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Inhibitor of Oocyte Maturation from Porcine Follicular Fluid: Further Purification and Evidence for Reversible Action

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ABSTRACT

The *in vitro* culture of porcine oocytes was used as a bioassay for inhibitory fractions from PM-10 membrane filtration, Sephadex G-25 column chromatography and paper electrophoresis of porcine follicular fluid (FFl). A 100-fold purification of the inhibitor of oocyte meiosis from the PM-10 concentrate was achieved. The major inhibitory fraction had a molecular weight of less than 2,000. Freshly collected, unfractionated FFl also contained inhibitory activity, with significant inhibition exhibited by fluids from small and medium sized follicles. The inhibition of maturation by unfractionated FFl, the PM-10 filtrate and the inhibitory peak from the Sephadex column could be reversed when the inhibitory fraction was removed from oocyte cultures by 20-24 h after the initiation of culture. The modifications from previously reported purifications of the inhibitor from FFl and the finding of a reversible arrest of porcine oocyte maturation are discussed.

INTRODUCTION

Several studies have shown that porcine follicular fluid (FFl) (Tsafriri and Channing, 1975a), granulosa cells (GC) (Tsafriri and Channing, 1975a), and extracts of GC (Tsafriri et al., 1976) inhibit the spontaneous maturation of porcine oocytes in culture. Porcine FFl and a partially purified fraction of FFl also inhibit the spontaneous maturation of cultured mouse (Channing and Tsafriri, 1976) and rat oocytes (Tsafriri et al., 1977a) and bovine follicular fluid inhibits the maturation of hamster oocytes (Gwatkin and Andersen, 1976), thus showing that the inhibitor(s) is not species specific. Physiological studies revealed that luteinizing hormone (LH) (5 μg/ml) partially or completely reversed the inhibitory action of porcine FFl (Tsafriri et al., 1976) and that an antiserum (Tsafriri et al., 1977b) prepared against a partially purified fraction of porcine FFl also reversed the inhibitory action of the partially purified fraction on rat oocytes.

An initial partial purification and chemical characterization of the inhibitor from porcine FFl has been reported (Tsafriri et al., 1976). It was found that the inhibitor in crude FFl was stable to 60°C for 20 min, but that trypsin destroyed the inhibitory activity. This paper reports further purification of the inhibitor and demonstrates that the inhibitory action is reversible.

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MATERIALS AND METHODS

Oocyte Collection and Culture

Oocytes and follicular fluid were collected from porcine follicles as previously reported (Tsafriri and Channing, 1975a,b). Cumulus enclosed oocytes from medium sized follicles (3-5 mm) were cultured in Microtest II Plates (Falcon) in compartments containing 12-15 oocytes in 0.2 ml of modified Medium 199 (Tsafriri et al., 1976). This served as the bioassay system for following the purification of the inhibitor. After a culture period of 44-48 h, the oocytes were stained with aceto-orcein stain (1% GIBCO) and were examined by light microscopy. Oocytes in which an intact germinal vesicle or nucleolus could not be identified and which did not show signs of degeneration were classified as mature.

When the inhibitor was being tested an appropriate volume of the sample was diluted with modified Medium 199 and sterilized by filtration through a sterile 0.22 μm cellulose Millipore membrane. The potency of a fraction was expressed as the percent inhibition of oocyte

maturation, which was calculated as follows:

$$\% \text{ inhibition} = 100 \frac{\% \text{ maturation in control} - \% \text{ maturation in the inhibitory fraction}}{\% \text{ maturation in control}}$$

For those studies in which fresh porcine follicular fluid was tested, the fluids from small (1-2 mm), medium (3-5 mm) and large (6-12 mm) follicles were aspirated at the abattoir within 30 min after the animals were killed. The fluids were kept separately in covered sterile tubes on ice until final preparation for use in culture. The fluids were centrifuged at 1,000×g for 5 min to remove cells and debris and were sterilized by filtration through the sterile filter immediately prior to addition to culture. In all of the analyses of fresh follicular fluid, the samples were used within 3 h of collection from the animals.

In order to pool fluids for the purification of the inhibitor, follicular fluids from small, medium and large follicles were collected separately and were stored for periods of 3-6 months in a freezer at −35°C before they were used.

Purification of the Inhibitor

Each step in the procedure has been replicated 3 or 4 times and a typical purification is described. All steps were carried out at 4°C unless otherwise indicated. A volume of 425 ml follicular fluid from small, medium and large follicles was pooled and filtered under N_2 through PM-10 membranes (Amicon Corp.) fixed in two 200 ml Amicon pressure cells. This required approximately 24 h. The filtrate (325 ml) was lyophilized, resuspended in water to a volume of 10.5 ml and centrifuged at 105,000×g for 1 h to remove a small amount of solid material. Both the concentrate of the filtered fraction and the material retained by the filter (PM-10 retentate) were assayed in oocyte culture. The PM-10 concentrate was further assayed in culture at several dilutions to establish a dose response to the inhibitor. The PM-10 concentrate was stored in liquid N_2.

An aliquot of the PM-10 concentrate (9 ml) was applied to a column of Sephadex G-25 (Pharmacia) (5×70 cm) which was suspended in 0.01 M NH_4HCO_3. The column had been calibrated with Blue Dextran (exclusion volume 615 ml) and porcine β-MSH (mol. wt. 2173; peak volume 840 ml). The column was eluted with 0.01 M NH_4HCO_3 at the rate of about 90 ml/h and each tube contained approximately 15.7 ml. The fractions were lyophilized to remove the volatile buffer, redissolved in 2 ml of quartz-distilled water and were assayed in culture at dilutions of 1:100. After the bioassay, Peak A (tubes 80-87), representing the bulk of the inhibitory material and Peak B (102-105) were lyophilized and the residues were dissolved in water to a final volume of 5.5 ml (Peak A) and 2.0 ml (Peak B). Tubes 60-70 were combined to serve as a dummy peak and after lyophilization the residue from these tubes was dissolved in 2.0 ml of water.

The inhibitory peak (Peak A) from the Sephadex fractionation was subjected to electrophoresis as follows. One ml was applied along a line (18 cm) at the origin of Whatman 3 mm paper that had been washed overnight in water to remove soluble contaminants. Electrophoresis was performed at 1500 volts for 3.5 h on a Savant flat plate cooled by circulating ethylene glycol-water from a refrigeration bath kept at 4°C. The buffer used was formic acid (0.37 M) acetic acid (1.17 M), pH 1.9. Tyrosine was run along the side of the paper as an external standard. After completion of the run the paper was cut into pieces 18 cm×3 cm or 18 cm×1.5 cm and each of these fractions was cut into small pieces and eluted twice at room temperature with a total of 20 ml H_2O. The extracts from each large piece were combined, lyophilized to dryness and the residues were dissolved in 2 ml H_2O and stored in liquid N_2. These fractions were assayed in oocyte cultures at dilutions of 1:50.

Peptide Measurement

The peptide contents of fractions were estimated with 4-phenylspiro [furan-2 (3H), 1'-phthalan]-3, 3'-dione (Fluorescamine) (Udenfriend et al., 1972) using porcine β-MSH as a standard. An aliquot (50 μl) was mixed with 1.60 ml of 0.2 M sodium borate, pH 9.0. To this rapidly mixed solution, 0.40 ml of fluorescamine (0.1 mg/ml in acetone) was added. The fluorescence was activated at 395 nm and read at 490 nm on an Aminco-Bowman spectrophotofluorometer.

Reversibility of Inhibition in FFl

A series of experiments was done to determine the ability of oocytes to resume meiosis *in vitro* after inhibition *in vitro* in FFl or in the partially purified inhibitory fractions of FFl. After 20-24 h or 28-36 h of oocyte incubation in a 1:2 dilution of FFl, the medium surrounding half of the oocyte cultures was gently aspirated with a sterile syringe and was replaced with freshly prepared inhibitory medium. In the analysis of data, these cultures served as the inhibitory controls. The other cultures were replenished with noninhibitory control medium. After the change of medium the incubations were continued for an additional 24 h to allow time for germinal vesicle breakdown. The oocytes were examined for nuclear maturation.

The same experimental design was used in testing the reversibility of the inhibition by the PM-10 concentrate (1:50 dilution) and by the pooled fractions of Sephadex Peak A (1:400 dilution). As in all other studies, the oocyte cultures in modified Medium 199 were the noninhibitory controls.

Statistical Analysis

The Chi-square test was used to determine the significance of the difference between the levels of maturation, nonmaturation and degeneration for an experimental fraction and those observed in the control. The standard error (SE) was calculated according to the following expression (Kendall and Stewart, 1969):

$$SE = \sqrt{\text{variance}} = \sqrt{\frac{P_2}{P_1}^2 \frac{P_1(1-P_1)}{n_1} + \left(\frac{1}{P_1}\right)^2 \frac{P_2(1-P_2)}{n_2}}$$

where P_1 and P_2 are the fractions of oocytes matured in the control and experimental cultures, respectively and n_1 and n_2 are the total numbers of oocytes in those cultures.

In order to calculate the amount of activity recovered at each step in the purification, a unit of inhibitory activity was defined as the amount of material present in a given dilution which causes 50% inhibition of oocyte maturation.

RESULTS

Inhibition by Fresh FFl

After oocyte culture in freshly collected follicular fluids, significant inhibition of maturation was observed in the fluids from two sizes of follicles (Fig. 1). The strongest activity (47.4% inhibition; $P<0.0005$) was exhibited by the fluid from small follicles. Fluid from medium sized follicles showed a level of inhibition (30.0%; $P<0.025$) similar to that of a standard frozen-and-thawed follicular fluid preparation (26.8%; $P<0.025$), while fluids from large follicles demonstrated insignificant inhibition. These data indicate, therefore, that the inhibition of maturation by FFl *in vitro* is not due to artifacts derived from freezing and thawing the fluid. The inhibitory activity is as strong in freshly collected fluid, especially that from small follicles, as in fluids pooled and preserved by freezing.

Purification of the Inhibitor

The concentrate from PM-10 filtration was assayed at several dilutions. The dose response curve obtained is displayed in Fig. 2. However, the PM-10 retentate, at a high concentration (1:4) in culture, demonstrated no inhibition of maturation. The percent maturation of oocytes in those cultures was slightly higher than that in control Medium 199 (Table 1). The inhibitory fraction in porcine FFl thus has a molecular weight of less than 10,000 daltons.

A portion of the PM-10 concentrate was applied to the Sephadex column. The elution from the column (Fig. 3) showed two areas of inhibition. Tubes 80 - 87 (Peak A) represented the bulk of the inhibition and corresponded to a molecular weight of less than 2,000; some inhibition was also seen in fractions 102 - 105 (Peak B). The major portion of the peptide was eluted between the two inhibitory peaks. Fractions in tubes 60-70 were pooled to serve as a "dummy" peak. The assays for Peak A and the dummy peak are shown in Fig. 4. The absence of activity in the dummy peak indicates that the activity observed in Peak A was not the result of nonspecific inhibitory material washed from the Sephadex resin and later concentrated. A dose response curve for Peak A is given in Fig. 5.

Sephadex Peak A was subjected further to paper electrophoresis. The fractions collected from the eluates of the paper were assayed in culture and the results are shown in Fig. 6. Clearly, the bulk of the activity was in the fraction between 3 and 4.5 cm from the origin ($P<0.01$); no other regions of the electrophoresis demonstrated significant levels of inhibition.

Recovery of Activity and Fold Purification

The data in Table 2 summarize the steps in the purification. The recovery of activity at each step was calculated from the dose response curves shown in Figs. 2 and 5.

Reversibility of Inhibition by FFl

After 20-24 h of culture in unfractionated FFl,

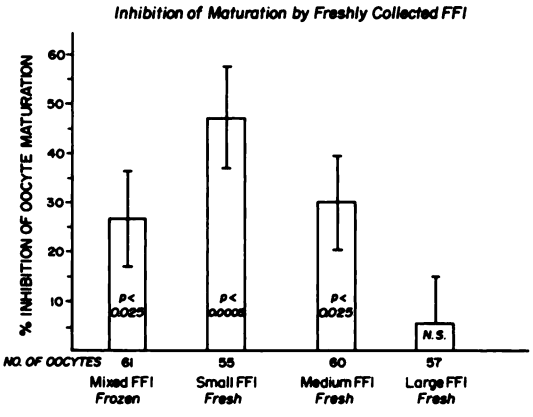

FIG. 1. Percent inhibition of maturation by freshly collected FFl. The experiments were conducted as described in Materials and Methods. The bars show ± SEM NS, not significant.

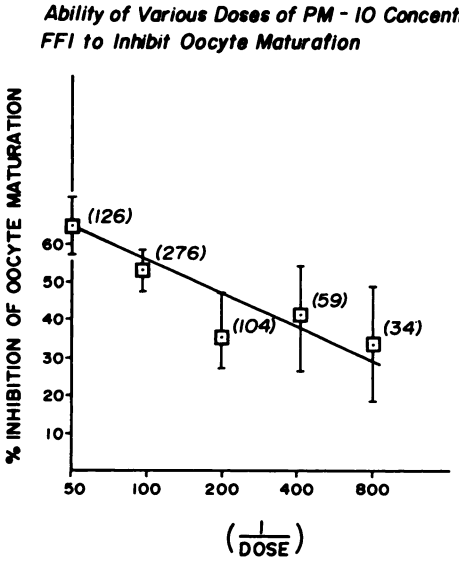

FIG. 2. Dose response curve of the PM-10 concentrate fraction. The experiments were conducted as described in Materials and Methods. The numbers in parentheses are the numbers of oocytes at each dilution. The line was drawn by the method of least squares. The bars show ± SEM.

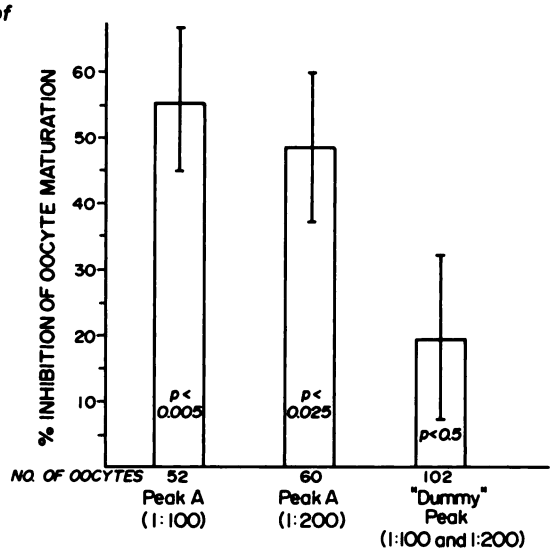

FIG. 4. Assays of Peak A and "Dummy" peak from Sephadex column. The fractions were collected and assayed as described in Materials and Methods. The bars show ± SEM.

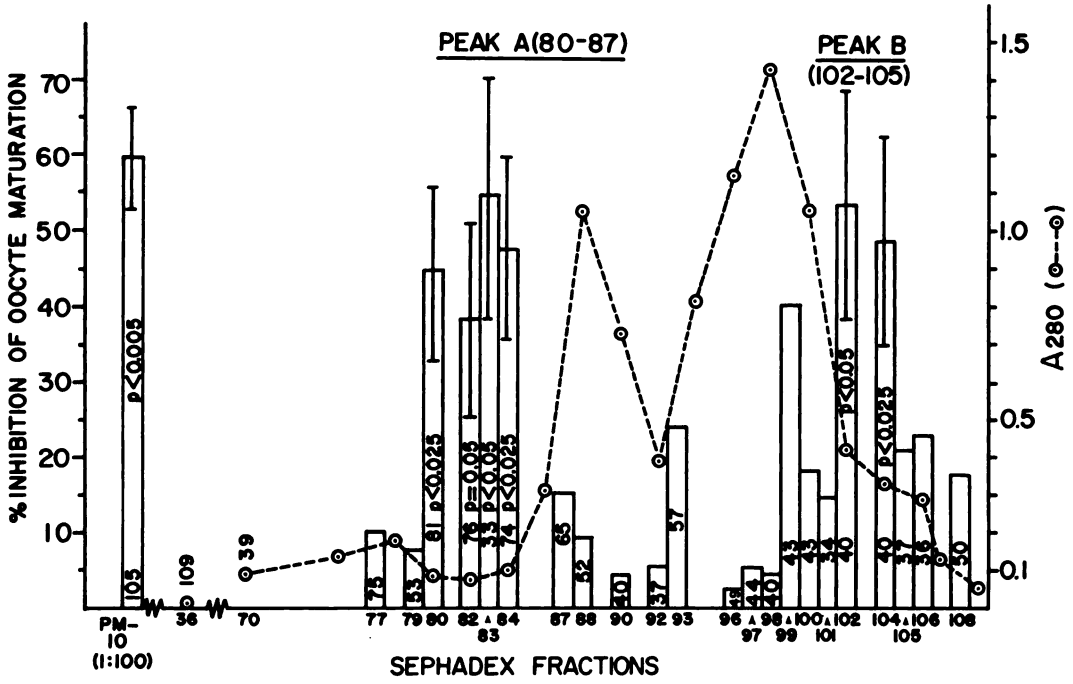

FIG. 3. Fractionation of PM-10 concentrate by chromatography on Sephadex G-25. The absorbency at 280 nm (---) was determined on the original fractions (15.7 ml). The fractions were then lyophilized, redissolved in 2 ml H_2O and the inhibitory activity assayed at a dilution of (1:100) as described in Materials and Methods. The numbers in the lower parts of the bars are the numbers of oocytes for each test. The bars show ± SEM. Inhibition values without error bars were not significantly different from controls.

TABLE 1. Effect of PM-10 membrane filtration of porcine follicular fluid on oocyte maturation.

Culture Medium	No. of oocytes	Mean % maturation	% inhibition ± SEM
Control-Medium 199	36	47.2	—
Pre-PM-10 FFl (1:2)	41	17.1	63.8 14.0*
PM-10 retentate (1:4)	46	54.3	None
PM-10 concentrate (1:50)	62	19.4	58.9 12.9*

The experiment was conducted as described in Materials and Methods. The Pre-PM-10 FFl was a portion of the FFl used to generate the PM-10 fractions used in the experiment. The PM-10 retentate (1:4) was equivalent in dilution to FFl (1:2).
*$P<0.025$

the oocytes were capable of resuming meiosis when the inhibitory culture medium was replaced with serum (1:2 in Medium 199) (Fig. 7). At 20-24 h, the maturation in the inhibitory controls was significantly different from that in the reversal, noninhibitory medium ($P<0.0001$). After 28-36 h in FFl, however, fewer oocytes were inhibited and fewer resumed maturation after the change of medium ($P<0.5$, N.S.). Furthermore, by the final day of culture (Day 3), oocytes in both the inhibitory controls and reversal cultures appeared to have begun degenerative changes. This finding indicates that the length of the culture period necessary to detect reversal after 28-36 h in FFl may have been too long for the maintenance of healthy oocytes. Therefore, with the use of the *in vitro* culture system, porcine oocytes can be held in the dictyate stage of meiosis for up to 24 h and still be capable of resuming meiosis after removal of the inhibitor.

The results of the reversibility studies using the partially purified inhibitor of FFl are shown in Fig. 8. After 20-24 h of incubation in the PM-10 concentrate, few of the oocytes underwent nuclear maturation when the inhibitory fraction was removed. The PM-10 concentrate, which contains all FFl components of <10,000 daltons, may contain low molecular weight moieties which, in high concentration, irreversibly affect the maturational ability of the oocytes. Inhibition of nuclear maturation by the Sephadex Peak A fraction, however, was partially reversed ($P<0.001$) when the inhibitory medium was replaced with noninhibitory medium.

DISCUSSION

By modifying and extending previous fractionations of porcine FFl, we have achieved a 100-fold purification of the inhibitory activity from the PM-10 concentrate. Several changes from previously reported purification procedures (Tsafriri et al., 1976) are noteworthy. 1) In order to purify larger batches of the inhibitor from FFl, greater volumes of fluid were applied to membrane filtration and to column chromatography; the size of the column was greatly increased from 2×43 cm

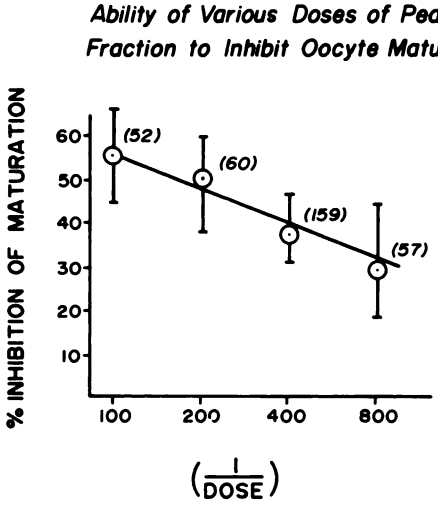

FIG. 5. Dose response curve of the Sephadex Peak A fraction. The experiments were conducted as described in Materials and Methods. The remaining information is given in the legend to Fig. 2.

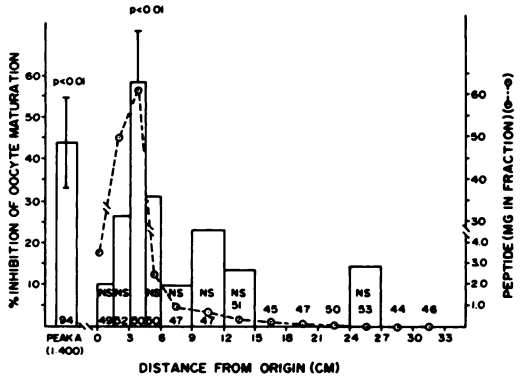

FIG. 6. Inhibitory activity of fractions derived by paper electrophoresis of Peak A. The paper was cut, the fracions eluted and the peptide content of each fraction (——) estimated by procedures described in Materials and Methods. The numbers along the lower part of the histogram are the number of oocytes used to test each fraction. The bars show ± SEM.

TABLE 2. Purification of oocyte maturation inhibitor from porcine follicular fluid.

Fraction	Vol ml	Peptide mg/ml	Dilution which produces 50% inhib.	Specific activity Units/mg	Fold purif.	Yield %
FFI*	425	1750	–	–	–	–
PM-10	9	4582	1:140	0.031	1	100
Peak A	5.5	102.6	1:170	1.66	53.6	74
	1 ml Peak A (170 units; 102.6 mg) subjected to electrophoresis					
Elect.	2.0	30.9	1:50	3.24	104	44

*It was not possible to calculate a starting specific activity because we did not do a dose response curve on FFI. A (1:2) dilution of the FFI used in this purification inhibited maturation by 64%.

to 5 × 70 cm. 2) The period of PM-10 membrane filtration was extended to assure that all low molecular weight material would pass through the membrane. Results of the bioassay of the filtrate and retentate unequivocally showed that all the inhibitory activity was in the filtrate. 3) The elution buffer of the Sephadex column was changed from 0.01 M sodium phosphate, 0.15 M NaCl to volatile 0.01 M NH_4HCO_3. This change minimizes the accumulation of buffer salts in the lyophilized fractions. In high concentrations, such material can dramatically affect the results of the bioassay and the further purification steps. As we demonstrated with the oocyte cultures in the "dummy" peak from the Sephadex column, lyophilized particles from the resin of the column had no effect on spontaneous oocyte maturation. As reported previously (Tsafriri et al., 1976), the principal inhibitory activity appears to be less than 2,000 daltons. An inhibitory component of similar size has also been isolated from bovine follicular fluid (Gwatkin and Andersen, 1976). Another inhibitory peak of lower molecular weight was also eluted in these experiments. This peak may represent material which, in earlier investigations, passed through the UM-2 membrane (nominal molecular weight cut-off of 1,000) that was used and was thus never applied to the Sephadex column. Alternatively, the second peak may represent breakdown products of Sephadex Peak A. Further studies are needed to resolve the nature of the second inhibitory peak.

An essential finding for our continued purification of the physiological inhibitory fraction from pooled FFI is that inhibitory activity is present in freshly collected FFI and, therefore, is not an artifact of the freezing and thawing of pooled fluids. It has been reported that porcine FFI aspirated from ovaries at the abattoir and FFI recovered from ovaries stored on ice differ with

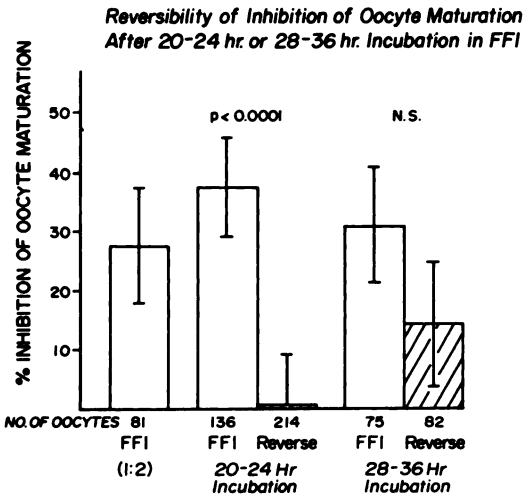

FIG. 7. Reversibility of inhibition of oocyte maturation after 20-24 h or 28-36 h incubation in FFI. The experiments were conducted as described in Materials and Methods. The bars show ± SEM.

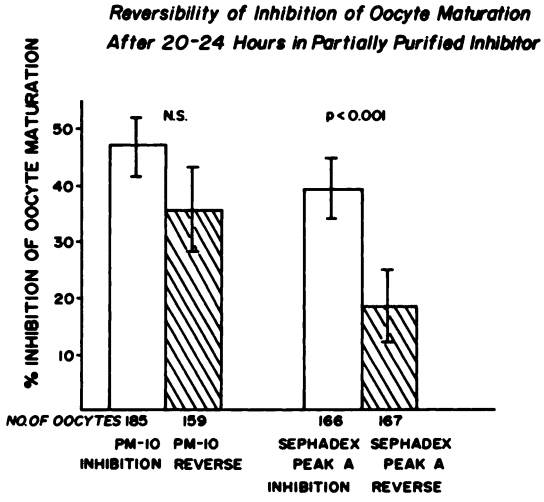

FIG. 8. Reversibility of inhibition of oocyte maturation after 20-24 h in partially purified inhibitor, PM-10 or Sephadex Peak A. The experiments were conducted as described in Materials and Methods. The bars show ± SEM.

respect to the levels of potassium, calcium, glucose and alkaline phosphatase (Chang et al., 1976). These differences, however, do not cause discrepancies in the oocyte maturation between fresh and stored FFl. Interestingly, the pattern of activity is almost identical to that exhibited by granulosa cells cocultured with oocytes (Tsafriri and Channing, 1975a). The granulosa cells and fresh FFl from small follicles most strongly inhibited maturation, but very slight inhibition was seen in the cultures with granulosa cells or fluid from large follicles. The close correlation of the data from the two studies is probably due to the sampling of fresh follicular components which were collected from ovaries immediately prior to the initiation of the cultures. These correlative findings support the contention (Tsafriri and Channing, 1975a) that the inhibitor in FFl is a product of the granulosa cells.

In vivo, the inhibition of oocyte meiosis is reversed by the gonadotropin surge prior to ovulation. *In vitro* oocytes resume meiosis when they are removed from the follicle and its fluid constituents (Pincus and Enzmann, 1935; reviewed by Donahue, 1972) or if LH is added to cultures of whole follicles (Tsafriri et al., 1972; reviewed by Channing and Tsafriri, 1977) or to oocytes cultured in follicular fluid (Tsafriri et al., 1976; Gwatkin and Andersen, 1976). Since the arrest of maturation by FFl *in vivo* and *in vitro* is, in some manner, removed by the action of LH, one would expect that the inhibition by FFl *in vitro* could be relieved by the removal of inhibitor from oocyte cultures. Indeed, the inhibition of maturation of oocytes cultured up to 24 h in FFl was completely reversed when the oocytes were given fresh, noninhibitory medium. Replenished with fresh, inhibitory medium, other oocytes remained in the dictyate stage. It is significant that the inhibition which we observed *in vitro* is not toxic to the oocytes, but can be reversed.

The *in vitro* culture system that was used was not adequate to support long term (28-36 h) inhibition *in vitro* with subsequent reversal *in vitro*. Many of the oocytes were extremely fragile and appeared to undergo degenerative changes by the third day of culture. The failure of oocytes to live for extended periods in culture has also been observed in the long term culture of whole follicles from rat ovaries. By the third day of culture *in vitro* all follicle-enclosed oocytes had degenerated (Stoklosowa and Nalbandov, 1972).

The PM-10 filtrate and the Sephadex Peak A fraction allowed partial reversal of meiotic arrest. The failure of these fractions to allow complete reversal cannot be fully explained. The PM-10 concentrate, particularly, is a very crude preparation and contains many other low molecular weight components. Any of these factors may irreversibly affect oocyte maturation in a culture system, such as ours, which provides minimal nutritional and macromolecular support. This is substantiated by the observation that the more highly purified Sephadex Peak A allowed significant reversal of inhibition.

The inhibitory activity found in porcine FFl is not specific only to porcine oocytes. Porcine FFl has been demonstrated to inhibit the spontaneous maturation of mouse (Channing and Tsafriri, 1976) and rat (Tsafriri et al., 1977a) oocytes and the partially purified fraction from porcine FFl (PM-10 concentrate) inhibits the germinal vesicle breakdown of rat oocytes (Tsafriri et al., 1977a). Inhibitory activity has also been shown in the FFl of the rabbit (Chang, 1955), hamster and cow (Gwatkin and Andersen, 1976). Both the bovine and the major porcine inhibitors appear to be approximately 2,000 daltons, but whether they are identical inhibitors is not resolved. How inhibitors affect the oocyte-cumulus complex and how LH relieves the arrest of meiosis remains to be elucidated. Certainly, both *in vivo* and *in vitro* approaches to these questions must be employed. An important facet of the studies is the purification of the reversible inhibitory activity from FFl. For potential application to fertility control, it is essential that a reversible activity be employed, so that oocytes, after inhibition, may complete maturation and undergo normal fertilization.

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