Proc. Natl. Acad. Sci. USA
Vol. 75, No. 5, pp. 2353–2357, May 1978
Cell Biology

Induction of maturation in *Xenopus laevis* oocytes by a steroid linked to a polymer

(mechanism of progesterone action/meiosis/plasma membrane/immobilized hormones/polyethylene oxide)

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Communicated by Clement L. Markert, March 9, 1978

ABSTRACT **A progesterone analog has been covalently linked via an amide bond to polyethylene oxide (molecular weight, 20,000). This macromolecular steroid molecule displays the biological activity of progesterone in inducing meiotic maturation when incubated with *Xenopus laevis* oocytes (stage VI) *in vitro*. Its efficiency (half-maximum effective concentration, 30 μM) is approximately 10 times lower than that of its low molecular weight homolog (3 μM). Control experiments with polyethylene oxide and an estradiol derivative (up to 1 mM) assessed the specificity of the progesterone macromolecular analog. Uptake experiments using radioactive derivatives revealed a small (if not negligible) intake of the macromolecular progesterone analog by the oocytes compared to that of free steroids, and no parallelism was found between radioactivity incorporation and effect. The possibility of cleavage of the macromolecular derivative during the incubation was ruled out. Furthermore, injection of the polymer-linked progesterone into the oocytes did not induce maturation. These observations suggest that the macromolecular progesterone analog itself is responsible for the biological effect and that the presence of this compound inside the cell is neither necessary nor sufficient for triggering reinitiation of meiosis. These conclusions are in agreement with the proposal that interaction with the plasma membrane of the oocyte is necessary for progesterone action in this particular system, in contrast to the case of somatic cells which have intracellular steroid receptors.**

Amphibian oocytes are arrested at the prophase of the first meiotic division. Meiosis is physiologically reinitiated by progesterone released by follicle cells under pituitary stimulation (1). During this period of "maturation" (2) the germinal vesicle undergoes dissolution and meiosis continues up to the second meiotic metaphase.

When incubated *in vitro* with progesterone, *Xenopus laevis* oocytes devoid of surrounding follicle cells undergo maturation (3, 4). A few hours before germinal vesicle breakdown (GVBD), an intracellular inducer of maturation [maturation promoting factor (MPF)] appears in the cytoplasm of progesterone-treated oocytes (5). Its appearance is dependent on protein synthesis but not on transcriptional activity (6, 7). So far, no soluble progesterone receptor has been described in this system although the particulate fraction, melanosomes, has been reported to bind progesterone specifically (8, 9). These observations on germinal cells are not compatible with the current models of mechanism of steroid action in somatic cells which require transcriptional events mediated by an intracellular hormone–receptor complex (10–12).

Because progesterone is inactive when injected into the oocyte (6, 13), it is conceivable that hormone action implies an interaction of the steroid with the oocyte plasma membrane. As suggested by experiments with ionophore A23187 (14) and specific ionophoresis (15), it could then lead to subsequent modifications of Ca^{2+} distribution. Recently La^{3+} (10 mM), which displaces Ca^{2+} from membrane sites (16), was also shown to induce maturation (17). A series of amphiphilic drugs that interact with membranes also had an effect on meiosis that was strongly correlated to their capacity to displace Ca^{2+} (18). Some organomercurials induce maturation, possibly through an interaction with SH groups of the membrane, because their capacity is inversely related to their ability to penetrate the cell (19). Finally, the inhibition of progesterone-induced maturation by theophylline (20) and γ-hexachlorocyclohexane (21) has been attributed to an effect at the membrane level.

The present work investigated whether a progesterone analog is able to promote oocyte maturation when linked to a macromolecule that does not enter the cell. This new approach was used to test the hypothesis that the decisive site of progesterone–oocyte interaction is located at the membrane level.

MATERIALS AND METHODS

Steroids were obtained from Roussel-Uclaf Romainville, France, and polyethylene oxide [poly(Eo); molecular weight, 20,000] was from Hoechst. [^{3}H]Deoxycorticosterone ([^{3}H]DOC) (44 Ci mmol^{-1}), [^{3}H]progesterone (90 Ci mmol^{-1}), and [^{14}C]poly(EO) 6000 (95 mCi mmol^{-1}) were obtained from the Radiochemical Centre, Amersham, England. Collagenase type I was purchased from Sigma and dialysis tubing (3887 H 45) was from Thomas Co. All reagents were analytical grade.

X. laevis females were obtained from South African Snake Farm (Fish Hoek, Cape Province, South Africa).

Synthesis of Polymer-Linked Steroid Derivatives. DOC was oxidized with periodic acid (22, 23) to yield 3-oxo-4-androstene-17β-carboxylic acid (ACA). Amidification with isopropylamine led to 3-oxo-4-androstene-17β-amidoisopropane (AAI). ACA was coupled to α,ω-bis-aminopropylamino-poly(EO) 20,000 by a mixed anhydride method involving amide bond formation (24). The resulting polymer-linked steroid was named ACA-poly(EO) (Fig. 1). After three precipitations by ether, the remaining uncoupled derivatives were removed by extensive washing with ethyl acetate in a Soxhlet

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Abbreviations: ACA, 3-oxo-4-androstene-17β-carboxylic acid; poly(EO), polyethylene oxide; ACA-poly(EO), α,ω-bis-3-oxo-4-androstene-17β-amido-poly(EO); E_2-poly(EO), α,ω-bis-estradiol 7α-butyramido-poly(EO); AAI, 3-oxo-4-androstene-17β-amidoisopropane; DOC, deoxycorticosterone; MPF, maturation promoting factor; GVBD, germinal vesicle breakdown; ED_{50}, half-maximum effective concentration.

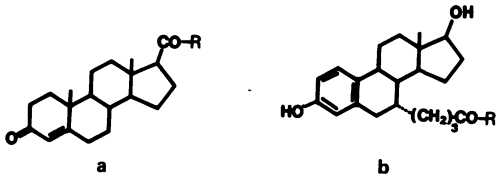

FIG. 1. Proposed structure of the steroid derivatives. (**a**) R =: $-CH_3$ (progesterone); $-CH_2OH$ (DOC); -OH (ACA); X [ACA-poly(EO)]; $-NH-CH(CH_3)_2$ (AAI). (**b**) R = X [E_2-poly(EO)]. For **a** and **b**, X = $-NH-(CH_2)_3-NH-(CH_2-CH_2-O)_n-CH_2-NH(CH_2)_3-NH-$. Note that in the case of E_2-poly(EO), poly(EO) had molecular weight 6000 and for ACA-poly(EO), it had molecular weight 20,000.

apparatus. The aqueous phase containing ACA-poly(EO) was dialyzed against water and evaporated under reduced pressure. Absorbance at 240 nm indicated that 0.65 mol of ACA was bound per mol of the polymer. This value is an average and does not take into account the probable simultaneous presence (in unknown proportions) of the three different species of α,ω-bis-aminopropylamino-poly(EO) binding to no, one, or two molecules of ACA.

When radioactive material was synthesized, 0.8 mCi of [^{3}H]DOC was added to unlabeled DOC (80 mg) and the same synthetic procedures were applied to yield [^{3}H]ACA, [^{3}H]AAI, and [^{3}H]ACA-poly(EO). The specific activity of [^{3}H]ACA-poly(EO) was 2.5 mCi mmol^{-1}.

The synthesis of estradiol-7α-butyric acid and its coupling via an amide bond to α,ω-bis-aminopropylamino-poly(EO) 6000 leading to E_2-poly(EO) were reported elsewhere (24).

Biological Assays. Ovarian fragments were digested with 0.2% collagenase in phosphate-buffered Barth's solution (pH 7.4) (25). After 1.5–2 hr at 28°, the oocytes were washed with the culture medium. In most of the experiments, sterile defined nutrient oocyte medium (26) was used, but in some experiments modified Barth's saline solution was used (27). Full-grown oocytes [stage VI (28)] were selected and incubated in petri dishes containing 2 ml of medium. In each experiment, oocytes were obtained from the same animal. Stock solutions of steroids in ethanol were diluted in the petri dish to the required concentrations. The final concentration of ethanol did not exceed 1% (vol/vol); at this concentration, ethanol had no effect on oocyte maturation. The poly(EO) derivatives were directly dissolved in the culture medium and their molar concentrations were expressed in terms of the steroid. Oocytes were continuously exposed to the inducer at 19–20° for 16 hr, and GVBD was scored. Criteria of maturation (GVBD) and cytological techniques have been described (5). The external concentration able to promote 50% GVBD was taken as the half-maximum effective concentration (ED_{50}).

MPF transfers were carried out as described (25). In microinjection experiments, 100 nl of a solution of the inducer in medium was injected into the oocytes which were further incubated in steroid-free medium and scored for maturation.

Protein Synthesis Analysis. Protein synthesis was analyzed after 16-hr incubation with the inducer, by using a double-labeling technique and sodium dodecyl sulfate/polyacrylamide gel electrophoresis (29, 30).

Column Chromatography of ACA-Poly(EO). A 60 × 2.4-cm column was packed with Ultrogel AcA 54 (LKB) and equilibrated in Barth's solution. Samples (2 ml) were loaded at 20° and fractions (1.7 ml) were obtained at a flow rate of 20 ml/hr. When radioactive material was chromatographed for analytical purposes, 0.1-ml aliquots were assayed for radioactivity in 10 ml of Instagel. When nonradioactive material was applied to the column for biological assays, absorbance at 240 nm was monitored. Pools of five fractions were subsequently incubated with oocytes, and GVBD was scored after 16 hr.

Uptake of ACA-Poly(EO) by Oocytes. Oocytes were incubated in culture medium containing the radioactive compounds at 100 or 10 μM. [^{3}H]Progesterone and [^{14}C]poly(EO) were diluted with the corresponding unlabeled compound in order to obtain the same final specific radioactivity (2.5 mCi/mmol^{-1}).

At various incubation times, 20 oocytes were removed, washed three times with 1 ml of medium, digested overnight in 1 ml of NCS solubilizer (Amersham, England), and assayed for radioactivity in a toluene-based scintillation mixture [0.4% (wt/vol) Omnifluor]. The radioactivity of the incubation medium was determined by simultaneous assay of 20-μl aliquots in Instagel (1 μl being the estimated volume of one oocyte). The uptake ratio was defined as the radioactivity of the oocytes measured at different incubation times, divided by the radioactivity of the incubation medium at time zero. After 20 hr of incubation, 20 oocytes were homogenized in 0.5 ml of medium by four freeze-thawing cycles, and 100 μl of the total homogenate was assayed for radioactivity in Instagel.

Double Assay Technique for Measuring Free and Polymer-Bound Radioactive Steroids. Aliquots of aqueous samples of radiolabeled free and polymer bound steroids were assayed in two different ways. First, the samples mixed with Instagel (10 ml) permitted determination of the total radioactivity of the sample. Second, to measure selectively the radioactivity of the free steroid molecules in the presence of the polymer-bound radioactive steroid, we used a two-phase system in which the aqueous sample (up to 0.1 ml) was agitated with 10 ml of a toluene-based scintillation liquid (4% Omnifluor). In this case, only the free steroid molecules (hydrophobic) extracted by toluene are assayed (the polymer-bound ones remain in the aqueous phase). The radioactivity detected in emulsion was called "total" whereas that detected in the two-phase system was refered to as "free." The ratio of free to total was calculated for each sample. The counting efficiencies were 0.45 in toluene and 0.40 in Instagel, and this difference was taken into account in the calculation of the ratios.

RESULTS

Chemical Characterization of ACA-Poly(EO). The UV spectrum displayed an absorbance maximum at 238 nm, characteristic of Δ_4-3-oxosteroids (not shown). No trace of the original ACA was detectable by thin-layer chromatography in the preparation of ACA-poly(EO). The elution profile of [^{3}H]ACA-poly(EO) on a gel filtration column (Fig. 2) was heterodisperse and showed one major peak of high molecular weight followed by a trail of lighter species. The main peak was eluted in the same volume as a globular protein of approximately 70,000 molecular weight. The discrepancy is likely due to the asymmetry of the molecule (31). However, the radioactivity detectable in the elution volume of free steroid (ACA) was <1% of the total radioactivity.

The free/total ratio for the different labeled derivatives in the aqueous phase was as follows: [^{3}H]ACA, 1.02; [^{3}H]AAI, 1.01; [^{14}C]poly(EO), 0.04; [^{3}H]ACA-poly(EO), 0.04. The results indicate that, in the ACA-poly(EO) preparation, all the hydrophobic steroid moieties are bound to the hydrosoluble, non-toluene-extractable polymer.

Biological Characterization of the Polymer-Bound Derivative. The two polymer-linked steroid derivatives were tested for biological activity in inducing maturation. Their

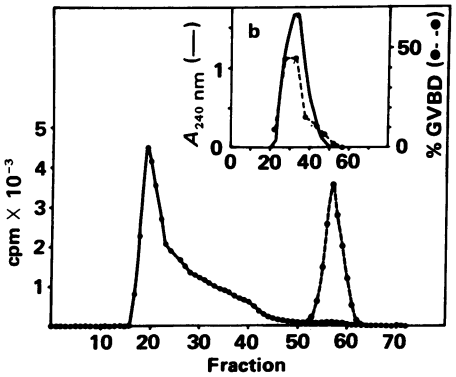

FIG. 2. Gel filtration of polymer-bound steroid derivative ACA-poly(EO). Column chromatography was performed at 20° on an Ultrogel AcA 54 column at a flow rate of 20 ml/hr. Either [^{3}H]-ACA-poly(EO) (2 mg) corresponding to 7.5×10^5 cpm (●—●) or [^{3}H]ACA (60 μg) (○-----○) was applied on the column. The radioactivity eluted was virtually 100% of the initial radioactivity. (*Inset*) Unlabeled ACA-poly(EO) (200 mg) was loaded on the column. Absorbance at 240 nm (—) was determined in the fractions. When the effluent was assayed for biological activity, pools of five fractions were incubated with oocytes, and GVBD (●-----●) was scored after 16 hr.

logarithmic dose-response relationships were compared to those of progesterone, ACA, and AAI (Fig. 3). ACA-poly(EO) was active, whereas E_2-poly(EO) and poly(EO) (not shown) were not. The ED_{50} for progesterone and ACA was 2 μM and 3 μM, respectively. The dose-response curve of ACA-poly(EO) was shifted to the right of ACA by 1 order of magnitude (ED_{50} ≃30 μM). AAI displayed a dose-response close to that of ACA-poly(EO). The maturation induced by ACA-poly(EO) was not abortive according to the following criteria. (*i*) By cytological examination, the presence of the meiotic spindle moving to the cortex was observed (Fig. 4). (*ii*) The cytoplasm of the oocytes induced to mature by ACA-poly(EO) displayed MPF activity after 2 hr, as expected for normal maturation (25). (*iii*) The electrophoretic pattern of protein synthesis in ACA-poly(EO)-induced maturation was similar to that obtained with progesterone (30) (data not shown).

When injected into the oocytes, neither 50 pmol of ACA-poly(EO), nor 50 pmol of ACA per oocyte induced maturation.

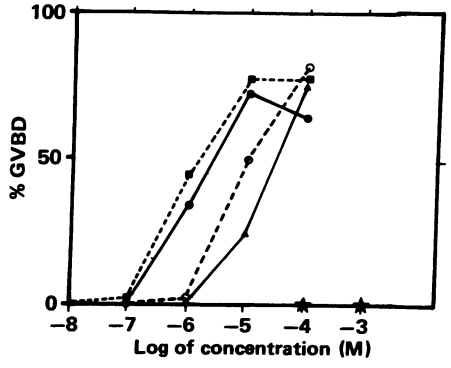

FIG. 3. Dose-response curves of ACA-poly(EO) and related compounds. At each experimental point, 100 oocytes were incubated continuously with the inducer (concentrations in incubation medium at zero time), and the percentage of GVBD was scored after 16 hr of exposure. Each point represents the mean of six different experiments on different females. ED_{50} values: ACA (●—●), 3 μM; ACA-poly(EO) (▲—▲), 30 μM; progesterone (■--■), 2 μM; and AAI (○---○), 20 μM. Poly(EO) or E_2-poly(EO) at 100 μM (★) did not induce maturation.

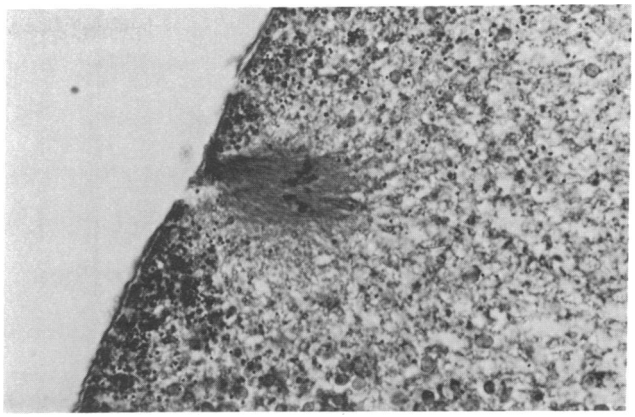

FIG. 4. Meiotic metaphase of an oocyte induced to maturation with ACA-poly(EO). After maturation, oocytes were fixed in Smith's solution. Sections (7 μm) were stained with Feulgen's reagent and counterstained with light green. (×800.)

As a positive control, cortisol[§] was injected and promoted GVBD in 60% of the oocytes.

In order to check whether the activity of ACA-poly(EO) could be due to the presence of low molecular weight components (i.e., free ACA) in the preparation, the biological activity was tested after gel filtration (Fig. 2 *inset*). The absorbance profile showed a lower resolution than that shown in the main figure, probably due to the overloading of the column necessary to obtain detectable biological activity in the fractions. The curve of the biological activity was parallel to that of the absorbance, with a maximum in a zone of high molecular weight. Less than 1.5% of the total biological activity of the preparation was found in the region where free steroids are eluted.

In order to exclude the possibility of hydrolysis occurring during the course of incubation with the oocytes, ACA-poly(EO) was first incubated with oocytes and then the whole incubation medium was subsequently ultrafiltered. The ultrafiltrate and the material remaining in the tubing were tested for their activity in inducing maturation. Most of the biologically active material remained in the tubing (data not shown). It was calculated from the dose-response curves that <0.2% of ACA could have been present in the medium during the maturation process.[¶] Estimation of the free steroid content of the oocytes was based on radioactive methods and is reported below.

Altogether these results indicate that the biological activity is associated with the high molecular weight material and not with free steroid contaminating the preparation or released in the medium during the incubation.

Uptake of the Poly(EO) Derivatives by the Oocytes. Free steroids (10 μM) were rapidly taken up by the oocytes (Fig. 5). In the case of [^{3}H]ACA, the uptake ratio reached 0.7 after 30 min and then increased more slowly. After 7 hr (i.e., after maturation had been attained), the ratio was 1.1. The uptake of [^{3}H]AAI was similar. [^{3}H]Progesterone was taken up more actively; after 1 hr, the uptake ratio was >2. The general pattern of the uptake was similar when concentrations 10 times higher (100 μM) were used and did not show any tendency to saturation, suggesting a nonspecific mechanism.

[§] This effect of *injected* cortisol, which is not obtained with progesterone, has been explained by leakage of this relatively hydrophilic hormone into the external medium (32).

[¶] The GVBD score recorded after incubation of fresh oocytes with the ultrafiltrate was 7%. The concentration of free ACA required to account for this effect would be only 0.2 μM or 0.2% of the initial ACA-poly(EO) concentration (100 μM).

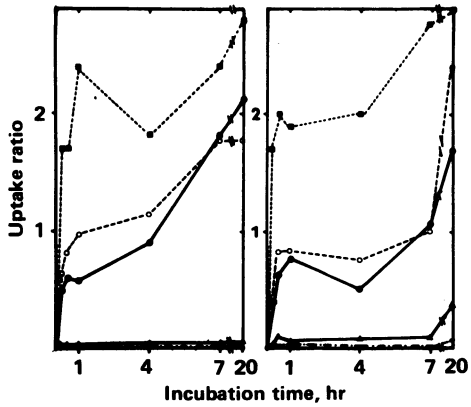

FIG. 5. Uptake of the maturation-inducing agents by oocytes. Uptake ratio was calculated as follows: radioactivity associated with the oocytes (determined by digestion of 20 oocytes in NCS) divided by radioactivity of the external medium (measured by assay of 20-μl aliquots in Instagel at zero time). Initial concentrations of inducing agents 100 μM (*Left*) and 10 μM (*Right*). The counting efficiencies in Instagel and NCS were sufficiently similar that the differences were not taken into account. ▽–·–·–▽, [^{14}C]poly(EO); ▲—▲, [^{3}H]-ACA-poly(EO); ●—●, [^{3}H]ACA; o------o, [^{3}H]AAI; ■------■, [^{3}H]progesterone.

Over the first 7 hr, the uptake ratio of [^{14}C]poly(EO) remained <0.03 and that of [^{3}H]ACA-poly(EO) remained <0.05. These results demonstrate that the linkage of ACA to poly(EO) prevents its uptake by the oocytes.

Estimation of Free Steroid Release during ACA-Poly(EO)-Induced Maturation. The double assay technique was used in order to discriminate between free and immobilized ACA in the incubation medium over the time of maturation and in the homogenate of the oocytes after GVBD had taken place.

In the presence of [^{14}C]poly(EO) 6000 for 20 hr, the free/total ratio was 0.11 in the medium and 0.12 in the homogenate (Table 1). Conversely, when [^{3}H]ACA was used, all ^{3}H radioactivity from the medium and the homogenate was detected in the toluene phase and the ratio was 1.02. When the oocytes were exposed to [^{3}H]ACA-poly(EO), the free/total ratio in the medium was 0.04. It remained constant over the incubation period and was always less than that obtained with [^{14}C]-poly(EO), indicating that no release of free steroid occurred in the medium. After 20 hr of incubation, the ratio obtained from the homogenate was again 0.04, indicating that the radioactivity associated with the oocytes was still in the bound form. On the basis of these results, the concentration of free steroid in the medium was estimated to be <0.2% that of the ACA-poly(EO) and the release of free steroid appeared to be insignificant in both medium and oocytes.

Table 1. Estimation of free steroid release after ACA-poly(EO)-induced maturation

	cpm/20 μl		
	[^{3}H]ACA	[^{14}C]poly-(EO)	[^{3}H]ACA-poly(EO)
Medium:			
Free	3,060	1090	250
Total	3,000	9880	5000
Free/total	0.90	0.11	0.04
Homogenate:			
Free	13,410	40	15
Total	12,300	280	300
Free/Total	0.97	0.12	0.04

The radioactivity of external medium and of oocyte homogenates was determined, after 16-hr incubation, by the double assay method. The free/total ratio was corrected for the difference in efficiencies between the two systems.

DISCUSSION

This report demonstrates that a high molecular weight progesterone analog is active in inducing meiotic maturation in *X. laevis* oocytes *in vitro*.

Because the polymer-bound steroid was only 1/10 as efficient as its low molecular weight precursor, it therefore was critical to be able to rule out the possible presence of traces of this compound in the polymer preparation; a contamination of 10% by ACA would be sufficient to account for the effect of the polymer derivative. Two controls were obtained which allow us to exclude this possibility. Gel filtration prior to biological assay indicated that the polymer-bound steroid is itself able to induce maturation and that the contamination by free steroid can be estimated as less than 0.15%. The detection of free steroid by the double assay method also showed a negligible level of such a contaminant. No increase of this level was detected during the course of incubation in the medium, making unlikely the release of free steroid during the maturation process. Furthermore, oocytes are able to concentrate such molecules, as revealed by uptake experiments, and if a release would occur it would have been possible to detect free steroid in the homogenate. On the basis of these results, it was calculated that no more than 0.2% of ACA was present which is in agreement with the chromatographic data (≃0.15%).

On the other hand, the absence of response to poly(EO) indicated that the polymeric structure alone could not be responsible for the effect. Moreover, the lack of effect of E_2-poly(EO) was an additional control.

Other studies have demonstrated that a biologically active compound linked to a polymer can interact specifically with a recognition structure and lead to the biological effect. Such a possibility underlies the method of affinity chromatography (33), and hormonal effects have been obtained with various polypeptide hormones linked to insoluble matrices (34). This field was recently extended to small molecules linked to soluble polymers and successful results have been obtained with steroids (24), acetylcholine antagonists (35), and isoproterenol (36). In this last case, the biological activity of the polymer–drug complex was retained, but a decrease of 2 orders of magnitude in efficiency resulted from the attachment of the drug to the polymer (36).

Poly(EO) is commonly used as a marker of the extracellular compartment and its hydrophilic properties are believed to prevent its incorporation into cells (37, 38). Evidence exists that high molecular weight proteins such as vitellogenin (39, 40) and cholera toxin (41) can go through the vitelline envelope and reach the plasma membrane. It therefore seems unlikely that poly(EO) of molecular weigh 20,000 would not be able to reach the plasma membrane. However, it was necessary to verify that this compound cannot penetrate across the oocyte membrane. The data obtained showed a restricted entry of poly(EO) into the oocytes, which was not modified by the attachment of the steroid. The entry of ACA-poly(EO) was less than 1/10 that of its low molecular weight homolog ACA. The low amount of ACA-poly(EO) associated with the oocytes may reflect entry into the oocytes by any mechanism, including endocytosis which is reduced at this developmental stage of the oocytes (28). In fact, it could be due as well to a trapping of the polymer in the glycoprotein layer of the vitelline membrane.

ACA and AAI, which have significantly different efficiencies in inducing maturation, display similar uptake kinetics, whereas ACA and progesterone have similar ED_{50} values but different levels of incorporation into the oocytes. Other studies have also indicated that there is no correlation between the amount of hormone acquired by the oocytes and its efficiency in inducing maturation (42). Estradiol, which is taken up with kinetics similar to those of progesterone, elicits no response (1, 42). Finally, the failure of ACA-poly(EO) to induce maturation when injected into the oocytes also is evidence against a correlation between uptake and action. Therefore, ACA-poly(EO), which induces maturation without entering the cell and which is inactive when injected into the cell, must exert its effect through an interaction with the plasma membrane.

We conclude from the present studies that the steroid–polymer complex exerts its effect in the conjugated form. Data dealing with the induction of meiosis by progesterone in amphibians (reviewed in the Introduction and in ref. 43) and by 1-methyladenine in starfish (44, 45)) have led to the hypothesis that a decisive step of meiosis reinitiation is the interaction of the hormone with the plasma membrane of the oocyte. We think that the present data provide more direct support for this model.

We acknowledge the interest and suggestions of Jean Neel and Pierre Pennequin and the partial support of the Ford Foundation, the Swiss National Science Foundation (no. 3.1710.73) and the Centre National de la Recherche Scientifique.

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