Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 5552–5556, September 1982
Cell Biology

Microinjected progesterone reinitiates meiotic maturation of *Xenopus laevis* oocytes

(meiosis/cAMP)

J. TSO*, C. THIBIER, O. MULNER, AND R. OZON†

Laboratoire de Physiologie de la Reproduction, Groupe Stéroïdes. E.R.A. Centre Nationale de la Recherche Scientifique 694, Université Pierre et Marie Curie, 4, Place Jussieu, 75230 Paris Cedex 05, France

Communicated by J. Brachet, May 27, 1982

ABSTRACT **Microinjection of progesterone dissolved in paraffin oil induces the reinitiation of meiotic maturation in the *Xenopus* oocyte; 50% maturation is obtained when 50 nl of a 50 μM solution is microinjected into the oocyte. The kinetics of the response to microinjected progesterone are similar to the kinetics of response to externally applied hormone. When an aqueous solution of progesterone is microinjected instead of an oil solution, maturation is never observed, a result which confirms previous work. Leakage of the steroid into the external medium was estimated to range from 1.6 pmol/hr when microinjection was performed in oil to 3.6 pmol/hr when it was performed in aqueous solution. Metabolism of the hormone microinjected in oil is weak (<20%) as compared to that after aqueous microinjection (>80%). Progesterone microinjected in oil decreases the cAMP content as does externally applied hormone. We therefore conclude that progesterone acts initially on an intracellular site in order to trigger meiotic maturation of the *Xenopus* oocyte.**

The classical theory is that steroid hormones act on their target cells via a common molecular mechanism in which the hormone first diffuses through the plasma membrane and then associates with a soluble receptor protein; the nuclear translocation of the hormone–receptor complex triggers an alteration of the transcription of specific genes (1). The full-grown amphibian oocyte offers an alternative experimental system in which this molecular scheme apparently does not work. In fact, progesterone, as well as many C_{21} and C_{19} steroids, can induce the first meiotic cell division in the prophase-blocked oocyte, at a post-transcriptional level (2–4).

In the oocyte system, the initial site of steroid action is unknown, although it has been suggested that the steroid primarily interacts with the oocyte surface. At least three lines of evidences support this view.

1. Although steroids do initiate maturation when present in the culture medium, at all concentrations tested they always fail to induce maturation when they are microinjected into the oocyte as an aqueous solution (2). Two reports, however, indicated that some steroids that are slightly more hydrophilic than progesterone (i.e., cortisol or testosterone) may induce maturation if microinjected (5, 6); because the possibility that injected steroids may have leaked out of the oocyte was not tested, it was later concluded that hormone that had leaked from the oocyte was responsible for the reported effect of the injected steroid (7).

2. When continuously incubated at 0.1 mM with *Xenopus laevis* oocytes, steroid covalently bound to a polymer was reported to be capable of inducing maturation under conditions that minimize uptake and cleavage (but not totally abolish them); it was therefore concluded that the steroid hormones interact with the outer plasma membrane to reinitiate meiosis (8, 9).

3. With the exception of a recent report (10), all attempts to isolate a soluble progesterone receptor based on methods used in somatic target tissues have been negative (11). Oocyte melanosomes have been shown to display a selective affinity for active steroids; until now, a physiological role for such a binding could not be established (12).

Recently the initial surface action of steroid has been questioned by experiments which show that the efficiency of progesterone stimulation is not dependent on its molar concentration in the incubation medium but rather on the actual quantity of hormone that reaches the oocyte (13, 14).

None of these experiments gives an unequivocal answer to the puzzling question, Does progesterone act initially on the outer surface of the oocyte plasma membrane?

In the present report we show that microinjection of steroid dissolved in paraffin oil does initiate meiotic maturation in the *X. laevis* oocyte; this experiment does not support the view that an interaction of the hormone with the outer surface membrane is a necessary step involved in this biological effect.

MATERIALS AND METHODS

Animals. *X. laevis* adult females (de Rover, The Netherlands) were bred and maintained under laboratory conditions.

Material. [1,2,6,7-^{3}H]Progesterone (82 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels) and [8-^{3}H]cAMP (30 Ci/mmol) were obtained from Amersham (France).

Progesterone, testosterone, cAMP, cholera toxin, and collagenase type I were purchased from Sigma. The synthetic steroid R 5020 was from Roussel-Uclaf (France) and dispase grade II was from Boehringer. Whitol paraffin oil was obtained from Igol (Paris).

Oocyte Preparation. Animals were anesthetized with MS 222 (Sandoz) at 1 g/liter. Ovaries were removed and transferred to medium A [88 mM NaCl/0.33 mM $Ca(NO_3)_2$/1 mM KCl/0.41 mM $CaCl_2$/0.82 mM $MgSO_4$/2 mM Tris, pH 7.4]. Penicillin (50,000 units/liter) and streptomycin (1 mg/liter) were added to the medium. After dispase digestion (0.4 mg/ml) for 4 hr at laboratory temperature and collagenase digestion (0.80 mg/ml) for 30–60 min at 20°C with continuous stirring, stage VI oocytes, 1.3 mm in diameter (15), were collected.

Oocyte Microinjection and Maturation. Steroid solutions for microinjection were prepared by drying the appropriate stock solution under nitrogen flow and dissolving the residue with paraffin oil or 5 mM 2(*N*-morpholino)ethanesulfonate buffer,

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviation: GVBD, germinal vesicle breakdown.

* On leave from Shanghai Institute of Cell Biology, Academia Sinaca.
† To whom reprint requests should be addressed.

pH 7.0, containing 1 mg of bovine serum albumin per ml, to the desired steroid concentration.

Approximately 50 nl of solution was injected into oocytes at the equatorial level. All injected oocytes were rinsed with 5 ml of medium A three times before incubation at room temperature. Usually 5–10 oocytes were incubated in 5 ml of medium A. Maturations with external progesterone were performed in 5 ml of medium A in the continuous presence of 1 μM progesterone. Maturation was evidenced by the appearance of a white spot surrounded by pigment at the animal pole of the oocyte. Germinal vesicle breakdown (GVBD) was ascertained by the absence of germinal vesicle determined by dissection of the oocyte after 5-min fixation in 10% trichloroacetic acid.

Progesterone Metabolism. Oocytes were microinjected with 50 nl of $[^3H]$progesterone adjusted to 1 mM with unlabeled progesterone. The oocytes were then incubated in medium A until maturation. At that time, the oocytes were removed, rinsed three times in medium A, and immediately homogenized in 1 ml of medium A in the presence of 50 μg of various unlabeled steroids. The homogenate was extracted three times with 10 ml ether/chloroform, 3:1 (vol/vol), each time. The ether/chloroform phase was evaporated to dryness, and the residue was resuspended in 0.3 ml of methanol and then chromatographed on thin-layer silica gel plates (Merck fluorescent silica gel GF 254) in chloroform/ethanol, 9:1 (vol/vol).

The radioactive metabolites were detected by using a Panax XY radiochromatogram scanner. The resulting metabolites were eluted with methanol and assayed in 15 ml of toluene-based system in a Packard Tri-Carb liquid scintillation spectrometer model 3320 with external standard. Aqueous solutions (0.1–1 ml) were assayed in 10 ml of a mixture containing 5.5 g of 2,5-diphenyloxazole (PPO) and 100 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) in 333 ml of Triton X-100 and 667 ml of toluene.

Radioactive metabolites were identified as described (16).

cAMP Determination. Usually four oocytes were manually homogenized quickly in 500 μl of boiling sodium acetate buffer (50 mM, pH 4) containing 1 mM theophylline. The tubes were vortexed and boiled for 4 min and then centrifuged at 4°C (24,000 × g, 15 min). The supernatant was removed for cAMP assay. The cAMP content of the extracts was determined by a binding assay method adapted from Gilman (17) by Thibier *et al.* (18). All buffers contained 1 mM theophylline. A standard curve for cAMP was obtained for each assay (0.2–5 pmol).

RESULTS

Induction of Meiotic Maturation by Microinjected Progesterone. *Xenopus* oocytes were stimulated by progesterone in three different types of experiments (Fig. 1). Addition of the steroid (1 μM) to the extracellular medium always reinitiated meiosis. When 1 mM progesterone was dissolved in an aqueous buffer solution containing albumin and microinjected into oocytes, reinitiation of meiosis was never observed, confirming that steroid microinjected in aqueous solution does not induce maturation (19, 20). When 50 nl of 1 mM progesterone in oil was microinjected into oocytes, meiotic maturation occurred in 10 experiments; the kinetics were similar to those of oocytes induced to mature by exposure to external progesterone. The mean (± SD) ratio of $GVBD_{50}$ (the time necessary for 50% GVBD) of the microinjected oocytes to $GVBD_{50}$ of the extracellularly exposed oocytes was 0.94 ± 0.06 (n = 10). In control experiments, microinjection of 50 nl of paraffin oil never induced maturation and did not inhibit maturation initiated by external progesterone. The efficiency of 1 mM progesterone in oil was independent of the site of microinjection; similar results were obtained by microinjection in either the animal or the

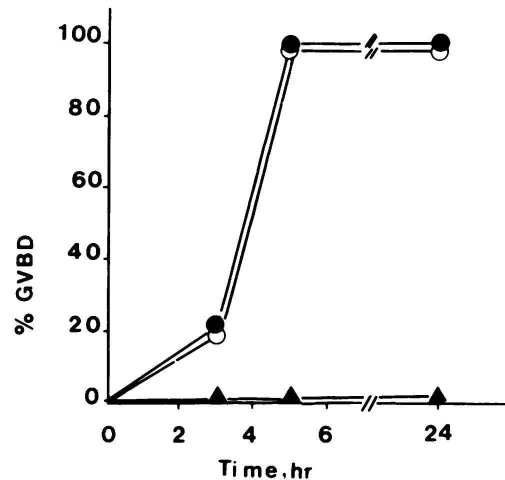

FIG. 1. Kinetics of oocyte maturation induced by microinjected or extracellular progesterone. Batches of five oocytes from the same female were incubated in 5 ml of medium A at room temperature either after microinjection of 50 nl of 1 mM progesterone in oil (○——○) or in aqueous albumin solution (▲——▲) or in the continuous presence of 1 μM external progesterone (●——●). Results from one typical experiment are presented; this experiment was repeated four times.

vegetal hemisphere. However, when progesterone in oil was microinjected in the vegetal hemisphere, the maturation was delayed compared to control oocytes (extracellular progesterone) or to oocytes microinjected into the animal hemisphere. In all further experiments the progesterone in oil was microinjected in the animal pigmented hemisphere just above the equator. Whatever the site of microinjection, the oil droplet (400 μm in diameter) was not miscible with the oocyte constituents and it remained intact during the whole maturation period. It is noteworthy that, before GVBD, the oil droplet migrated to the upper animal pole, suggesting a fluidization of the oocyte cytoplasm prior to GVBD. Cytological analysis revealed an apparently normal breakdown of the germinal vesicle and the condensation of the chromosomes, but the subsequent organization of metaphase I was abnormal because the metaphase chromosomes were found deep in the cytoplasm, associated with normal spindles or aster formations.

In some experiments, 10 microinjected oocytes were incubated in 5 ml of medium A together with 10 untreated full-grown oocytes; in no instance were these control oocytes induced to mature, indicating that, if steroids do leak out of the microinjected oocytes, the extracellular concentration never reaches a level sufficient to induce maturation.

The dose–response curves (Fig. 2) show that the concentration of progesterone dissolved in the oil droplet that induced GVBD in 50% of the microinjected oocytes was 50 μM (three experiments); this corresponds to a total amount of steroid inside the oocyte of 2.5 pmol. If progesterone diffused from the oil droplet to the whole oocyte (assuming its volume to be 1 μl) the final concentration would correspond to 2.5 μM. Other steroids microinjected in oil solution were also capable of reinitiating meiosis, although with a lower efficiency. Of particular interest is the observation that the efficiency of a given steroid correlates with its partition coefficient in oil/water; progesterone in oil solution is the most active steroid in inducing meiotic maturation and possesses the highest partition coefficient (unpublished data).

To determine if the maturation-promoting factor was present in oocytes microinjected with progesterone in oil, their cytoplasm was transferred into recipient oocytes at different times after microinjection; in all experiments, the maturation-pro-

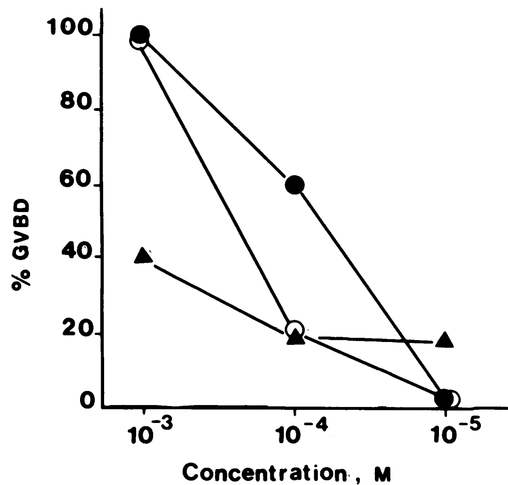

FIG. 2. Dose-dependent induction of maturation in the presence of different steroids injected into oocytes. Each experimental point corresponds to 10 oocytes incubated in medium A after microinjection of progesterone in oil (●——●), testosterone in oil (○——○), or R_{5020} in oil (▲——▲) (50 nl of each concentration). GVBD was scored after incubation for 24 hr.

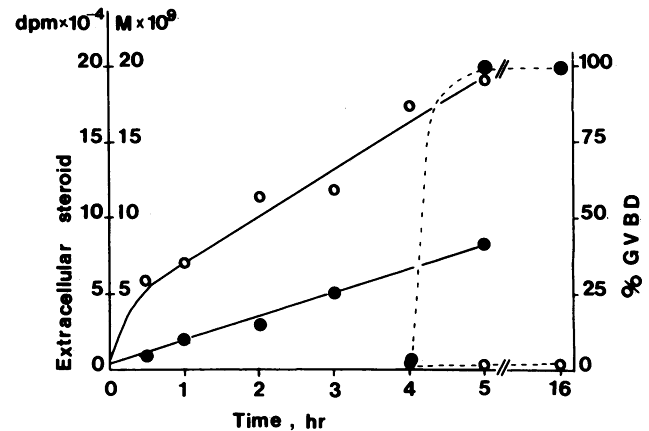

FIG. 3. Steroid released from microinjected oocytes. Radioactive progesterone (50 pmol corresponding to 98,000 dpm of ^3H) was dissolved in 50 nl of either paraffin oil or albumin buffer and microinjected into each oocyte. The oocytes were rinsed three times in medium A and then incubated (five oocytes per flask) in 5 ml of medium A. At the indicated time, an aliquot (100 μl) of the incubation medium was removed and radioactivity was measured; the extracellular concentration of the steroid was calculated. % GVBD of the oocytes were scored (broken lines) in parallel with it. ●——●, Progesterone in oil; ○——○, progesterone in albumin solution.

moting factor did appear with kinetics similar to those of oocytes incubated in 1 μM progesterone in the extracellular medium (data not shown).

Estimation of Steroid Leakage and Metabolism. Progesterone in oil diffused out of the oocyte much less than progesterone in albumin solution did (Fig. 3). After a 5-hr incubation, 8 pmol (16%) was released from each oocyte in the former case and 18 pmol (36%) was released in the latter. In both cases, five microinjected oocytes were placed in a Petri dish containing 5 ml of incubation medium. It can be calculated that, after 5 hr of incubation of oocytes injected with progesterone in oil, the final extracellular steroid concentration never reached 10 μM, an external progesterone concentration which, by itself, does not induce maturation. In other experiments, charcoal (1%) was added to the incubation medium immediately after microinjection of progesterone in oil. Although charcoal complexes nearly all extracellular steroids, maturation was not inhibited in these oocytes.

To determine the amount of progesterone that diffuses out of the oil droplet inside the oocyte after microinjection, the distribution of radioactivity between the oil droplet and the oocyte cytoplasm was estimated after microinjection of [^3H]-progesterone. After trichloroacetic acid treatment and dissection of the fixed oocyte, the oil droplet (which remained intact and never dissociated into liposomes) could be easily separated from the oocyte with a micropipette. In one typical experiment (Table 1), 50 nl of progesterone in oil (i.e., ≈50 pmol per oocyte) was microinjected and the oil droplet was recovered after fixation of the matured oocytes (5 hr after microinjection in this particular experiment). At that time the steroid concentration in the oil was only 45 μM or 2.5 pmol, indicating that 47.5 pmol (i.e., 95% of the steroid) was released from the oil during the 5-hr incubation; 85% of the released steroid remained bound to the oocyte. This experiment was repeated twice and similar results were obtained.

It is well established that steroids are metabolized in defolliculated amphibian oocytes whether they are present in the incubation medium or microinjected (21, 22). The metabolism of microinjected progesterone dissolved either in oil or in albumin solution was analyzed. The steroids were extracted from whole oocytes and chromatographed. At 5 hr after microinjection, the radioactivity associated with oil-microinjected oocytes remained mainly (>80%) in the form of progesterone (Fig. 4); conversely the radioactivity extracted from albumin-microinjected oocytes was associated with progesterone metabolites comparable to those observed in oocytes exposed to extracellular progesterone (16). This result demonstrates a striking dependence of progesterone half-life inside the oocyte on the solvent in which the hormone was microinjected. The possibility that progesterone may form a complex with paraffin oil, and

Table 1. Radioactivity and concentration of steroid in oocyte and culture medium after maturation

	Fractions							
	Whole oocyte		Oil droplet		Oocyte		Medium†	
Microinjected solution*	dpm	Conc., μM	dpm	Conc., μM	dpm	Conc., μM	dpm	Conc., μM
Progesterone in oil	68,060	3.5	4,460	45	63,600	32	83,500	8.5
Progesterone in albumin solution	56,300	29	—	—	—	—	199,300	20

The measurement of radioactivity (13) was done at the time of GVBD, 5 hr after injection of progesterone.

* Approximately 50 nl of 1 mM progesterone was injected—i.e., 50 pmol (98,000 dpm) per oocyte.

† Five oocytes in 5 ml of medium.

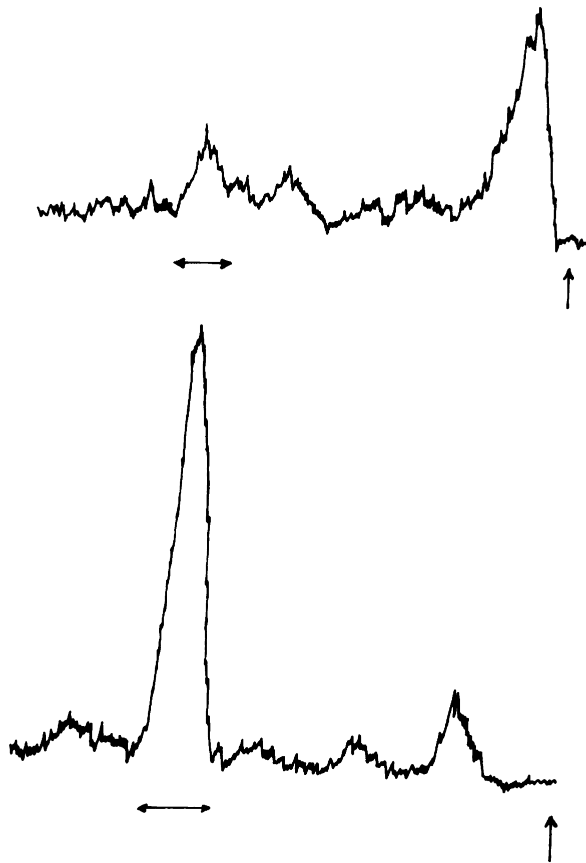

FIG. 4. Thin-layer chromatography profiles of radioactivity isolated from oocytes, showing metabolism of progesterone microinjected into the oocyte. Injection and incubation conditions were as in Fig. 3. Oocytes were removed from incubation medium at maturation (5 hr) and steroids were extracted. Upper tracing, progesterone in albumin; lower tracing, progesterone in oil; ↔, progesterone; ↑, starting line.

thus have an altered reactivity, seems unlikely because extracellular progesterone dissolved in oil is metabolized by the oocyte in the same manner as extracellular progesterone in aqueous medium.

Microinjected Progesterone Provokes a Decrease in Intracellular cAMP Concentration. It is now well established that extracellular progesterone provokes a decrease in the *Xenopus* oocyte cAMP concentration, probably via an inhibition of adenylate cyclase (23–26). In the following experiment, we compared the effect of extracellular and intracellular progesterone on the oocyte cAMP level. Microinjection of progesterone in oil into oocytes pretreated with 3-isobutyl-1-methylxanthine and cholera toxin induced, in 1 hr, a decrease in the cAMP content (Fig. 5) similar to that induced by external progesterone (18). At that time, the extracellular concentration of steroid that had leaked out of the oocyte would not be more than 2 nM (Fig. 3), a concentration far below that necessary to inhibit adenylate cyclase activity in the whole oocyte (23) or in its membrane fraction (24, 25). It was observed that the decrease in cAMP concentration was delayed (from 30 to 60 min) in oocytes injected with progesterone in oil compared to oocytes treated with progesterone extracellularly.

DISCUSSION

Our results show that microinjection of 50 nl of a solution of progesterone (0.1–1 mM) dissolved in paraffin oil induces meiotic maturation in defolliculated *Xenopus* oocytes, with kinetics comparable to those observed after addition of extracellular hormone (1 μM). Before one can draw any conclusion from these experiments, which are easily reproducible, possible leakage of the injected steroid must be carefully controlled because it is possible that release of progesterone into the incubation medium is responsible for the observed effect. At least four lines of argument strongly suggest that leakage of injected hormone out of the oocyte cannot account for our results.

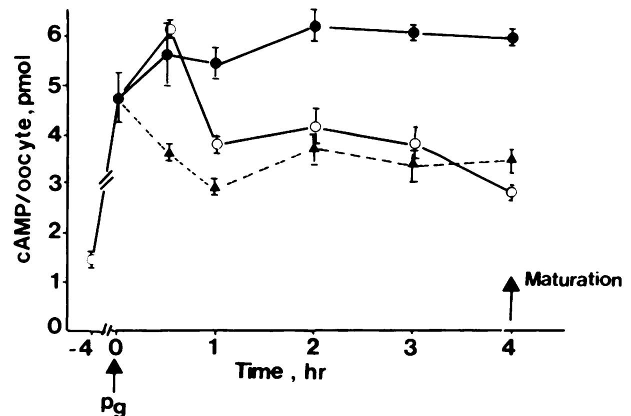

FIG. 5. cAMP levels after progesterone treatment of stimulated *Xenopus* oocytes. Oocytes were preincubated for 4 hr in medium A in the presence of 6 nM cholera toxin plus 1 mM 3-isobutyl-1-methylxanthine. Forty oocytes were then transferred to the same medium containing 1 μM progesterone (▲---▲) or microinjected with 50 nl of 1 mM progesterone in oil (○——○) and incubated in the same medium. At different times thereafter, four oocytes were taken out and assayed for cAMP content. Control oocytes untreated with hormone but incubated in the same medium were analyzed at the same times (●——●). Data are the mean of two experimental points (assayed in duplicate); error bars show SD.

1. The kinetics of maturation in oocytes microinjected with progesterone in oil and in oocytes incubated in the presence of progesterone are similar if not identical. In fact, one would have expected a significant delay in GVBD if the hormone had to leak out of the oocyte in order to initiate its biological effect.
2. Even after 5 hr of incubation—i.e., at the time of GVBD—the steroid in the extracellular medium never reaches a concentration sufficient to induce maturation. Furthermore, charcoal, which binds extracellular steroids, does not inhibit maturation.
3. Companion oocytes placed in the same dish as microinjected oocytes never underwent maturation.
4. It is known that progesterone is actively metabolized as soon as it enters the oocyte. Nearly 80% of progesterone microinjected in oil remains unmetabolized (Fig. 4) even 5 hr after microinjection. This suggests that the progesterone present in the cell does not come from the extracellular medium; otherwise it would have been transformed into metabolites. In contrast, our results demonstrate that the hormone that is released from the oil droplet is protected against metabolism compared to progesterone injected in aqueous solution or added to the medium. Whether the progesterone leaving the oil droplet is associated with an intracellular compartment that does not contain the enzymes of steroid metabolism or is bound to a specific molecule (an intracellular receptor?) remains to be determined.

Taken together these experimental facts indicate that, when the microinjection vehicle is oil, an intracellular hormone concentration sufficient to induce meiotic maturation is maintained.

Other experimental evidence supporting the view that the hormone is capable of acting intracellularly is the observation that microinjection of progesterone in oil induces a decrease in cAMP level. This decrease is observable 1 hr after microinjec-

tion, at a time when the external concentration of hormone that has leaked from the oocyte is no more than 1 nM, a concentration below that necessary to act on cyclase activity when the hormone is applied externally (23).

However, our experiments do not exclude the generally accepted conclusion that progesterone acts near the cell surface or the possibility that the hormone works initially on cell membranes (inner plasma membrane or intracellular membranes). They only indicate that an interaction of the steroid hormone with a receptor present on the outer cell surface, as described for water-soluble hormones, is now questionable as a mechanism for the induction of *Xenopus* oocyte maturation. This conclusion apparently disagrees with the interpretation given to experiments performed with steroid-bound polymers (8, 9).

Preliminary experiments have shown that the biological efficiency of microinjected steroids in oil solution parallels the solubility of the steroid in oil (partition coefficient); it is tempting to speculate that the steroid must reach a lipophilic site located near the oil droplet (lipid bilayer of plasma or intracellular membranes) in order to produce its biological activity.

In conclusion, we favor the view that, in *Xenopus* oocytes as in all other target cells, progesterone acts through an initial site accessible from the intracellular compartment, in contrast to the general opinion (reviewed in ref. 2). This implies that a steroid hormone, which is known to work post-transcriptionally, is capable, *via* intracellular mechanisms, of regulating the cellular cAMP level.

This work was supported by Institut National de la Santé et de la Recherche Médicale (Grant 814016) and by Délégation Générale à la Recherche Scientifique et Technique (Grant 81.E.1077).

1. Gorski, J. & Gannon, F. (1976) *Annu. Rev. Physiol.* **38**, 425–450.
2. Smith, L. D. (1975) in *Biochemistry of Animal Development*, ed. Weber, R. (Academic, New York), Vol. 3, pp. 1–46.
3. Masui, Y. & Clarke, M. (1979) *Int. Rev. Cytol.* **57**, 185–223.
4. Maller, J. L. & Krebs, E. G. (1980) *Curr. Top. Cell. Regul.* **16**, 271–311.
5. Schorderet-Slatkine, S. (1972) *Cell Diff.* **1**, 179–189.
6. Jacobelli, S., Hanocq, J., Baltus, E. & Brachet, J. (1974) *Differentiation* **2**, 129–135.
7. Drury, K. C. & Schorderet-Slatkine, S. (1975) *C. R. Hebd. Seances Acad. Sci. Ser. D* **280**, 1273–1275.
8. Ishikawa, K., Hanaoka, Y., Kondo, Y. & Imai, K. (1977) *Mol. Cell. Endocrinol.* **9**, 91–100.
9. Godeau, J. F., Schorderet-Slatkine, S., Hubert, P. & Baulieu, E. E. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2353–2357.
10. Kalimi, M., Ziegler, D. & Morrill, G. A. (1979) *Biochem. Biophys. Res. Commun.* **86**, 560–567.
11. Ozon, R. (1978) *Mém. Soc. Zool. France* **41**, 83–92.
12. Bellé, R., Marot, J. & Ozon, R. (1977) *Life Sci.* **20**, 849–856.
13. Bellé, R., Marot, J. & Ozon, R. (1976) *C. R. Hebd. Seances Acad. Sci. Ser. D* **283**, 175–178.
14. Cloud, J. G. & Schuetz, A. W. (1977) *Dev. Biol.* **60**, 359–370.
15. Dumont, J. N. (1972) *J. Morphol.* **136**, 153–179.
16. Thibier-Fouchet, C., Mulner, O. & Ozon, R. (1976) *Biol. Reprod.* **14**, 317–326.
17. Gilman, A. G. (1970) *Proc. Natl. Acad. Sci. USA* **67**, 305–312.
18. Thibier, C., Mulner, O. & Ozon, R. (1982) *J. Steroid Biochem.*, in press.
19. Smith, L. D. & Ecker, R. E. (1971) *Dev. Biol.* **25**, 233–247.
20. Masui, Y. & Markert, C. (1971) *J. Exp. Zool.* **177**, 129–146.
21. Reynhout, J. K. & Smith, L. D. (1973) *Dev. Biol.* **30**, 392–402.
22. Fouchet, C., Serres, C., Bellé, R. & Ozon, R. (1975) *Comp. Biochem. Physiol.* **52**, 205–210.
23. Mulner, O., Huchon, D., Thibier, C. & Ozon, R. (1979) *Biochim. Biophys. Acta* **582**, 179–184.
24. Sadler, S. E. & Maller, J. L. (1981) *J. Biol. Chem.* **256**, 6368–6373.
25. Finidori-Lepicard, J., Schorderet-Slatkine, S., Hanoune, J. & Baulieu, E. E. (1981) *Nature (London)* **292**, 255–257.
26. Baltus, E., Hanocq-Quertier, J. & Guyaux, M. (1981) *FEBS Lett.* **123**, 37–40.