hor-

none. Nuclear disintegration, as well as separation of the vitelline envelope from the oocyte plasmalemma, was also observed in all the steroid-treated oocytes. Clearly then, radioactive vitellogenin obtained from *X. laevis* is incorporated into *R. pipiens* oocytes. Simultaneous incubation of oocytes with steroids and vitellogenin further inhibits this incorporation, as well as initiating nuclear and cortical differentiation. This demonstrates, therefore, that the oocytes incubated in vitellogenin retain their responsiveness to the steroid hormones. On the basis of these data, we also conclude that oocytes which are sensitive to the steroid, as reflected in the initiation of nuclear breakdown, retain the capacity to incorporate vitellogenin.

Time-Course of Vitellogenin Incorporation

The question arises as to whether steroid inhibition of incorporation of labeled vitellogenin into amphibian oocytes is the result of a reduced rate of incorporation over the duration of incubation or is due to a time-dependent alteration in the rate of incorporation. Previous studies have shown (Schuetz, 1967, 1972 *a*) that steroid induction of nuclear maturation occurs after a lag period of several hours. Thus a time-course of vitellogenin incorporation by oocytes in the presence or absence of steroid was carried out to investigate this question. Oocytes were incubated with ^3H -labeled protein mixture (diluted 1:3 with amphibian Ringer's) and at designated intervals were collected, washed, and processed, without fixation, for radioactivity measurement. Other oocytes also incubated with labeled protein and DOCA were fixed and examined at the designated times to determine if and when nu-

TABLE I
In Vitro Effect of DOCA on Incorporation of [^3H]Vitellogenin into Amphibian Oocytes and Oocyte Maturation

Animal*	Control			DOCA		
	No. of oocytes	cpm/Oocyte	GVBD†	No. of oocytes	cpm/Oocyte	GVBD†
A	5	8,355 ± 235	0	5	1,946 ± 136	5
B	5	2,030 ± 97	0	5	534 ± 54	5
C	5	1,886 ± 79	0	5	211 ± 41	5

* Oocyte diameter of A, 1.70 mm ± 0.01; B, 1.45 mm ± 0.01; C, 1.49 mm ± 0.01.

† Germinal vesicle (nucleus) breakdown.

clear breakdown occurred. Results of this experiment are presented in Fig. 1 and Table II.

Incorporation of isotope occurred over the 25-h period in the control oocytes, whereas the amount of radioactivity in steroid-treated oocytes was essentially the same at all time periods. Considerable incorporation of vitellogenin in the presence of the steroid was evident; however, this had occurred within the initial 9-h incubation period. In fact, in animal B, after 9 h of incubation, the amount of radioactivity in the steroid-treated oocytes was not statistically different from the controls, indicating an essentially identical rate of vitellogenin incorporation during this 9-h incubation period. Inhibition of vitellogenin incorporation into the oocytes of animal A was statistically significant ($P < 0.01$) even at the 9-h sample. These animal differences were strikingly correlated with the nuclear maturation process and the separation of the vitelline envelope from the oocyte. Oocytes from animal A had or were undergoing nuclear disintegration at 9 h. Nuclear maturation was not observed in oocytes of animal B after 9 h of incubation but had occurred before 19.5 h of incubation. Time-course studies were carried out on oocytes of three additional animals in which the individual oocytes were both examined for nuclear disintegration and assayed

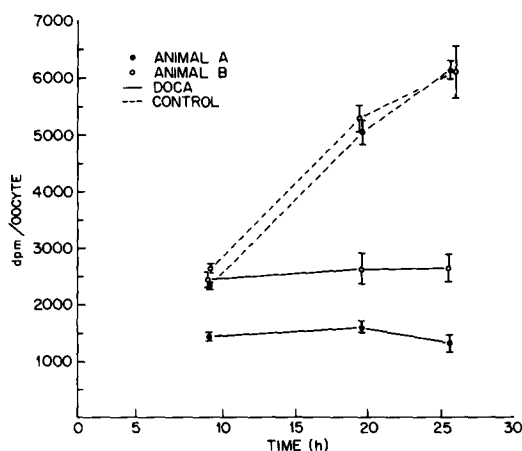


FIGURE 1 Time-course effect of deoxycorticosterone acetate on incorporation of [^3H]vitellogenin into denuded *R. pipiens* oocytes in vitro. Individual oocytes were simultaneously incubated in labeled vitellogenin and either the steroid or the hormone vehicle. Each group consisted of five denuded oocytes obtained from an individual animal. Each point on the graph represents the mean, and the bars represent the standard error of the mean.

TABLE II
Incorporation of [^3H]Vitellogenin into Amphibian Oocytes in Vitro: Time-Course*

Incubation time	DOCA	No. of oocytes	Vitellogenin incorporated	
			dpm/oocyte	dpm/mm ²
Animal A				
9	+	5	1,456 ± 63	170.5
19.5	+	5	1,629 ± 92	190.7
25.5	+	4	1,530 ± 261	179.2
9	-	5	2,357 ± 54	276.0
19.5	-	5	5,059 ± 256	592.4
25.5	-	5	5,973 ± 253	699.4
Animal B				
9	+	5	2,465 ± 153	339.1
19.5	+	5	2,673 ± 256	367.7
25.5	+	5	2,669 ± 247	367.1
9	-	5	2,512 ± 96	345.5
19.5	-	5	5,328 ± 230	732.9
25.5	-	5	6,137 ± 485	844.6

*After incubation, oocytes were washed three times in 15 ml of fresh amphibian Ringer's for 45 min before digestion with protosol. Surface area: animal A, 8.54 mm²; animal B, 7.27 mm².

for radioactivity. A similar, approximately 9-h, lag period for steroid inhibition was seen in all animals.

Effect of Preincubation on Incorporation

In order to confirm that the lag period for inhibition was due to the time of exposure of the oocytes to steroid rather than to the duration of incubation, oocytes were preincubated for 9 h with or without steroid and subsequently transferred to media containing labeled vitellogenin. Oocytes were incubated in the labeled vitellogenin for 16 h and then assayed for radioactivity. These results are presented in Table III and demonstrate that preincubation essentially obliterates the presence of a lag phase for inhibition. The amount of protein incorporation (disintegrations/minute/oocyte) in the control oocytes was greater than 50 times that which was seen in the steroid-treated oocytes.

Effect of Follicle Cells on Incorporation

The role of the follicle cells which remain attached to the vitelline envelope on the incorporation of vitellogenin was also investigated. Denuded oocytes were preincubated in amphibian Ringer's

containing 3 mM EDTA for a 1-h period. This treatment loosens the follicle cells from the vitelline envelope, and they can then be removed from the oocytes after agitation or by manipulation with forceps. Table IV presents the results of such an experiment, which indicate that, after removal of the follicle cells, oocyte incorporation is nearly comparable to that seen in oocytes not treated with EDTA (Table III). These results suggest that the follicle cells play no role in the transfer of the labeled vitellogenin into the oocyte. In addition, oocytes preincubated in EDTA for 1 h and subsequently incubated in the presence of DOCA exhibited inhibition of vitellogenin incorporation and underwent nuclear disintegration. Prolonged incubation (9 h) in EDTA before incubation with vitellogenin, however, does markedly inhibit protein incorporation.

Effect of DOCA on the Oocyte Surface

The surface of control oocytes cultured for 25.5 h is highly irregular or contoured and, like that of oocytes *in vivo*, is characterized by many ridges and valleys or crypts (Fig. 2). Microvilli project from the surface and extend outward to interdigitate with the vitelline envelope. The oolemma is further characterized by the presence of micropinocytotic pits. In the subjacent ooplasm are micropinocytotic vesicles or pinosomes and larger bodies (PYP, Fig. 2) similar to the primordial yolk platelets found in *Xenopus* oocytes that are actively incorporating yolk precursors (Dumont, 1972).

TABLE III
Uptake of [³H]Vitellogenin by Isolated Oocytes of *R. pipiens* after Preincubation in the Presence or Absence of Steroid Hormones*

Animal	No. of oocytes	dpm/oocyte	dpm/mm ²
Preincubation with DOCA			
A	5	76 ± 7	8.9
B	5	62 ± 5	7.3
Preincubation controls			
A	5	4,427 ± 110	608.9
B	5	3,653 ± 568	502.5

* Oocytes were preincubated for 9 h in amphibian Ringer's solution containing either DOCA or steroid vehicle (propylene glycol:ethanol, 1:1) before being transferred to media (50 μl) containing [³H]vitellogenin. Incubation in [³H]vitellogenin was for 16.5 h.

TABLE IV
Effect of Preincubation in 3 mM EDTA on Incorporation of [³H]Vitellogenin into *R. pipiens* Oocytes *In Vitro*

Preincubation time in EDTA	No. of oocytes	Presence or absence of DOCA	Vitellogenin incorporated
<i>h</i>			<i>dpm/oocyte</i>
1	5	—	5,063 ± 692*
1	5	+	1,885 ± 192*
9	5	—	72 ± 18†

* Oocytes incubated in [³H]vitellogenin for 25.5 h.

† Oocytes incubated in [³H]vitellogenin for 16.5 h.

DOCA-treated oocytes, on the other hand, have lost not only their contoured surface but also their microvilli (Fig. 3). The surface becomes flattened, with only a few bulbous projections remaining. All evidence of micropinocytotic activity (both pits and pinosomes) disappears. There does not appear to be any alteration in the configuration of the vitelline envelope after DOCA treatment.

DISCUSSION

Data presented here demonstrate that incorporation of the major macromolecular yolk precursor (vitellogenin) into the amphibian oocyte is sensitive to inhibition by DOCA. Significantly, it has been shown for the first time that inhibition of vitellogenin incorporation is delayed after steroid treatment and is closely linked in time to steroid-induced breakdown of the germinal vesicle and a structural rearrangement of the oocyte-vitelline envelope complex.

Clearly then, a major question concerns the mechanism of steroid inhibition of protein incorporation. Continuous exposure of the oocytes is probably not required for inhibition of incorporation, since nuclear maturation occurs subsequent to a short exposure of oocytes to the steroid (Schuetz, 1967). Although nuclear maturation is induced by steroids, the nucleus is probably not involved in the mediation, since morphological changes in the cortex, including separation of the vitelline envelope from the oocyte, and cortical granule alterations occur in hormone-treated enucleated oocytes (Smith and Ecker, 1969). Cytoplasmic factors have been implicated in mediating maturation processes within the oocyte and may also be involved here (Masui and

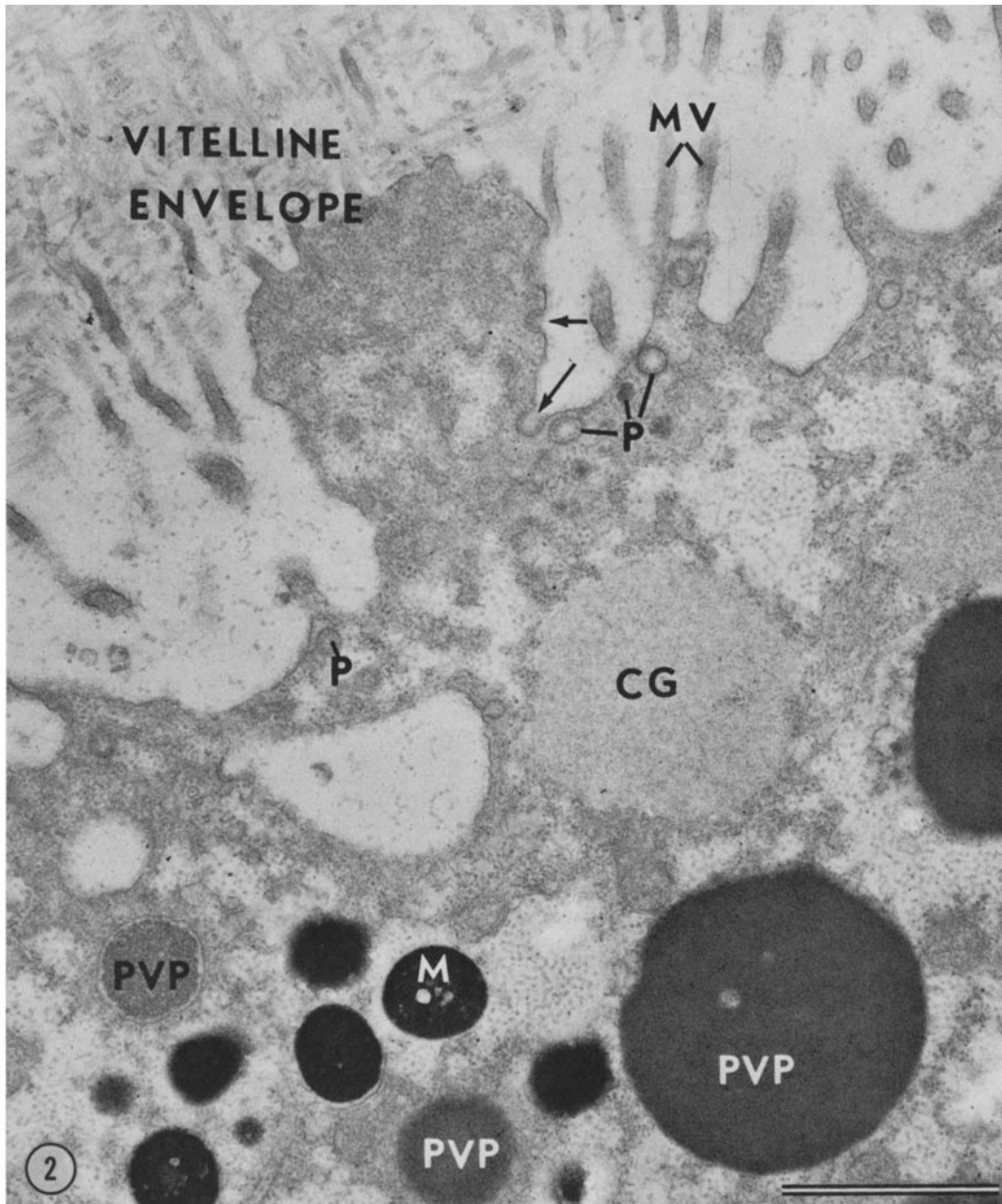


FIGURE 2 A portion of a control oocyte cultured 25.5 h. The irregular surface is characterized by microvilli (*MV*) and micropinocytotic pits (arrows). In the ooplasm are pinosomes (*P*), primordial yolk platelets (*PYP*), melanosomes (*M*), and cortical granules (*CG*). $\times 29,400$.

Markert, 1971; Smith and Ecker, 1971). Substantial morphological and biochemical evidence indicates that vitellogenin incorporation in the amphibian oocyte is selective, and is accomplished by a micropinocytotic process which occurs in

the oocyte cell membrane and cortex (Wallace and Dumont, 1968; Wallace et al., 1970; Dumont, 1972; Dumont and Wallace, 1972).

Vitellogenin is presumably sequestered in micropinocytotic pits (Fig. 2), and transferred

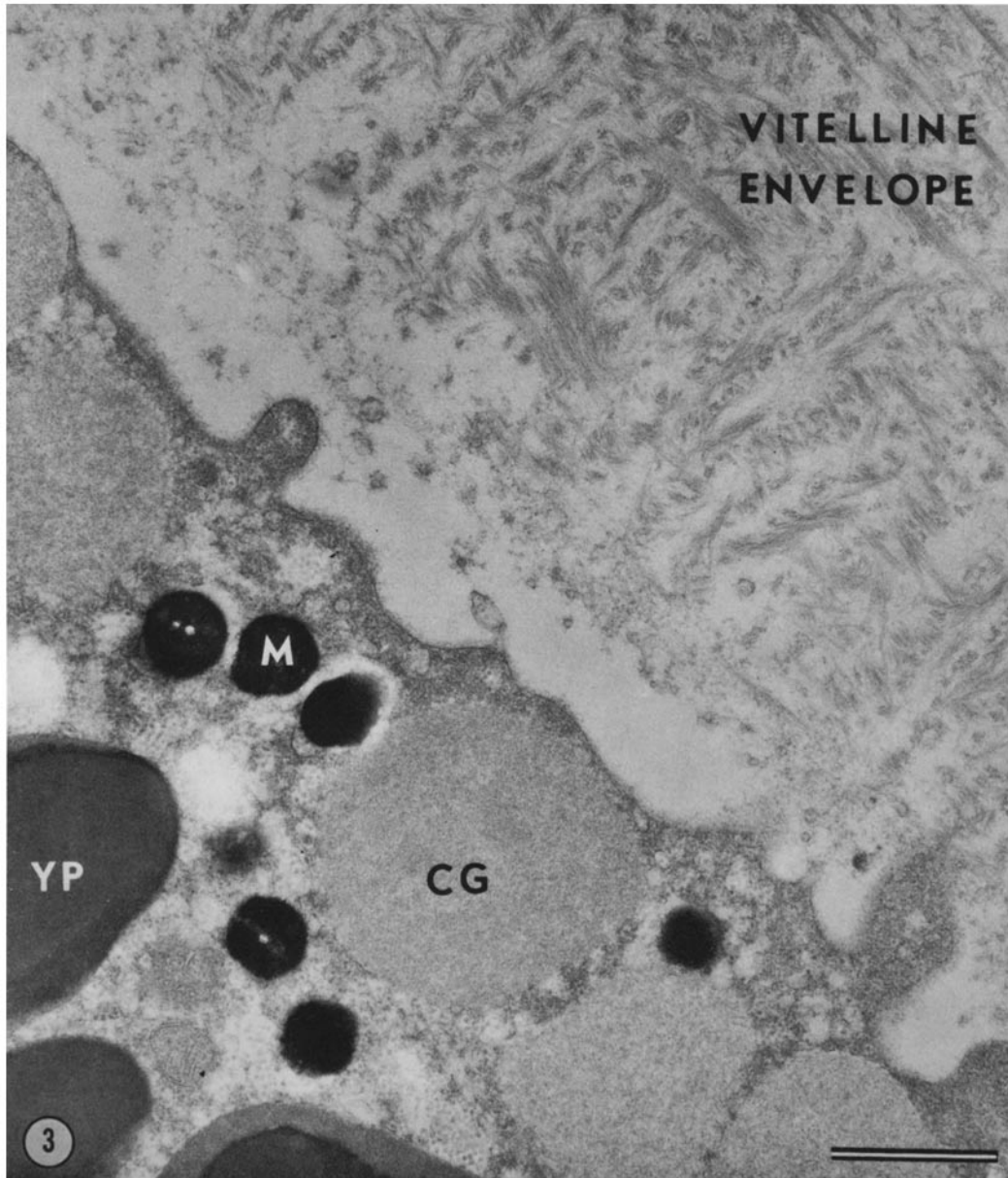


FIGURE 3 A portion of an oocyte cultured 25.5 h in the presence of DOCA. Note the flattened surface and the absence of microvilli, micropinocytotic pits, and pinosomes. Melanosomes, *M*; yolk platelet, *YP*; cortical granules, *CG*. $\times 23,000$.

to the pinosomes within the oocyte. Inhibition of vitellogenin incorporation by the steroid can be envisioned to occur, therefore, either by preventing vitellogenin access to the oocyte surface or by altering the functional capacity of the cell surface. Altering the function of follicle cells or vitelline envelope permeability would most

simply interfere with vitellogenin access to the oocyte surface. Evidence for a role of the follicle cells and vitelline envelope in vitellogenin incorporation, however, is exceptionally weak (Wallace et al., 1972, 1973; Jared and Wallace, 1969). The effects of EDTA (Table II) and the rapid passage of substances such as trypan blue,

and tracers such as ferritin, peroxidase, and iron dextran through channels between the follicle cells and through the vitelline envelope, strongly suggest that there is no need for the vitellogenin to traverse the follicle cell cytoplasm or that the vitelline envelope acts as a barrier (Wallace and Dumont, 1968). These results, therefore, indicate that the steroid interrupts the protein incorporation process (micropinocytosis) rather than hinders vitellogenin access to the oocyte. The relative importance of the structural alterations in the oocyte cortex (microvilli, micropinocytotic elements, microtubules, plasmalemma) to vitellogenin incorporation and its inhibition by steroids, however, is presently unclear (Figs. 2, 3; Dumont and Wallace, 1972).

An integrated series of pituitary and apparently steroid-induced events occur within the ovarian follicle and oocyte in mediating extrusion of the oocyte from the ovary and in preparation for its subsequent fertilization and embryonic development (Schuetz, 1967, 1972, *a, b*; Masui, 1967; Smith et al., 1968). Steroid inhibition of vitellogenin incorporation thus represents a marked alteration in the function of this cell and has major implications concerning the normal physiology of the ovarian follicle and of oocyte differentiation. The lag phase to steroid inhibition of vitellogenin incorporation is clustered or synchronized to other events in the oocyte and follicle wall close to the time of oocyte extrusion from the follicle (Schuetz, 1972). Clearly, if exchange between the ovarian environment and the oocyte is of importance for oocyte function, it would be advantageous for interruption of this process to occur close to the time of actual expulsion of the oocyte. Alterations in the normal integrated processes could be expected to have deleterious effects on follicular functions. It has previously been demonstrated that asynchrony of ovulation and oocyte maturation occurs to a large extent in ovarian follicles (Schuetz, 1967, 1972 *a, b*), and this has been suggested to be a causative factor in the process of oocyte and follicular atresia (Schuetz, 1972 *a, b*). Similar alterations are also observed in numerous mammalian species (Ingram, 1962). Thus oocytes which have undergone nuclear maturation, and in which the mechanism of ovarian-oocyte exchange has been markedly inhibited in the absence of ovulation, may possibly undergo subsequent degenerative changes associated with oocyte atresia.

The present data indicate that a steroid can interfere with what appears to be a vital cellular function and at the same time initiate other physiological events. It remains to be elucidated whether there is a strict relationship between the capacity of a steroid to inhibit vitellogenin incorporation and its capacity to induce the nuclear and cortical alterations. In particular, the effects of the estrogenic steroids that are biologically inactive with respect to inducing the cortical and nuclear changes, but which are responsible for the synthesis of vitellogenin, require investigation (Schuetz, 1972 *c*). Preliminary experiments of Wallace and Ho (1972) indicated no inhibition of protein incorporation into *Xenopus* oocytes by β -estradiol. Likewise, the investigation of the effects of other steroids on the incorporation of vitellogenin at the various stages of oogenesis, or when nuclear maturation is not responsive to steroids, may provide clues to the process of oocyte differentiation.

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